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RESEARCH ARTICLE

Characterization and biological function of milk-derived miRNAs

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Scope: Breastfeeding is associated with reduced risk of infection, immune-mediated disorders, obesity, and even cancer. Recently it was found that breast milk contains a variety of microRNAs (miRNAs) in the skim and fat layer that can be transferred to infants, and appear to play important roles in those biological functions.
Methods and results: This study applied next generation sequencing and quantitative real-time PCR analysis to determine the miRNA expression profile of the skim and fat fraction of human, goat, and bovine milk as well as infant formulas. Human and mammalian milk were found to contain known advantageous miRNAs in exosomes and also in the fat layer. These miRNAs are highly conserved in human, bovine and goat milk. However, they were not detected in several infant formulas. Further, miRNAs present in milk were able to enter normal and tumor cells and affect their biological functions. Following incubation of milk derived human miRNA with normal and cancer cells, the expression of miRNA-148a was upregulated and the expression of the DNA methyltransferase1 target gene of miRNA-148a was down regulated.
Conclusion: These results reinforce previous findings on the importance of miRNA in breast milk. Future studies should concentrate on the addition of miRNA to infant formulas.

Keywords:
Exosomes / Fat layer / Infant nutrition / Micro RNA / Milk

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Milk is the main source of nutrition for newborn mammals, and breastfeeding is known to be one of the most valuable contributors to infant health [1]. Infants who are breastfed enjoy significant health benefits in comparison to those fed with infant formulas. These benefits include protection against a whole range of diseases including, diabetes [2], cancer [3], and obesity [4]. Breast milk is also thought to play an important role in the development of children's immune system, based on evidence that the composition of breast milk can influence immune responses in neonates [5, 6]. However, the mechanisms governing the immune modulation and anti-infection properties of milk are poorly understood and may arise from biological macromolecules present in breast milk such as growth factor antibodies and nucleic acid. Breast milk contains micro RNAs (miRNAs) in particular in the exosomes [7, 8], but their putative biological role has not been fully elucidated.

MiRNAs are small non-coding RNAs involved in post-transcriptional gene regulation. They control a wide range of cellular functions such as cell differentiation, proliferation, and cell death [9–11]. Dysregulated expression of miRNAs may play an important role in the development of diseases such as cancer [12, 13] and diabetes [14]. During the fetal period, miRNA regulates organogenesis and morphogenesis [15]. MiRNAs have been found in extracellular fluids such as plasma [16], serum [16], saliva [17], urine [18], and milk [19]. Studies have reported miRNA expression in human [20],

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Abbreviations: **DNMT1**, DNA-methyltransferase 1; **miRNA**, microRNA; **NGS**, next generation sequencing; **qRT-PCR**, quantitative real time PCR

bovine [7], porcine [21], and goat [22] milk and found that a significant number of milk-derived miRNAs are related to immune regulation. Furthermore, over 60% of all immune-related pre-miRNAs are present and enriched in breast milk [19]. Based on the expression profile of milk-derived miRNAs, it was suggested that these miRNAs support the immune system of the infant and may have other beneficial effects on childhood health [23, 24]. Moreover, milk miRNAs may play an important role against development of allergy and autoimmune disease [25, 26].

Although it has been claimed that miRNA derived from ingested food do not survive in the human gastrointestinal tract [27], milk-derived miRNAs withstand degradation. Milk miRNAs were found to be stable to acidic conditions and resistant to RNase and freeze-thaw cycle degradation [8, 28–30], which led us to speculate that these miRNAs can transfer from the mother's milk and impact infant intestine and immune system development. There is a previous study that demonstrate that porcine milk exosomes and their miRNAs are taken up by intestinal epithelial cells, modify target gene expression and promote proliferation of intestinal cells [31]. The skim fraction of milk was found to contain exosomes which are membranous vesicle packaging proteins, RNA and miRNAs [8, 19] that shield miRNAs from degradation [32]. In addition, exosomes are crucial to the transfer of miRNA into the intestine conferring protection against degradation and facilitating uptake by endocytosis [33, 34]. Cow and goat milk are widely used as dairy products and most infant formulas are based on cow milk proteins. Findings indicate that milk-derived miRNAs in commercial pasteurized milk are absorbed by adults in meaningful amounts [35]. In addition, studies have reported the incorporation of milk exosomes into colon cancer cells [33], intestinal cells [33], kidney cells [35], macrophages [36], and in human peripheral blood mononuclear cells [35].

To better understand the role of miRNAs in breast milk, we simultaneously determined the miRNA expression profile of the skim and fat fractions of human, goat, and bovine milk. Then, we studied the biological effects of miRNA derived from the fat layer of human milk and human miRNA derived from the skim layer, on normal and transformed cells.

2 Materials and methods

2.1 Ethical approval

All the mothers who participated in the study signed informed consent forms. The Ethics Committee at Hadassah Hospital approved this study (HM0-0101-13).

2.2 Milk sample collection

Human milk samples were collected from healthy mothers at one month of lactation. The samples were transported to the laboratory and stored at −80°C until further analysis. Milk samples were collected before and after pasteurization from a pool of cow and goat milk. The samples were transported to the laboratory and stored at -80°C until further analysis.

2.3 Infant formulas

The infant formulas were commercially available formulas that are marketed in Israel (Materna extra care stage 1, Maabarot products Ltd. Smilac advance plus stage 1, Abbott nutrition Ltd. Nutrilon stage 1, Nutricia Ltd.). All the three tested formulas are intact protein, cow's-milk-based, infant formula (67 kcal/100 mL reconstituted formula).

2.4 Milk fractionation

Human, bovine, goat milk and the infant formula were fractionated by centrifugation at 6500 × *g* for 30 min at 4°C. Three fractions were obtained from each sample: cells, lipids and skim milk. The skim milk and the lipid layer were transferred separately to a new tube. The skim milk was centrifuged at 12 000 × *g* for 1 h at 4°C to remove debris. The defatted supernatant was then passed through 5 and 0.45 μm filters to remove residual debris. The skim and fat layer were subjected either to Next Generation Sequencing or quantitative real time PCR (qRT-PCR). The skim layer was also used for exosome isolation.

2.5 MiRNA extraction from milk samples

TRI reagent (INVITROGEN, Carlsbad, USA) was added to the skim milk (1/3 v/v) and to the lipid layer (1/5 v/v). Chloroform and Lysophosphatidic acid (LPA) (6 μL of 1:20 of LPA to 1 mL of TriReagent) were added, shaken vigorously and incubated for 15 min at room temperature and then centrifuged at 12 000 × *g* for 15 min at 4°C. The aqueous phase was carefully transferred to a new tube. The same volume of chloroform as the aqueous phase was added and incubated for 3 min at room temperature and then centrifuged at 12 000 × *g* for 15 min at 4°C. The upper phase was carefully transferred to a new tube. Ethanol 70 % (the same volume as the chloroform) was added. MicroRNAs were isolated using the miRNeasy mini kit and the RNeasy MinElute Cleanup kit, according to the manufacturer's instructions. Briefly, following the addition of the ethanol, the samples were transferred into an RNeasy Mini column centrifuged at 8000 × *g* for 15 s, and the flow through was collected. Ethanol 100% (0.65 volume) was added to the flow through from the RNeasy column. The sample was transferred to an RNeasy MinElute spin column and centrifuged at 8000 × *g* for 15 s. Following the addition of the RPE buffer the column was centrifuged at 8000 × *g* for 15 s. Ethanol 80% was added to the column and the column was centrifuged at 8000 × *g* for 2 min and then at full speed for 5 min to dry the membrane. To collect the miRNAs 14 μL of

RNase free water was added to the column and centrifuged at full speed for 1 min.

2.6 Extraction of total RNA

2.6.1 From milk

TRI reagent (INVITROGEN, Carlsbad, USA) was added to the skim milk (1/3 v/v) and to the lipid layer (1/5 v/v). Chloroform and LPA (6 μL of 1:20 LPA to 1mL of TriReagent) were added, shaken vigorously and incubated for 15 min at room temperature and then centrifuged at 12 000 × *g* for 15 min at 4°C. The aqueous phase was carefully transferred to a new tube. Subsequently isopropanol (0.5 mL per 1 mL TriReagent) was added to precipitate the RNA mixed by inversion. Following incubation for 10 min at room temperature the samples were centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatant was discarded, the pellet washed with 75% ethanol (1 mL per Tri Reagent) and centrifuged at 12 000 × *g* for 5 min at 4°C. The pellet was air dried and re-suspended in RNase-free water.

2.6.2 From cells

TRI reagent was added to the cells and treated as described above.

RNA quantity and quality was assessed by measurement of the absorbance at different wavelengths using a NanoDrop spectrophotometer of the RNA samples. We use RNA with a 260/280 ratio of at least 1.85 in nuclease-free water. 260/230 ratio was also used to estimate the presence of other contaminants. Quantitation and quality control of small RNA isolated for NGS was performed also using an Agilent Bioanalyzer 2100 system.

2.7 Small RNA library construction and sequencing

Small RNA library construction from the extracted miRNA of each of the 12 samples was carried out using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Set 1), according to the manufacturer's instructions. Briefly, a 3′ adapter was ligated to 100 ng RNA using RNL2, followed by 5′-adapter ligation, using T4 ligase. Reverse transcription with SuperScript II generated cDNA, which was PCR-amplified and size-selected on a gel was purified. Sequencing was carried out on the NextSeq 500 System (Ilumina).

2.8 Computational analysis of sequencing data

Bioinformatics analysis of the miRNA was carried out as previously described http://rnajournal.cshlp.org/content/early/2013/02/18/rna.036475.112.full.pdf with some minor changes.

2.9 MicroRNA detection by qRT-PCR

For total RNA samples (isolated from milk or from cells), 400 ng of total RNA was used to prepare cDNA using the qScript microRNA cDNA Synthesis Kit (Quanta). After cDNA synthesis, an equivalent of 2.4 ng of the original RNA sample was mixed with Perfecta SYBR Green SuperMix (Quanta) and Universal PCR Primer (Qauanta) in 15 μL qPCR reactions. Three cDNA samples were run in adjacent wells of each 96-well qPCR plate. The qPCR plates were run using the StepOnePlus Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using a two-step cycling protocol (95°C for 5 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s) and concluded by a melting curve. After the reactions C_t values were determined using fixed-threshold settings (C_t Threshold RNU6 = 2.2, miRNA-148a = 2.7, miRNA-206 = 0.9, miRNA-375 = 2.2, miRNAs-320 = 1.8, miRNA-146a = 1.3, and miRNA-146b = 2.6). The delta-delta C_t method was used to determine the relative amount of miRNAs.

2.10 Exosome isolation

Exosomes were isolated from the skim layer of the milk following a series of centrifugations and filtrations as described in the milk fractionation paragraph. Exosomes isolation used ExoQuick reagent according to the manufacturer's instructions. Briefly, 63 μL ExoQuick (System Biosciences) were added to 250 μL of the skim layer of the milk and the mix was incubated overnight at 4°C with no rotation. Then, two centrifugation steps were performed at 1500 × *g* for 30 and 5 min, respectively, to sediment the exosomes and the pellet was re-suspended in 200 μL of PBS. We analyze the expression of two known proteins that characterized exosomes: CD81 and CD9 (Supporting Information Fig. 5). Exosomes isolated from human milk were lysed in RIPA buffer and analyzed for total CD81 and CD9 protein expression. Lysate was separated by SDS-PAGE and transferred onto a PVDF membrane. The membranes were probed with antibodies, and detected using enhanced chemiluminescence detection. Primary antibodies were as follows: anti-CD81 (1:200;Abcam); and anti-CD9 (1:200;Santa Cruz Biotechnology). Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit (1:5000; Zymed Laboratories).

2.11 RNA labeling

RNA derived from exosomes and the fat layer of the milk were labeled using Exo-Glow Exosome Labeling Kits. ***Exosomes***: We added 20μL of 10 X Exo-Red to a 200 μL volume of a re-suspended exosome suspension in PBS. The mixture was mixed well by flicking/inversion and incubated for 10 min at 37°C. To stop the labeling reaction we added 40 μL of the ExoQuick-TC reagent to the labeled exosome sample suspension and mixed by inverting six times. The labeled

exosome samples were incubated on ice for 30 min. Then, the samples were centrifuged 12 000 × *g* for 3 min, to sediment the exosomes and the pellet was re-suspended in 200 μL of PBS. ***Fat layer:*** 50 μL of the fat layer were re-suspended in 150 μL of PBS and 20 μL of10X Exo-Red were added. The mixture was mixed well by flicking/inversion and incubated for 10 min at 37°C. The labeled fat layer was centrifuged at 6500 × *g* for 5 min at 4°C. The PBS was discarded and the fat layer was washed twice and re-suspended in PBS and centrifuged at 6500 × *g* for 5 min at 4°C to discard the labeling solution. The labeled exosomes and fat layer were added to the target cells in culture and incubated with them.

2.12 Quantitation of mRNA by qRT-PCR

Complementary cDNA for the quantitation of mRNA was generated using the high capacity RNA-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Total RNA isolated from cells (1mg) was used to generate cDNA. The cDNA was subjected to qPCR. The mRNA levels of DNMT1 were performed by qRT-PCR with Fast SYYBR Green master mix (Applied Biosystems) using the StepOne Plus Real-Time PCR System (Applied Biosystems) machine. Primers: *DNMT1*: For: CCATCAGGCATTCTACCA Rev: CGTTCTCCTTGTCTTCTCT *β -actin* For: AGAAAATCTGGCACCACACC; Rev: AGAGGCGTACAGGGATAGC. The PCR reaction steps were 1 cycle at 95°C for 5 min, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The delta-delta C_t method was used to determine the relative amount of miRNAs.

2.13 Cells culture

The cell line FCH (CRL 1831; American Type Culture Collection), was derived from primary cultures of normal colon epithelial cells. The cells were grown in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 1:1 with 2.5 mM L-glutamine, 25 mM HEPES supplemented with 10% fetal calf serum (FCS), 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 100U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator (37°C, 5% CO_2). LS123 (American Type Culture Collection) colonic cancer cells were grown in Eagle's Minimum Essential Medium supplemented with 10% FCS, 100U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator (37°C, 5% CO_2). The K562 cells were cultured in RPMI1640 medium supplemented with 10% FCS, 100U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator (37°C, 5% CO_2).

3 Results

3.1 Expression of miRNA in bovine and goat milk

To determine the expression profiles of miRNA in the fat and skim fraction of bovine and goat milk, before and after pasteurization we utilized Next Generation Sequencing **(NGS)**. Small RNAs including mature miRNA were sequenced in the fat and skim fractions of the milk samples using Ilumina small RNA next generation sequencing. The number of reads of small RNA with length 17–25 for each sample is shown in the supplementary data (Supporting Information Table 1). The NGS results indicated a high similarity between the miRNA expressed in human, bovine and goat milks: 95% of the miRNAs expressed in bovine milk were also expressed in goat milk and 91% of the miRNAs expressed in goat milk were also expressed in bovine milk (Fig. 1A). The miRNA expressed in the fat and skim layers of pasteurized and non- pasteurized goat and bovine milk were similar (Fig. 1B and 1C). Furthermore, pasteurization did not affect the profile expression of miRNA in either the bovine or goat milk, as shown in Fig. 1D and 1E. Rather, the ten most highly expressed miRNA were found in the different layers of both the bovine and goat milk (Fig. 2A–D). For example

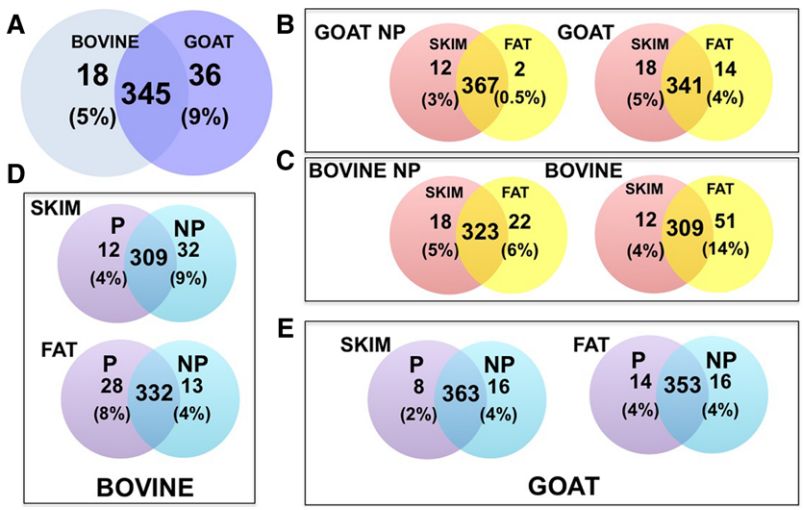


Figure 1. MicroRNA expressed in cow and goat milk. MicroRNA expressed in cow milk compared to goat milk. MicroRNA expression was performed by Next Generation Sequencing in RNA isolated from cow and goat milk. Venn diagram depicting miRNAs expressed in cow-goat milk (A). miRNA expressed in the fat layer of the milk versus the skim milk of pasteurized (P) and unpasteurized (NP) goat and cow milk. Venn diagram depicting miRNAs expressed in fat and skim layer of goat (B) and cow milk (C). miRNA expressed in the skim and fat layer of pasteurized (P) vs. unpasteurized (NP) cow and goat milk. Venn diagram depicting miRNAs expressed in fat and skim layer of pasteurized and unpasteurized cow (D) and goat (E) milk.

A cow milk not pasteurized

SKIM MILK		FAT	
miRNA	%	miRNA	%
miR-148a-3p	23.26	miR-148a-3p	60.53
miR-6073	20.40	miR-30a-5p	7.72
miR-200c	5.96	miR-21-5p	7.11
miR-200b	4.11	miR-6073	2.48
miR-99a-5p	3.91	miR-26a-5p	1.54
miR-30a-5p	3.43	let-7f-5p	1.37
miR-30d	3.24	let-7g-5p	1.21
miR-320-3p	3.23	miR-99a-5p	1.19
let-7a-5p	2.74	miR-200a	0.98
miR-26a-5p	2.53	miR-22-3p	0.93

B cow milk pasteurized

SKIM MILK		FAT	
miRNA	%	miRNA	%
miR-148a-3p	16.09	miR-6073	48.95
miR-6073	12.69	miR-148a-3p	7.16
miR-200c	6.52	miR-30a-5p	5.15
miR-3135b	4.62	miR-21-5p	4.27
miR-30d	3.88	chi-miR-200c	2.74
let-7b-5p	3.86	miR-320-3p	2.58
let-7a-5p	3.73	let-7b-5p	2.39
miR-200b	3.44	miR-200b	2.26
miR-4492	3.35	miR-26a-5p	1.70
miR-718	3.06	miR-1246	1.62

C goat milk not pasteurized

SKIM MILK		FAT	
miRNA	%	miRNA	%
miR-148a-3p	43.21	miR-148a-3p	60.95
miR-6073	6.14	miR-30a-5p	5.00
miR-200b	3.75	miR-26a	3.46
miR-200c	3.60	miR-21-5p	3.09
miR-30a-5p	3.45	let-7g-5p	1.89
miR-21-5p	2.96	miR-22-3p	1.35
miR-26a-5p	2.44	miR-378-3p	1.28
miR-30d	2.29	miR-146b-5p	1.25
miR-378-3p	1.85	miR-200a	1.24
miR-146b-5p	1.71	miR-30d	1.18

D goat milk pasteurized

SKIM MILK		FAT	
miRNA	%	miRNA	%
miR-148a-3p	23.96	miR-148a-3p	28.08
miR-6073	17.06	miR-6073	8.62
miR-200c	5.09	miR-21-5p	6.68
miR-30d	5.03	miR-26a-5p	4.52
miR-1246	4.06	miR-30a-5p	3.50
miR-200b	3.77	let-7a-5p	3.10
miR-375	3.59	miR-200c	2.78
miR-30a-5p	3.31	let-7f-5p	2.53
miR-320-3p	2.61	miR-200b	2.41
miR-146b-5p	2.57	let-7b-5p	2.16

Figure 2. Highly expressed miRNA in cow and goat milk. The ten highly expressed miRNA in cow and goat milk. MicroRNA expression was performed by Next Generation Sequencing in RNA isolated from the skim and fat fraction of pasteurized and unpasteurized cow (A, B) and goat milk (C, D). Abundance profile of miRNA in percentages (%) for each miRNA, from the total miRNA reads.

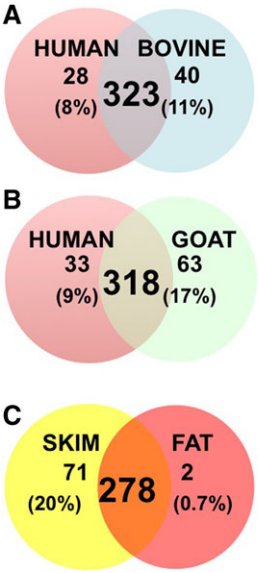
miR-148a-3p was highly expressed in the fat and skim fractions of goat and bovine milk before and after pasteurization (Fig. 2A–D and Supporting Information Fig. 2). The number of reads and expression levels of all the detected miRNAs in the fat and skim fractions of bovine and goat milk are shown in the Supporting Data (2).

3.2 Expression of miRNA in breast milk

To obtain the expression profiles of miRNA in the fat and skim fractions of breast milk, NGS analysis was performed as described above. Small RNAs including mature miRNAs were sequenced in the fat and skim fractions from breast milk. The total reads for each sample are shown in the supplementary data (Supporting Information Table 1). A comparison of miRNA expression in human and other mammalian milks indicated that 91–92% of the miRNAs expressed in human milk was also expressed in bovine and goat milk, 89% of the miRNAs expressed in bovine milk was also expressed in human milk and 83% of the miRNA expressed in goat milk was also expressed in human milk (Fig. 3A and B). The miRNA expression in the fat and skim layers of human milk were similar (Fig. 3C). Moreover, the most highly expressed miRNA were similar in the human, bovine and goat fractions of the milk. (Fig. 2 and 3D). MiR-148a-3p, one of the most highly expressed miRNAs in the fat and skim layers of goat and bovine milk before and after pasteurization (Fig. 2) was also highly expressed in breast milk (Fig. 3D). The expression of miR-148a-3p in the skim and fat layers of milk of 13 mothers (Supporting Information Table 3) indicated that this miRNA was highly expressed in both layers compared to the other milk miRNAs (Fig. 4A and B). The expression of miR-148a-3p was similar in the two layers of the milk (Fig. 4C). MiR148a-3p is considered to have been conserved throughout evolution (Fig. 4D) as are the other highly expressed miRNA in milk (Supporting Information Fig. 4). The number of reads and expression levels of all the detected miRNAs in the fat and skim fractions of human milk are shown in the Supporting Data (2).

3.3 Expression of miRNA in milk-based infant formulas

We analyzed the expression of miR-148a-3p in three different commercial milk-based infant formulas described in materials and methods section, compared to two human milk samples, one with high and one with low expression of mir-148-3p. Total RNA was isolated from the fat (**F**) and skim fractions



D human milk

SKIM MILK

miRNA	%
miR-148a-3p	37.59
miR-6073	11.77
miR-30a-5p	5.92
miR-99a-5p	3.68
miR-146b-5p	3.51
miR-200a	3.10
miR-21-5p	2.77
miR-30d	1.72
let-7b-5p	1.56
let-7a-5p	1.30

FAT

miRNA	%
miR-6073	30.22
miR-148a-3p	7.59
miR-320-3p	4.48
miR-378-3p	4.33
miR-146b-5p	3.60
miR-22-3p	3.54
miR-99a-5p	3.52
let-7b-5p	3.21
miR-30a-5p	2.76
miR-184	2.01

Figure 3. MicroRNA expressed in human milk. Expression of miRNA in human milk compared to other mammalian milks. MicroRNA expression was performed by Next Generation Sequencing in RNA isolated from human, cow and goat milk. Venn diagram depicting miRNAs expressed in human-cow milk (A), and human-goat milk (B). miRNA expressed in the fat vs. the skim layers of human milk. Venn diagram depicting miRNAs expressed in the fat and skim layers of human milk (C). The ten highly expressed miRNA in human milk. Abundance profile of miRNA in percentages (%) for each miRNA, from the total miRNA reads by NGS (D).

(W). The expression of miR-148a-3p, one of the most highly expressed miRNAs in milk was significantly lower in the infant formula than the miRNA level in human milk (Fig. 5).

3.4 Milk derived miRNAs are able to enter normal and cancer cells and change their miRNA expression profile

Milk-derived miRNAs are crucial because they are thought to enter target cells and regulate the expression profile of miRNA and hence gene expression. The miRNAs found in the skim fraction of breast milk are encapsulated by milk exosomes. Isolated labeled exosomes from breast milk were incubated with CRL 1831 cells (human normal intestine cell line), K562 (leukemia cells) and Lim 1215 (colon cancer cells). All the normal and cancer cells visualized by fluorescent microscopy were positively labeled, which indicates that the exosomes and their RNA content were taken into the cells (Fig. 6A). The RNA content of the lipid fraction of the milk was also labeled and then incubated with CRL 1831 cells. Cells incubated with the fat layer of the milk visualized by fluorescent microscopy were positively labeled, which indicates that the RNA content of the fat layer was also taken into the cells (Fig. 6C). The RNA content from the skim and fat layers from the human milk entered the cells and the expression of miR-148a-3p was found to be up- regulated compared to control cells (Fig. 6B, 6D).

3.5 Milk-derived miRNAs enter the cells and change their mRNA expression profile

MiRNAs regulate gene expression post-transcriptionally by either inhibiting protein translation or degrading target mRNAs. For example, DNA-methyltransferase 1 (DNMT1) [37] is a target gene of miR-148a. The expression of DNTM1, a target gene of miR-148a, was found to be down- regulated in cells with up- regulation of miR-148a after incubation with human milk-derived miRNA from the skim and fat layers (Fig. 7).

4 Discussion

In recent years several studies have reported the existence of miRNA in human breast milk as well as in other mammalian milks such as bovine [8] porcine [21] and goat [22] milk. The current notion is that milk-derived miRNAs could be transferred from mother to infant and regulates gene expression in target tissues and cells [31, 35]. Based on the studies in milk exosomes and their miRNA functions the notion is that milk derived miRNAs exert systemic functions in the milk recipient: the infant [38, 39]. We show in this study as previously was found that the highly expressed miRNAs in milk are mainly immune- related miRNAs such as miR-146a, miR-200 and miR-30 [19]. Some miRNAs that exist in milk may be involved in various physiological functions including regulation of cell growth and differentiation [20], and could influence the development and maturation of the infants gut and other organs. There are experimental proof that milk exosomes are taken up by intestinal epithelial [40] cells and promote intestinal cell growth and proliferation of intestinal epithelial cells [31]. In addition, we found that miRNA-148a is highly expressed in human milk. Micro RNA-148a have been shown to exhibit tumor suppressor activity by regulating the expression of genes such as DNMT1 [37], DNMT3 [41], ERBB3 [42] and ROCK1 [43], which are involved in tumor development, proliferation and metastasis. Highly expressed miRNAs in milk such as miR-148a and others may be able to protect the infant from the development of cancer. Micro RNA-148a was found to be less expressed in leukemia [44, 45]. Therefore we speculated that miRNAs such as miR-148a in breast milk might protect against childhood leukemia [3]. Whereas milk miRNA appears support the immune system of the infant and may have other beneficial effects on childhood health persistent abuse of this signaling system may promote diseases such as cancer [46]. For example miRNA-21-5p one of the top miRNA expressed in cow milk (Fig. 2B) is a well-known "oncomiR", which questions its "beneficial effect" for long-term intake [47]. However MiRNA-21 is an excellent driver of cell growth

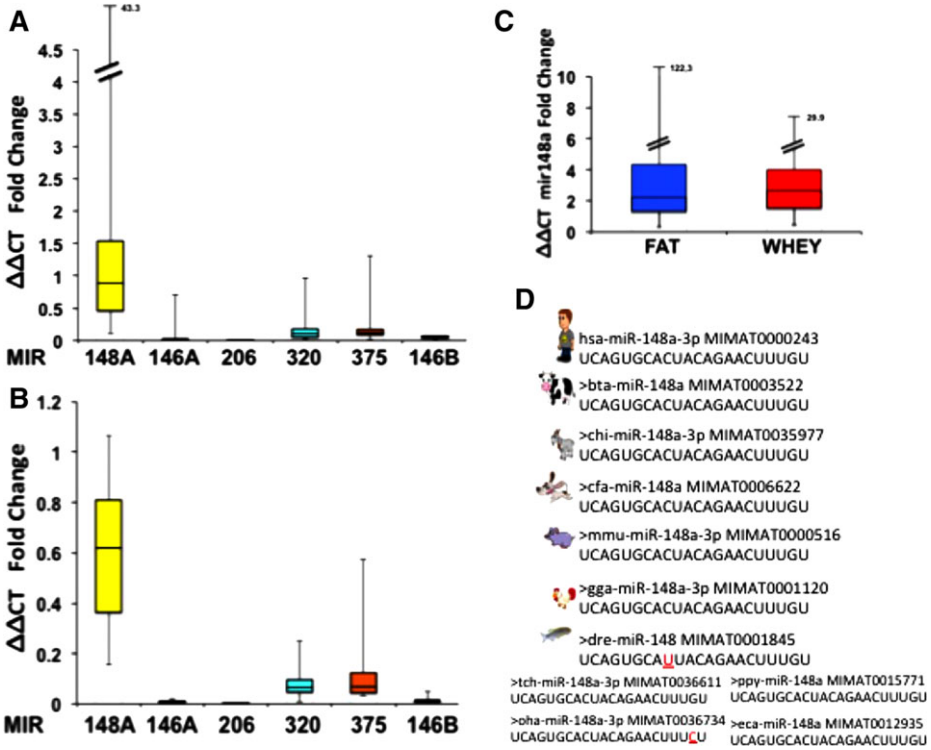


Figure 4. Expression of selected miRNAs in human milk. The total RNA from the skim and fat fraction of the milk of 13 different mothers at one month of lactation was isolated and the expression of miRNA-148a (148A), miRNA-146a (146A) miRNA-146b (146B), miRNA-206 (206), miRNA-375 (375) and miRNA-320 (320) were analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt) and normalized against RNU6B. The ΔΔCt values of miRNA in the fat fraction (A) and skim milk (B) are shown in box-and-whisker plots. The expression of miRNA-148a in the skim and fat fraction of the milk of 13 different mothers at one month of lactation was analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt) and normalized against RNU6B. The ΔΔCt value of miRNA-148a (mir148a) in each fraction is shown in a box-and-whisker plot (C). The sequence of miRNA-148a in different species (D).

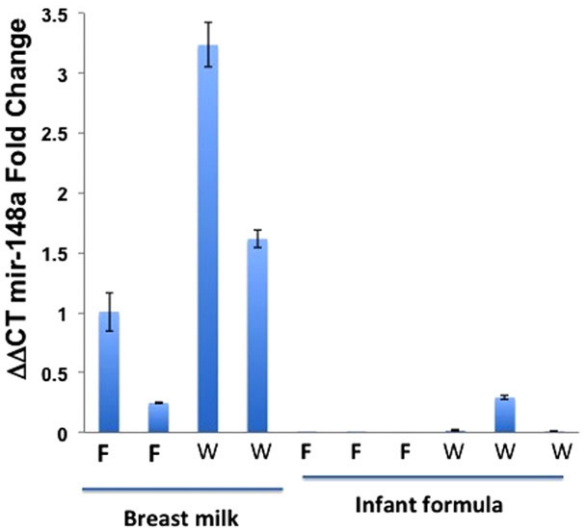


Figure 5. Expression of miRNA-148a-3p in the whey and fat fraction of human milk from term and infant formula. Total RNA from the skim (W) and fat fraction (F) of two samples of the breast milk and three different types of cow based infant formulas were isolated. Expression of miRNA-148a-3p (mir-148a) was analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT method (ΔΔCt), and values were normalized against RNU6B.

and proliferation, applied for postnatal growth of mammals [48].

Studies investigating milk-derived miRNAs have focused mainly on the skim and not the lipid fraction [18, 19, 34]. Several studies have demonstrated that miRNAs may also be present in the lipid fraction [38, 49, 50] of the milk and have higher quantities of miRNA than skim milk [38]. Based on these results we focused as well on the fat layer of the milk. One of the important findings of the current study is that highly expressed miRNAs in the fat layer of the milk are also highly expressed in the skim fraction. Nevertheless, in contrast to skim milk where miRNAs are protected from degradation by exosomes, it is unclear how miRNAs remain stable in the lipid fraction. One of our hypotheses is that miRNAs in the fat layer are protected and transported into the fat globules. Exosomes have been observed in cytoplasmic crescents of milk fat globules of human milk indicating that maybe milk fat globules transport exosomes carrying miRNA [51]. Another possibility is that miRNAs in milk are associated with RNA-binding proteins such as Agronaute-2 that protect them from degradation [52].

Since bovine and goat milk are widely used as dairy products and in particular bovine milk is the main component of most infant formulas, it is crucial to determine the levels of

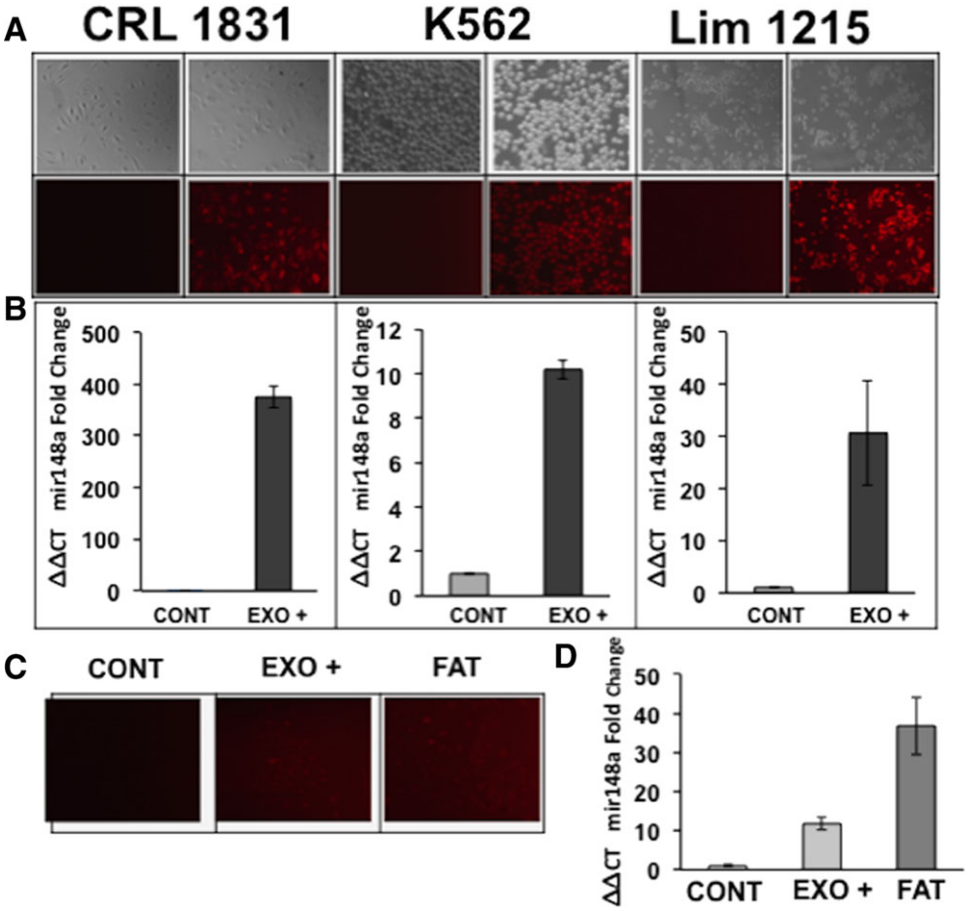


Figure 6. MicroRNA uptake by different types of cells. Labeled milk exosomes and the fat layer isolated from human milk were incubated with normal intestinal cells (CRL 1831), colon cancer cells (Lim 1215) and leukemia cells (K562). Images were obtained by fluorescent microscope analysis after incubation of the cells for 2 h with labeled milk exosomes (A) and fat layer (C). Expression of miRNA-148a (mir148a) in the different cells incubated with (EXO +) or without (CONT) exosomes was analyzed by qRT-PCR (B). Expression of miRNA-148a (mir148a) in CRL 1831 incubated with exosomes (EXO+), lipid fraction (FAT) or without (CONT) was analyzed by qRT-PCR (D). The qRT-PCR results were calculated by the Delta-Delta CT method (ΔΔCt), and values were normalized against RNU6B.

miRNA expression. Milk-derived miRNAs can be transferred from human but also from bovine milk to humans and regulate gene expression in target tissues and cells (Fig. 6 and 7) [35]. Several studies have aimed to determine the expression profile of miRNA in human and other types of milk [20–22]. However, these studies have examined the miRNA content in bovine [28] and human milk samples separately [19]. Here, we compared milk-derived miRNA expression from the skim and fat layer of the milk simultaneously. We found that highly expressed miRNAs are conserved in different types of milk (Fig. 1). The biological components of milk can differ across species; for example the molecular composition of lipid varies [53]. Interestingly, we found that the miRNAs that are highly expressed in milk are totally or strongly conserved across species. For example miR-148a-3p is conserved in mammalians throughout evolution (Fig. 4). Other highly expressed miRNAs in milk such as miR-320, 375, 99 and others are also conserved in different species (S4).

Previous studies have reported that the milk-derived miRNAs in the skim fraction are stable and resistant to degradation under harsh conditions such as freeze-thaw, prolonged room temperature incubation, RNA digestion and even boiling [19]. Most of the milk products consumed in developing countries require pasteurization to sterilize the milk. Here, we found similar miRNA profile expression in the skim and fat layers of pasteurized and unpasteurized bovine and goat milk. Thus pasteurizing milk appears to have a minor effect in the distribution of miRNA profile expression. Nevertheless we (Supporting Information Fig. 6) and others [25] found that pasteurized cow´s milk resulted in loss of miRNA-148a-3p. These finding may explain the allergy/asthma-preventive effect of unprocessed farm milk consumption in children [54]. Importantly, the MiRNA content in infant formula (Fig. 5) was significantly lower than that found in human milk as was also found by other groups [28]. Although the highly expressed miRNAs that we found in milk are also expressed in infant formulas, their expression is much lower than in human and mammalian milk. The low miRNA content in infant formulas can partially explain why infants fed with breast milk are better protected against infections than infants fed with formula [55].

Accumulating evidence in the last few years confirms that miRNAs are present in all food sources including milk. A number of studies have investigated whether food-derived miRNAs can survive the gastrointestinal tract and be absorbed in the intestine to influence gene expression in animals [56, 57]. Food specific miRNAs such as miR-168a ingested orally have been found to be present in the tissue and

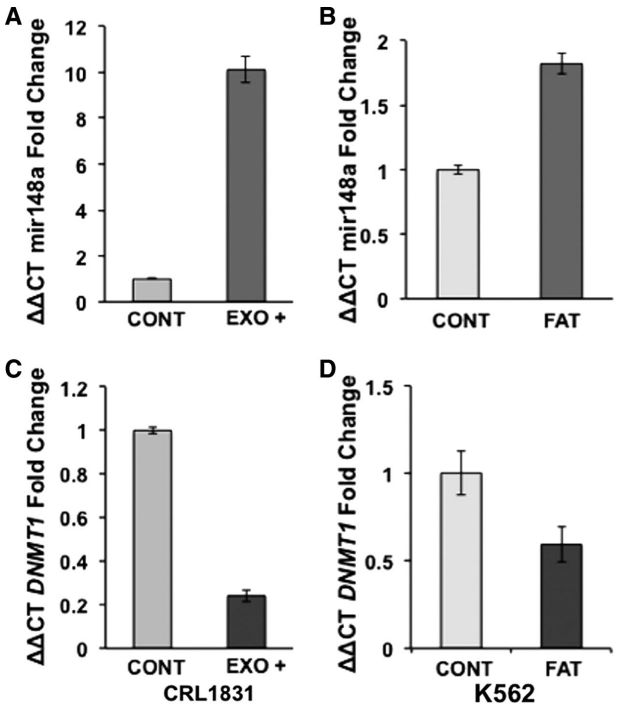


Figure 7. Expression of DNMT1 in cells incubated with the fat layer and exosomes isolated from human milk. Exosomes isolated from human milk were incubated with normal colon cells (CRL1831). Expression of miRNA-148a (mir148a) in cells incubated with (EXO+) or without (CONT) exosomes was analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt), and values were normalized against RNU6B (A). Expression of DNMT1 in cells described in (A) was analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt), and values were normalized against beta actin (C). The fat layer of human milk was incubated with K562 leukemia cells. Expression of miRNA-148a (mir148a) with (FAT) or without (CONT) fat was analyzed by qRT-PCR. The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt), and values were normalized against RNU6B (B). Expression of the DNMT1 in cells shown in (B) was analyzed by qRT-PCR.The qRT-PCR results were calculated by the Delta-Delta CT Method (ΔΔCt), and values were normalized against beta actin (D).

sera of different animals [56]. The transfer of milk- derived miRNAs in exosomes and other vesicles may help miRNAs survive in the gastrointestinal tract, and permit their subsequent uptake into the intestine and transfer to the bloodstream [35, 40]. MicroRNAs derived from bovine milk consumed by humans have been shown to be transferred to the plasma and have a regulatory effect on cellular biological functions [35]. Given that only milk exosomes has been investigated as a potential route to transfer milk miRNAs to infants or human adults, other ways such as fat globules or lipoprotein-nucleic acid interactions could also be involved in milk miRNA protection and transport. It was shown that extracellular miRNAs in body fluids could be delivered to target cells. For example, although it is known that HDL transports endogenous and exogenous miRNAs in plasma and delivers them to target cells [58], there have been no experimental studies up to date. It is also remains unclear whether miRNAs derived from the lipid fraction are able to enter the cells and regulate their biological functions. We showed here that human miRNAs present in the fat milk fraction are able to enter the cells and affect their biological functions. Previous works which demonstrated that exosomes and milk miRNAs enter cells have used exosomes and miRNAs derived from bovine [33, 35]. Here we examined the ability of miRNA derived from the skim and fat layer of human milk to enter normal and tumor cells. We showed that miRNAs present in either the exosomes or the fat layer can enter normal and tumor cells and can regulate cellular biological functions. For instance, following incubation of milk- derived human miRNA with normal and cancer cells the expression of DNMT1, a target gene of miR-148a [37], was down regulated in these cells and potentially induced DNA demethylation, which could regulate epigenetic changes in the cells.

Thus overall, human and mammalian milk contain beneficial miRNAs in the exosomes but also in the fat layer. These miRNAs are highly conserved in human, bovine and goat milk. Milk derived miRNAs can enter normal and malignant cells and can regulate biological signals. Our results reinforce previous findings on the importance of miRNA in milk. Further studies are needed to better understand the role of miRNAs in human and infant health.

R.G.-G. participated in the design of the study, supervised the study, carried out the experimental studies, performed the data analysis, contributed to discussions and wrote the manuscript. Y.E participated in the design of the study, carried out the experimental studies, performed the data analysis and contributed to discussions. V.L-M. participated in the design of the study, D.S. carried out part of the experimental studies. D.L. performed the bio-informatics data analysis. S.R participated in the design of the study, supervised the study, contributed to discussions, and wrote the manuscript.

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5 References

[1] Hoddinott, P., Tappin, D., Wright, C., Breast feeding. *BMJ* 2008, *336*, 881–887.

[2] Mayer-Davis, E. J., Dabelea, D., Lamichhane, A. P., D'Agostino, R. B, Jr. et al., Breast-feeding and type 2 diabetes in the youth of three ethnic groups: the SEARCh for

diabetes in youth case-control study. *Diabetes Care* 2008, *31*, 470–475 (2008).

[3] Amitay, E. L., Keinan-Boker, L., Breastfeeding and Childhood Leukemia Incidence: a Meta-analysis and Systematic Review. *JAMA Pediatr.* 2015, *169*, e151025.

[4] Gale, C., Logan, K. M., Santhakumaran, S., Parkinson, J. R. et al., Effect of breastfeeding compared with formula feeding on infant body composition: a systematic review and meta-analysis. *Am. J. Clin. Nutr.* 2012, *95*, 656–669.

[5] Verhasselt, V., Milcent, V., Cazareth, J., Kanda, A. et al., Breast milk–mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat. Med.* 2008, *14*, 170–175.

[6] Yamamoto, T., Tsubota, Y., Kodama, T., Kageyama-Yahara, N., et al., Oral tolerance induced by transfer of food antigens via breast milk of allergic mothers prevents offspring from developing allergic symptoms in a mouse food allergy model. *Clin. Dev. Immunol.* 2012, *2012*, 1–9.

[7] Hata, T., Murakami, K., Nakatani, H., Yamamoto, Y. et al. Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. *Biochem. Biophys. Res. Commun.* 2010, *396*, 528–533.

[8] Izumi, H., Kosaka, N., Shimizu, T., Sekine, K. et al., Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions. *J. Dairy Sci.* 2012, *95*, 4831–4841.

[9] Ambros, V., The functions of animal microRNAs. *Nature* 2004, *431*, 350–355.

[10] De Guire, V., Caron, M., Scott, N., Menard, C. et al., Designing small multiple-target artificial RNAs. *Nucleic Acids Res.* 2010, *38*, e140.

[11] Sylvestre, Y., De Guir, V., Querido, E., Mukhopadhyay, U. K. et al., An E2F/miR-20a autoregulatory feedback loop. *J. Biol. Chem.* 2007, *282*, 2135–2143.

[12] Stahlhut Espinosa, C. E., Slack, F. J., The role of microRNAs in cancer. *Yale J. Biol. Med.* 2006, *79*, 131–140.

[13] Eis, P. S., Tam, W., Sun, L., Chadburn, A. et al., Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc. Natl. Acad. Sci. U. S. A.* 2005, *102*, 3627–3632.

[14] Feng, J., Xing, W., Xie, L., Regulatory roles of microRNAs in diabetes. *Internat. J. Mol. Sci.* 2016, *17*, 1729.

[15] Laurent, L. C., MicroRNAs in embryonic stem cells and early embryonic development. *J. Cell. Mol. Med.* 2008, *12*, 2181–2188.

[16] Chen, X., Ba, Y., Ma, L., Cai, X. et al., Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 2008, *18*, 997–1006 (2008).

[17] Michael, A., Bajracharya, S. D., Yuen, P. S., Zhou, H. et al., Exosomes from human saliva as a source of microRNA biomarkers. *Oral Dis.* 2010, *16*, 34–38.

[18] Weber, J. A., Baxter, D. H., Zhang, S., Huang, D. Y. et al., The MicroRNA Spectrum in 12 Body Fluids. *Clin. Chem.* 2010, *56*, 1733–1741.

[19] Zhou, Q., Li, M., Wang, X., Li, Q. et al., Immune-related microRNAs are abundant in breast milk exosomes. *Int. J. Biol. Sci.* 2012, *8*, 118–123.

[20] Kosaka, N., Izumi, H., Sekine, K., Ochiya, T., microRNA as a new immune-regulatory agent in breast milk. *Silence* 2010, *1*, 7.

[21] Gu, Y., Li, M., Wang, T., Liang, Y. et al., Lactation-related microRNA expression profiles of porcine breast milk exosomes. *PLoS One* 2012, *7*, e43691.

[22] Na, R. S., E, G. X., Sun, W., Sun, X. W. et al., Expressional analysis of immune-related miRNAs in breast milk. *Genet. Mol. Res* 2015, *14*(3), 11371–11376.

[23] Admyre, C., Johansson, S. M., Qazi, K. R., Filen, J. J. et al., Exosomes with immune modulatory features are present in human breast milk. *J. Immunol.* 2007, *179*, 1969–1978.

[24] Admyre, C., Telemo, E., Almqvist, N., Lotvall, J., et al., Exosomes – nanovesicles with possible roles in allergic inflammation. *Allergy* 2008, *63*, 404–408.

[25] Kirchner, B., Pfaffl, M. W., Dumpler, J., von Mutius, E., et al., microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma. *J. Allergy Clin. Immunol.* 2016, *137*, 1893–1895.

[26] Melnik, B. C., John, S. M., Carrera-Bastos, P., Schmitz, G., Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation. *Clin. Transl. Allergy* 2016, *6*, 18.

[27] O'Neill, M. J., Bourre, L., Melgar, S., O'Driscoll, C. M., Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models. *Drug Discov. Today* 2011, *16*, 203–218.

[28] Chen, X., Gao, C., Li, H., Huang, L. et al., Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. *Cell Res.* 2010, *20*, 1128–1137.

[29] Pieters, B. C. H., Arntz, O. J., Bennink, M. B, Broeren, M. G. et al., Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-?? *PLoS One* 2015, *10*, e0121123.

[30] Benmoussa, A., Lee, C. H., Laffont, B., Savard, P. et al., Commercial dairy cow milk microRNAs resist digestion under simulated gastrointestinal tract conditions. *J. Nutr.* 2016, *146*, 2206–2215.

[31] Chen, T., Xie, M. Y., Sun, J. J., Ye, R. S. et al., Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. *Sci. Rep.* 2016, *6*, 33862.

[32] Lonnerdal, B., Du, X., Liao, Y., Li, J., Human milk exosomes resist digestion in vitro and are internalized by human intestinal cells. *FASEB J* 2015, *29*, 121–123.

[33] Wolf, T., Baier, S. R., Zempleni, J., The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma Caco-2 cells and rat small intestinal IEC-6 cells. *J. Nutr.* 2015, *145*, 2201–2206.

[34] Kusuma, R. J., Manca, S., Friemel, T., Sukreet, S. et al., Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis. *Am. J. Physiol. - Cell Physiol.* 2016, *310*, C800–C807.

[35] Baier, S. R., Nguyen, C., Xie, F., Wood, J. R., et al., Microrna are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, hek-293 kidney cell cultures, and mouse livers. *J. Nutr.* 2014, *144*, 1495–1500.

[36] Izumi, H., Tsuda, M., Sato, Y., Kosaka, N. et al., Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. *J. Dairy Sci.* 2015, *98*, 2920–2933.

[37] Long, X.-R. R., He, Y., Huang, C., Li, J., MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in hepatocellular carcinogenesis. *Int. J. Oncol.* 2014, *45*, 1915–1922.

[38] Alsaweed, M., Hartmann, P. E., Geddes, D. T., Kakulas, F., Micrornas in breastmilk and the lactating breast: potential immunoprotectors and developmental regulators for the infant and the mother. *Int. J. Environ. Res. Public Health* 2015, *12*, 13981–4020.

[39] Zempleni, J., Aguilar-Lozano, A., Sadri, M., Sukreet, S. et al., Biological activities of extracellular vesicles and their cargos from bovine and human milk in humans and implications for infants 1–3. *J. Nutr.* 2017, *147*, 3–10.

[40] Wolf, T., Baier, S. R., Zempleni, J., The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma caco-2 cells and rat small intestinal iec-6 cells. *J. Nutr.* 2015, *145*, 2201–2206.

[41] Duursma, A. M., Kedde, M., Schrier, M., le Sage, C., et al., miR-148 targets human DNMT3b protein coding region. *RNA* 2008, *14*, 872–877.

[42] Yu, J., Li, Q., Xu, Q., Liu, L. et al., MiR-148a inhibits angiogenesis by targeting ERBB3. *J. Biomed. Res.* 2011, *25*, 170–177.

[43] Li, J., Song, Y., Wang, Y., Luo, J. et al., MicroRNA-148a suppresses epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. *Mol. Cell. Biochem.* 2013, *380*, 277–282.

[44] Zhang, H., Luo, X. Q., Zhang, P., Huang, L. B. et al., MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. *PLoS One* 2009, *4*, e7826.

[45] de Oliveira, J. C., Brassesco, M. S., Scrideli, C. A., Tone, L. G., et al., MicroRNA expression and activity in pediatric acute lymphoblastic leukemia (ALL). *Pediatr. Blood Cancer* 2012, *59*, 599–604.

[46] Lu, W., Chen, H., Niu, Y., Wu, H. et al., Dairy products intake and cancer mortality risk: a meta-analysis of 11 population-based cohort studies. *Nutr. J.* 2016, *15*, 91.

[47] Melnik, B. C., John, S. M., Schmitz, G., Milk is not just food but most likely a genetic transfection system activating mTORC1 signaling for postnatal growth. *Nutr. J.* 2013, *12*, 103.

[48] Kumarswamy, R., Volkmann, I., Thum, T., Regulation and function of miRNA-21 in health and disease. *RNA Biol* 2011, *8*, 706–713.

[49] Alsaweed, M., Lai, C. T., Hartmann, P. E., Geddes, D. T. et al., Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. *Sci. Rep.* 2016, *6*, 20680.

[50] Munch, E. M., Harris, R. A., Mohammad, M., Benham, A. L. et al., Transcriptome profiling of microRNA by next-gen deep sequencing reveals known and novel miRNA species in the lipid fraction of human breast milk. *PLoS One* 2013, *8*, e50564.

[51] Gallier, S., Vocking, K., Post, J. A., Van De Heijning, B. et al., A novel infant milk formula concept: Mimicking the human milk fat globule structure. *Colloid. Surf. B Biointerf.* 2015, *136*, 329–339.

[52] Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K. et al., Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. USA* 2011, *108*, 5003–5008.

[53] Dreiucker, J., Vetter, W., Fatty acids patterns in camel, moose, cow and human milk as determined with GC/MS after silver ion solid phase extraction. *Food Chem.* 2011, *126*, 762–771.

[54] von Mutius, E., Maternal farm exposure/ingestion of unpasteurized cow's milk and allergic disease. *Curr. Opin. Gastroenterol.* 2012, *28*, 570–576.

[55] Melnik, B. C., John, S. M., Schmitz, G., Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? *J. Transl. Med.* 2014, *12*, 43.

[56] Zhang, L., Hou, D., Chen, X., Li, D. et al., Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res.* 2012, *22*, 107–126.

[57] Yang, J., Farmer, L. M., Agyekum, A. A. A., Hirschi, K. D., Detection of dietary plant-based small RNAs in animals. *Cell Res.* 2015, *25*, 517–520.

[58] Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. et al., Micrornas are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* 2011, *13*, 423.

