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MiRNA-320a is less expressed and miRNA-148a more expressed in preterm human milk compared to term human milk

Yaffa Elbaum Shiff[a], Shimon Reif[b,1], Ronella Marom[c], Kobi Shiff[d], Ram Reifen[e], Regina Golan-Gerstl[b,*]

[a] The School of Nutritional Sciences, Hebrew University of Jerusalem, Israel
[b] Department of Pediatrics, Hadassah Medical Center, Hebrew University, Jerusalem, Israel
[c] Neonatology Department Aviv Medical Center, Sackler Faculty, Tel Aviv University, Israel
[d] Maccabi Health Service, Israel
[e] School of Nutritional Sciences, Institute of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, Israel

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ABSTRACT

Objectives: To investigate whether there is a difference in the profile of miRNAs between human milk (HM) from mothers of preterm versus HM from mothers of full-term infants. Second goal is to assess biological functions or implication related to those differences in miRNAs expression.
Methods: Four of the highly expressed miRNAs in milk were detected by qRT-PCR. Milk derived exosomes were incubated with cells. The expression of miRNAs and target gene were detected by qRT-PCR.
Results: MiRNA-320 was more highly expressed in the colostrum of fullterm than in preterm HM. The expression of MiRNA-148 was higher in preterm mother's milk than of full-term colostrum. MiRNA-320 and MIRNA-148a expression were upregulated in cells incubated with milk exosomes, which lead to a decrease in their target genes FASN1 and DNMT1 respectivilly.
Conclusions: Alterations in miRNAs expression in HM can affect biologic function in infants and may serve as a nutritional therapeutic target.

1. Introduction

Human milk (HM) is the optimal source of nutrition for full-term infants. In addition, it is widely recognized that HM-fed infants gain significant health benefits compared to those fed with infant formulas (Slusser, 2007). These benefits include decreased risks for infections, sudden infant death syndrome, diabetes, cancer, and obesity (Lessen & Kavanagh, 2015). In addition, HM-fed infants achieve higher performance in neurocognitive behavior testing and higher intelligence scores (Lessen & Kavanagh, 2015). These effects are mediated by HM-specific regulatory factors including immunologic, growth and nutritional factors (Gartner et al., 2005; Lessen & Kavanagh, 2015).

Premature infants have different nutritional needs than full-term infants. Indeed, the macronutrient profile of HM varies with the time of delivery. These differences are adapted to the needs of preterm infants and are associated with improvement of their health outcomes, making HM even more important for preterm feeding (Carney et al., 2017). Indeed, artificial infant formulas lack some of the bioactive factors found in HM that have roles in the development and accelerated growth of preterm infants (Lessen & Kavanagh, 2015).

Recently, numerous studies have shown that human bodily fluids contain microRNAs (miRNAs), and, in fact, HM was found to have a high concentration of miRNAs in comparison to most other fluids (Alsaweed, Lai, Hartmann, Geddes, & Kakulas, 2016a; Weber et al., 2010; Zhou et al., 2011).

MiRNAs are small, noncoding RNAs (20–22 nucleotides) that regulate gene expression, control protein synthesis at the posttranscriptional level in eukaryotic cells and play important roles in a wide range of physiological and pathological processes (Zhou et al., 2011). Several previous studies, including some by our group, have demonstrated that miRNAs are present in exosomes, which are nanoparticles with a bilayer membrane (Admyre et al., 2007; Golan-Gerstl et al., 2017; Kusuma et al., 2016). In addition, exosomes were found in the milk lipid fraction (Golan-Gerstl et al., 2017; Munch et al., 2013).

* Corresponding author.
E-mail addresses: yaffa.elbaumshif@mail.huji.ac.il (Y.E. Shiff), shimon@hadassah.org.il (S. Reif), ronellam@tlvmc.gov.il (R. Marom), ram.reifen@mail.huji.ac.il (R. Reifen), reginag@hadassah.org.il (R. Golan-Gerstl).
[1] Equal contribution.

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Table 1
Clinical characteristics of the mothers and infants.

	Full term (n = 23)	Preterm (n = 15)
Maternal age (year)	32.09 ± 5.41	31.31 ± 5.81
Gestational age (week)*	39.82 ± 1.26	31.67 ± 2.82
Birth weight (grams)*	3435.65 ± 320.74	1637 ± 484
C-section (%)	23	62
Sex (% males)	64	80

* $p < 0.05$.

The aim of the present study was to investigate if the highly expressed miRNAs in HM differ between HM from mothers of preterm infants and HM from mothers of full-term infants. In addition, the miRNA patterns during different periods of lactation (colostrum, and 1 month) were studied in the skim and fat fractions of HM. Finally, we investigated the biological effects of miRNAs derived from the skim fraction of human milk on normal colonic cells and tumor cells.

2. Methods

2.1. Milk sample collection

Milk samples were collected from 38 healthy mothers from preterm (n = 15) and full-term infants (n-23) (Table 1) during the first 48 h after delivery and at 30 days postpartum. Milk samples were manually or electronic breast pumps expressed by participants into 15 ml sterilized, Polypropylene collection tubes (MIniplast, Ein Shemer, Israel). The samples were transported to the laboratory in container at 4 °C and stored at −80 °C pending further analysis.

2.2. Milk fractionation

The milk samples were fractionated by centrifugation at 6500*g* for 30 min at 4 °C. Two fractions were obtained from each sample: the fat and skim milk. The skim milk and the lipid layer were transferred separately to different tubes. 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 µm and 0.45 µm filters to remove residual debris. The skim and fat layers were subjected to quantitative real-time PCR (qRT-PCR).

2.3. Extraction of total RNA from milk fractions

Trizol 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 (linear polyacrylamide) (6 µl of 1:20 LPA to 1 ml of Trizol reagent) were added, and the mixture 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 Trizol reagent) was added to precipitate the RNA, and the solution was mixed by inversion.

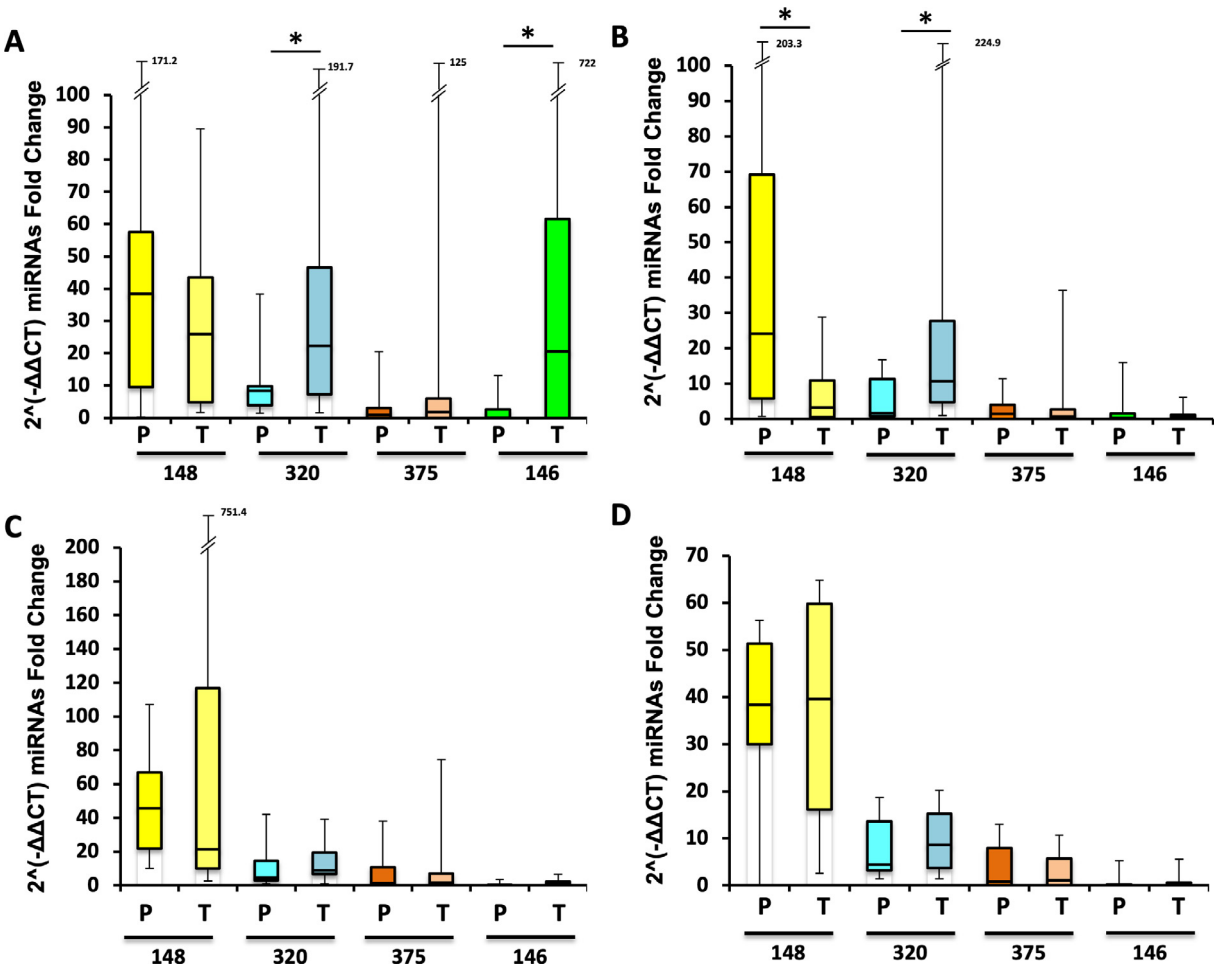

Fig. 1. Expression of selected miRNAs in the skim (A) and fat fractions (B) of colostrum and in the skim (C) and fat fractions (D) at one month of lactation from preterm (P) and full-term (T) breast milk. Total RNA from milk at 0 and 1 month of lactation was isolated, and the expression levels of miRNA-148 (148), miRNA-320 (320), miRNA-375 (375), and miRNA-146a (146a) were analyzed by qRT-PCR. The qRT-PCR results were calculated using the 2^(−ΔΔ CT) method, and the values were normalized to RNU6 (*$p < 0.05$). The 2^(−ΔΔ CT) values in the skim milk and fat fraction are shown in box-and-whisker plots.

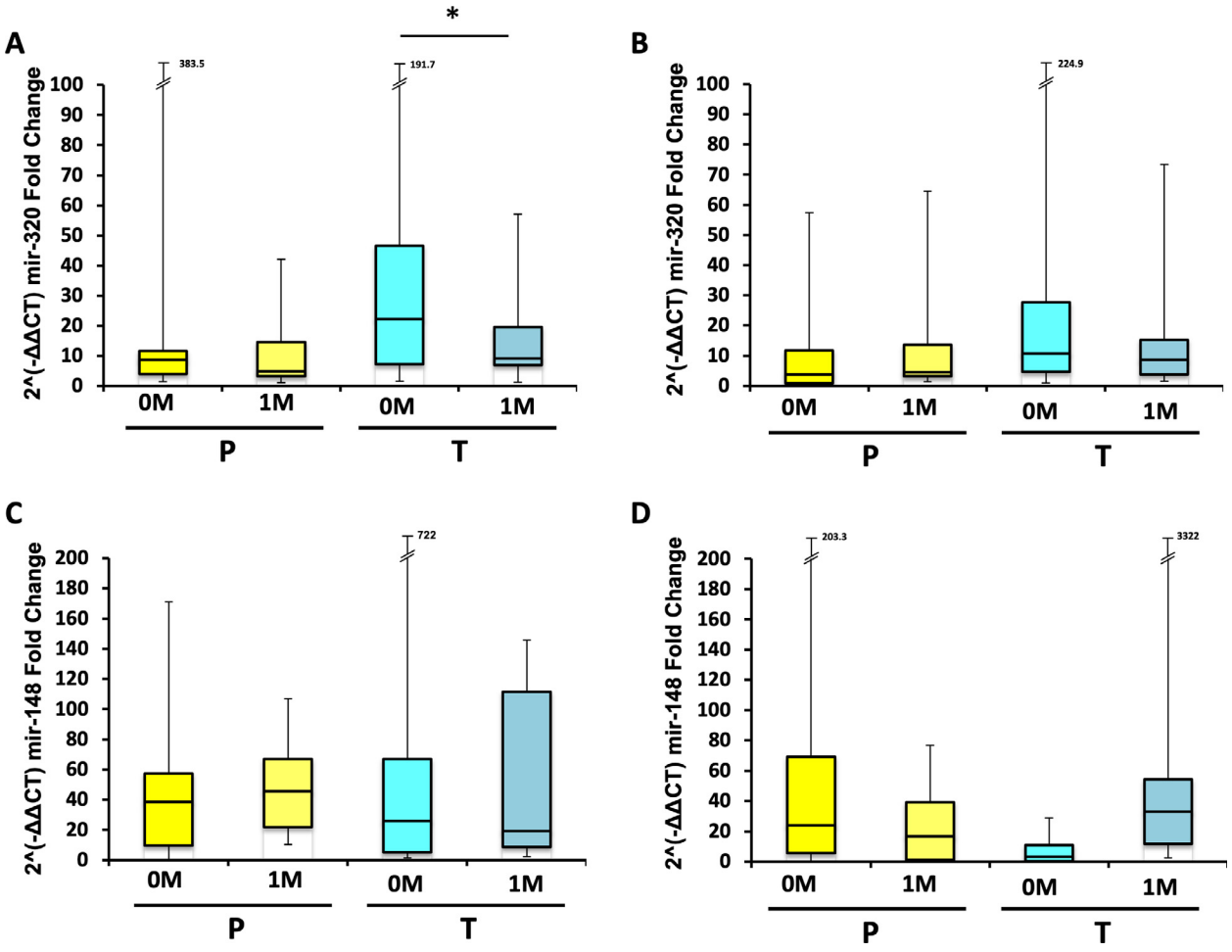

Fig. 2. Expression of miRNA-320 (mir-320) and miRNA-148a (mir-148) in the skim (A, C) and fat fraction of milk (B and D) from preterm (P) and full-term (T) breast milk. Total RNA from the skim fraction of the colostrum and milk and at one month of lactation of mothers from preterm and full-term infants was isolated, and the expression level of miRNA-320 and miRNA-148a were analyzed by qRT-PCR. The qRT-PCR results were calculated using the 2ˆ(−ΔΔ CT) method, and the values were normalized to RNU6 (*p < 0.05). The 2ˆ(−ΔΔ CT) values of the miRNAs in the skim and fat fractions are shown in box-and-whisker plots.

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, and the pellet washed with 75% ethanol (1 ml) and centrifuged at 12,000*g* for 5 min at 4 °C. The pellet was air dried and resuspended in RNase-free water (20 µl). RNA quantity and quality were 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 1.8 in nuclease-free water.

2.4. MicroRNA detection by qRT-PCR

For the total RNA samples, 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 (Quanta). The pre-designed and validated perfecta microRNA assay primer (Quanta) were used, for hsa-miR-320a: CTGGGTTGAGA GGGCGAAA, hsa-miR-148a-3p: CGCTCAGTGCACTACAGAACTTTT, hsa-miR-375: GTTCGGCTCGCGTGAAA, hsa-miR-146a-5p: CTCTGAAA TTCAGTTCTTCAGAAAA and RNU6 (NR_002752.1): GCAAATTCGTGA AGCGTTCC 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), concluding with a melting curve (The dissociation temperature range extends from 60 °C to 90 °C). After the reactions Ct values were determined using fixed-threshold settings (Ct Threshold RNU6 = 2.2, miRNA-148a = 2.7, miRNA-375 = 2.2, miRNA-320a = 1.8, miRNA-146a = 1.3). The delta-delta Ct method was used to determine the relative amount of miRNAs. The 2ˆ(−ΔΔ CT) method was used to determine the relative amounts of miRNAs.

2.5. Exosome isolation

Exosomes were isolated from the skim layer of the milk following a series of centrifugations and filtrations as described above in the "milk fractionation" paragraph. Exosome isolation was performed as described previously by us (14) with ExoQuick reagent according to the manufacturer's instructions. Briefly, 63 µl ExoQuick (System Biosciences) was added to 250 µl of the skim layer of the milk, and the mixture was incubated overnight at 4 °C with no rotation. Next, two centrifugation steps were performed at 1500*g* for 30 and 5 min to sediment the exosomes (pellet). Following isolation, exosomes were stored at −80 °C until they were added to the cells. The pellet was resuspended in 200 µl of phosphate-buffered saline (PBS).

2.6. Cell culture

LS123 (colonic cancer cells) and CCD 841 (normal colon epithelial cells) cells were grown in Eagle's Minimum Essential Medium supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL

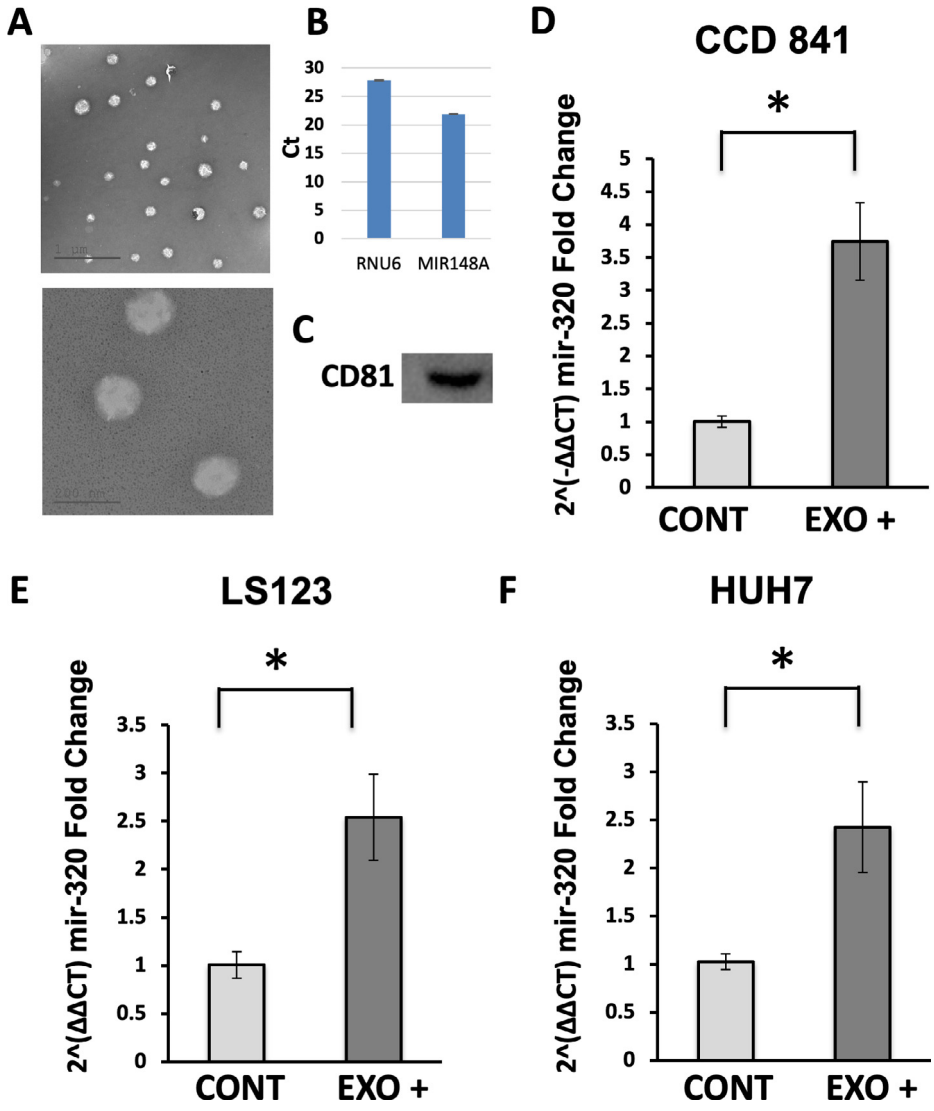

Fig. 3. Milk derived exosomes were analyzed by transmission electron microscopy using negative staining (A); miRNA-148a and RNU6 expression were analyzed by qRT-PCR (B); and protein expression of CD81 as a marker of milk derived exosomes (C). Human milk exosomes were incubated with CCD 841 (D) LS123 (E) and HUH7 (F) cells. The expression levels of miRNA-320 in the cells incubated with (EXO+) or without (CONT) exosomes were analyzed by qRT-PCR. The results were calculated using the 2ˆ(−ΔΔ CT) method, and the values were normalized to RNU6 ($^{*}p < 0.05$).

streptomycin in a humidified incubator (37 °C, 5% CO_2). HUH7 cells (hepatocarcinoma cells) were cultured in RPMI1640 medium 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, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator (37 °C, 5% CO2). The cells were cultivated for no more than 1 month.

2.7. Incubation of cells with exosomes

Milk-isolated exosomes resuspended with PBS were incubated with the 3 cell lines in starving medium (0% FCS) for 24 h. After 24 h, cells were washed with PBS and were detached from the plate using trypsin. The cells were collected and centrifuged at 1600 rpm for 5 min. The pellets were resuspended with 1 ml of Trizol reagent for total RNA isolation as describe above.

2.8. Quantitation of mRNA by qRT-PCR

Total RNA isolated from cells (1 mg) was used to generate cDNA. Complementary cDNA for the quantification of mRNA was generated using the high capacity RNA-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was subjected to qPCR. The mRNA levels of fatty acid synthase (FASN) were measured using qRT-PCR with Fast SYBR Green master mix (Applied Biosystems) using a StepOne Plus Real-Time PCR System (Applied Biosystems) machine. Primers: FASN: For: 5′ CTTCCGAGATTCCATCCTACG 3′ Rev: 5′ CTAT GAGGCCTATCTGGATGG 3′. *hGAPDH* For: 5′GATCATCAGCAATGCCT CCT 3′; Rev: 5′ TGTGGTCATGAGTCCTTCCA 3′. Primers were designed and unspecific amplification was avoided by using the In-Silico PCR (The UCSC Genome Browser).

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, concluding with a melting curve (The dissociation temperature range extends from 60 °C to 90 °C). The 2ˆ (−ΔΔ CT) method was used to determine the relative amounts of mRNAs.

Ethical approval information: This study was approved by the Investigational review board (IRB) of Hadassah-Hebrew University Hospital (HM0-0101-13). All mothers participating in the study signed an informed consent form approved by the IRB.

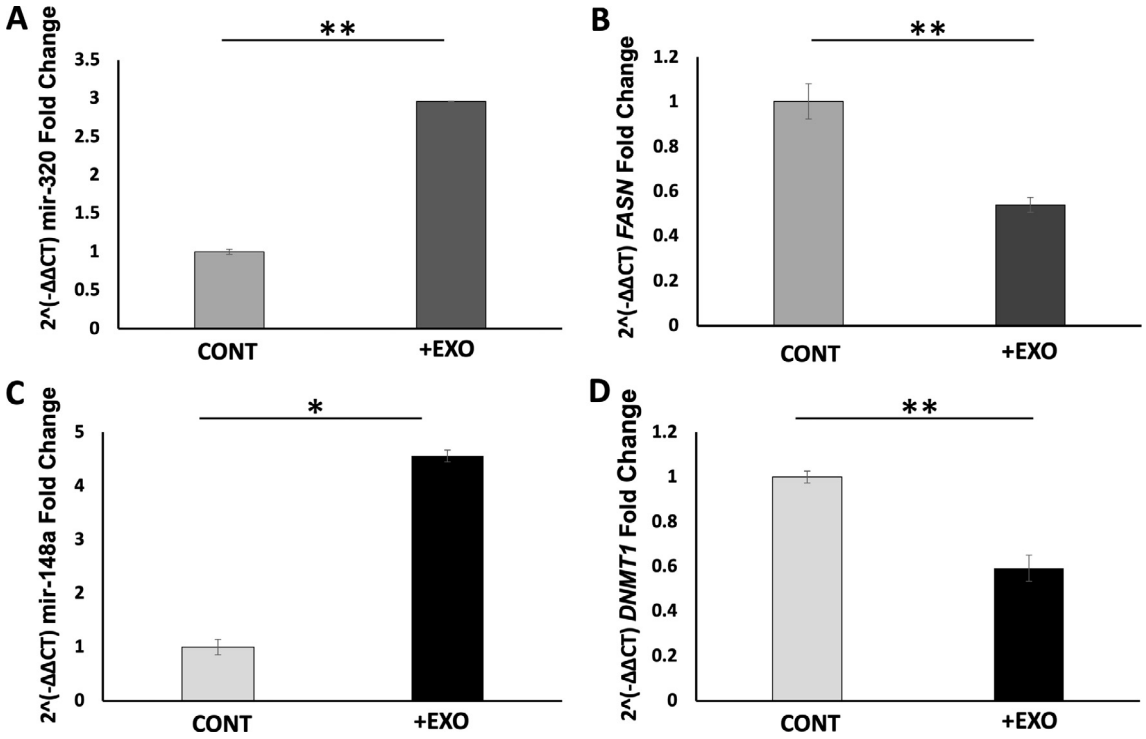

Fig. 4. Exosomes were incubated with CCD 841 cells, and the expression level of miRNA-320 (A) and miRNA-148a (C) were analyzed by qRT-PCR. The results were calculated using the 2^(−ΔΔ CT) method, and the values were normalized to RNU6. The expression level of FASN in the cells described in (A) and DNMT1 levels in cells described in (C) were analyzed by qRT-PCR. Results were calculated using the 2^(−ΔΔ CT) method, and the values were normalized to beta actin and GAPDH (B and D) ($^{**}p < 0.01$, $^{*}p < 0.05$).

2.9. Electron microscopy

Exosomes were analyzed by electron microscopy using negative staining. Isolated exosomes were stained with 2% phosphotungstic acid (PTA) in water. Briefly 5 µl of diluted exosomes in PBS were placed on Formvar/carbon coated copper 200 mesh grids (EMA) and mixed with 5 µl PTA for 10–20 sec. Excess stain was blotted off and the grids were dried. Samples were examined with a Jem-1400 *Plus* transmission electron microscope (Jeol, Peabody, MA, USA).

3. Results

MiRNA expression was analyzed in human milk from mothers of preterm infants born between 26 and 36 weeks of gestation and mothers of full-term infants within the first 48 h and at 30 days after birth. Maternal age and gender distribution did not differ between the two groups (Table 1).

Based on our previous study, in which we analyzed the expression profiles of miRNAs in different mammalian milk sources, we measured the expression of 4 of the highly expressed miRNAs in milk (Golan-Gerstl et al., 2017). We compared the expression of miRNA-148a-3p (mir-148a), miRNA-320-3p (mir-320), miRNA-375 and miRNA-146a in the skim and fat fractions of HM from mothers of preterm and full-term infants (Fig. 1). Mir-148a was the most highly expressed in the colostrum of preterm mothers, in the skim and fat fraction, and its expression was significantly higher than that of the other miRNAs: miRNA-320 ($p < 0.01$), miRNA-375 ($p < 0.01$) and miRNA-146a ($p < 0.01$) (Fig. 1A and B). In full-term colostrum miRNA-148 was only more abundantly expressed compared to miRNA-146 in the fat fraction ($p < 0.01$) and to mRNA-375 in the skim fraction ($p < 0.05$) (Fig. 1A and B). In contrast, in full-term HM, miRNA-320 was more abundantly expressed compared to miRNA-148 ($p \leq 0.05$), miRNA-375 ($p \leq 0.05$) and miRNA-146a ($p \leq 0.05$) in the fat fraction and to mRNA-375 in the skim fraction ($p < 0.05$).

At one month, MiRNA-148a was highly expressed in the milk of preterm (miRNA-320 ($p < 0.05$), miRNA-375 ($p < 0.05$) and miRNA-146a ($p < 0.05$)) and term (NS) mothers (Fig. 1C and D). These findings were observed in the skim milk as well as in the fat fraction. In addition, miRNA-148 was significantly higher expressed in preterm colostrum than in full-term colostrum ($p < 0.05$) (Fig. 1B) in the fat fraction, and not significant in the skim fraction (Fig. 1A). In contrast, we found that in colostrum, miRNA-320 was significantly more expressed in full-term milk than in preterm milk, both in the fat and skim milk fractions (Fig. 1A and 1B). This trend was also observed at 1 month of age, but the difference was not statistically significant (Fig. 1C and D). We found a persistent decline in miRNA-320 expression from colostrum to 1-month milk (Fig. 2).

MiRNA-320 expression in human of full-term mothers after one month was decreased compared to colostrum ($p < 0.05$ in the skim fraction and NS in the fat fraction) (Fig. 2A and B).

Due to the difference in miRNA-320 expression that we found between preterm and full-term infants, we further explored the biological role of miRNA-320 in human milk by incubation of cells with milk derived exosomes. The isolated vesicles from human milk were identifiable as exosomes based on electron microcopy examination (Fig. 3A), expression of miRNA (Fig. 3B) and proteins known as exosomes markers (Fig. 3C). Following incubation with human milk-derived exosomes resuspended in PBS, the expression of miRNA-320 was found to be upregulated more than 2-fold compared to that in the control cells in all three cell lines: CCD 841 (human normal intestine cells), LS123 (colon cancer cells), and HUH7 (hepatocarcinoma cells) (Fig. 3D–F).

Fatty acid synthase is a key enzyme in the metabolism of long-chain fatty acids, and it is a major target of miRNA-320. Incubation of CCD 841 cells with milk-derived exosomes increased mir320 expression by 3-fold, whereas it caused a 2-fold downregulation of FASN (Fig. 4A and B).

Due to the difference in miRNA-148 expression that we found

between preterm and full-term infants, we further explored the biological role of miRNA-148 in human milk by incubation of cells with milk derived exosomes. DNMT1 is a major target of miRNA-148a. Incubation of CCD841 cells with milk-derived exosomes increased mi148a expression by 4-fold, whereas it caused a 2-fold downregulation of DNMT1 (Fig. 4B and C). The results of incubation of cells with exosomes are representative of two different experiments.

4. Discussion

In recent years, HM as well as other mammalian milks, such as bovine and goat milk, were found to contain a wide spectrum of miRNAs (Golan-Gerstl et al., 2017; Izumi et al., 2012; Mobuchon et al., 2015; Weber et al., 2010). One speculation is that milk-derived miRNAs may be transferred from mother to infant to regulate gene expression in target tissues of the infant, thus providing beneficial biological effects, such as cell differentiation, tissue maturation, and enhancement of immunologic function (Alsaweed, Lai, Hartmann, Geddes, & Kakulas, 2016b). We and others have previously reported that miRNAs that are highly expressed in human and mammalian milk are largely immune-related miRNAs, such as miRNA-148a-3p, miRNA-146a, miRNA-200 and miRNA-30 (Golan-Gerstl et al., 2017; Zhou et al., 2011). We and others have also identified the highly expressed miRNAs in HM by next-generation sequencing (Alsaweed et al., 2016; Carney et al., 2017; Golan-Gerstl et al., 2017). Base on those results, we chose to compare the expression of 4 of the highly expressed miRNAs in milk, miRNA-148a-3p miRNA-320, miRNA-146a and miRNA-375, between preterm and full-term babies during different lactation stages. We found that miRNA-320 was less expressed in preterm milk, mainly in the colostrum, in comparison to full-term milk. This difference was observed in both the skim milk and fat fractions (Fig. 2). Carney et al. found that the expression of 9 miRNAs differ between preterm and full-term HM at 1 month postdelivery, including miRNA 378, miRNA 1260, miRNA 4783 (Carney et al., 2017). In contrast, we looked at two different times: colostrum and 1 month. We found significant changes in the expression level of miRNA-320 in the colostrum and at 1 month, but the differences were not statistically significant.

Another finding of this study is related to miRNA-148a-3p. MiRNA-148a-3p was the most highly expressed miRNA in preterm milk compared to the other miRNAs (mainly in colostrum) (Fig. 1), both in the skim and fat fractions in HM from mothers of preterm and at one month in preterm and term infants. Nevertheless, miRNA-148 was more highly expressed in preterm milk than in full-term milk (mainly in the colostrum). MiRNA-148a has been implicated in immune-related functions such as the transformation of B cells into plasma cells (Porstner et al., 2015). Furthermore, miRNA-148 has an important role in the regulation of adipocyte differentiation (Tian et al., 2017). These processes are crucial in preterm infants because their immune system and adipocyte numbers and functions are not fully developed. Moreover, miRNA-148a downregulated target genes involved in lipid metabolism and feeding control (WNT10B, CPT1A, PRKAA1, ABCA1 and others) (Melnik & Schmitz, 2017). DNA methyltransferase 1 (DNMT1) is a direct target gene of miRNA-148a (Pan et al., 2010), that modifies epigenetic regulation via demethylation of many genes involved in growth cell and metabolism. More expression of miRNA-148 in preterm human milk can regulate long term metabolic process based in the function of their target genes and epigenetic regulation via DNMT1.

MiRNAs, like other bioactive molecules in human milk, are important components of the innate immune system. Differences in cytokines, growth factors and lactoferrin between preterm and full-term milk are most dramatic in colostrum and early milk, and these differences last mostly up to 4 weeks after delivery. Therefore, we are not surprised that the main differences in miRNA expression levels in our study were also most pronounced in colostrum and for less than 1 month.

One main finding of this study is that miRNA-320 can enter into intestinal cells to regulate gene expression. MiRNA-320 has a role in adipogenesis and glucose metabolism and has been reported to have a protective effect against obesity and metabolic syndrome (Ling et al., 2009; Tang, Tang, & Özcan, 2008). MiRNAs play a role in adipocyte differentiation (Engin, 2017; Shi et al., 2015). Indeed, miRNA-320 was found to induce and regulate pancreatic islet development, β cell differentiation, insulin secretion and glucose and lipid metabolism (Ling et al., 2009; Tang et al., 2008). Prematurity is known to be associated with a higher risk of insulin resistance and metabolic syndrome during childhood and later life (Kajantie, Osmond, Barker, & Eriksson, 2010). Based on our study, we propose that the lower expression of miRNA-320 in the HM of mothers of preterm infants may somehow be related to the occurrence prematurity (Kajantie et al., 2010). In a systematic review, breastfeeding was associated with a range of short- and long-term health outcomes, including reduced risks for obesity and diabetes (Slusser, 2007). Other investigators reported a protective effect of breastfeeding against rapid weight gain and obesity risk in comparison to feeding with artificial formula (Gartner et al., 2005).

We propose that one of the possible mechanisms is via the beneficial miRNAs contained in HM, such as miRNA-320 and miRNA-148a.

One of the target genes of miRNA-320 is the enzyme fatty acid synthase. Fatty acid synthase is a key enzyme in the synthesis of long-chain saturated fatty acids. Indeed, two previous studies showed that inhibition of FASN using FASN inhibitors caused reduced food intake and weight loss in the treated animals. This effect was mediated by the regulation of energy balance (Loftus, 2000; Proulx, Cota, Woods, & Seeley, 2008). In our study, we indeed showed that the expression of FASN was down regulated in CCD 841 cells with a corresponding up-regulation of miRNA-320. We assume that the level of miRNA-320 in the colostrum of mothers of preterm infants is relatively low to enable fatty acids synthesis for the short-term needs of the rapidly growing preterm infant. However, this positive effect has a long-term deleterious function by influencing the long-term programing of obesity and diabetes. This possibility is consistent with our finding that infant formula contains much lower amounts of miRNAs (including miRNA-320 and miRNA-148) than HM (Golan-Gerstl et al., 2017).

5. Conclusions

In our view, different miRNA expression may potentially contribute to the discovery of a new aspect of the beneficial effects of human milk on gut maturation, immune system development, and the prevention diseases that threaten to devastate humanity, such as diabetes and obesity. Furthermore, the different expression of miRNAs in various stages of lactation may have implications on future recommendations for the duration of breastfeeding. Finally, studies such as the present one could form the basis for the addition of beneficial miRNAs detected in HM to artificial infant formulas, thereby enhancing the health of infants who are not fortunate enough to benefit directly from nature's best food.

Conflict of interest

No conflict of interests.

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