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RESEARCH PAPER

Detection of long non-coding RNAs in human breastmilk extracellular vesicles: Implications for early child development

Oskar Karlsson[a,b], Rodosthenis S. Rodosthenous[b], Calvin Jara[c], Kasey J. Brennan[b], Robert O. Wright[c,d], Andrea A. Baccarelli[b,*], and Rosalind J. Wright[c,d,*]

[a]Center for Molecular Medicine, Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden; [b]Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA; [c]Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA; [d]Kravis Children's Hospital, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA

ABSTRACT

Breastmilk has many documented beneficial effects on the developing human infant, but the components of breastmilk that influence these developmental pathways have not been fully elucidated. Increasing evidence suggests that non-coding RNAs encapsulated in extracellular vesicles (EVs) represent an important mechanism of communication between the mother and child. Long non-coding RNAs (lncRNAs) are of particular interest given their key role in gene expression and development. However, it is not known whether breastmilk EVs contain lncRNAs. We used qRT-PCR to determine whether EVs isolated from human breastmilk contain lncRNAs previously reported to be important for developmental processes. We detected 55 of the 87 screened lncRNAs in EVs from the 30 analyzed breastmilk samples, and CRNDE, DANCR, GAS5, SRA1 and ZFAS1 were detected in >90% of the samples. GAS5, SNHG8 and ZFAS1 levels were highly correlated (Spearman's rho > 0.9; $P < 0.0001$), which may indicate that the loading of these lncRNAs into breastmilk EVs is regulated by the same pathways. The detected lncRNAs are important epigenetic regulators involved in processes such as immune cell regulation and metabolism. They may target a repertoire of recipient cells in offspring and could be essential for child development and health. Further experimental and epidemiological studies are warranted to determine the impact of breastmilk EV-encapsulated lnRNAs in mother to child signaling.

Abbreviations: Ct, cycle threshold; EVs, extracellular vesicles; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; TEM, transmission electron microscopy

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Introduction

Breastmilk has many beneficial effects on the developing human infant[1,2], including decreased risk of sudden infant death syndrome,[3-5] reduced neonatal infections,[5-9] more optimal metabolic development throughout childhood,[10-13] reduced cancer risk,[5,14] and better cognitive outcomes.[15] In addition, some epidemiologic studies suggest that breastfeeding protects against asthma and allergies in offspring, especially when there is a family history for allergic rhinitis,[16] atopic allergies,[17] and asthma[5]; although the protective effect is contested in breastfed infants.[18,19] The specific components of breastmilk influencing developmental processes in infants are not fully elucidated.

Breastmilk contains a diverse mixture of components including nutrients, milk fat globules, hormones, growth factors, immune component cells, antibodies, cytokines, antimicrobial peptides, and extracellular vesicles (EVs) that may play a role in infant development.[1,20, 21] Increasing attention has been given to EVs, which are small double-lipid membrane vesicles that are released into the extracellular environment from a variety of cells, enabling cell-cell communications through specific interaction with target recipient cells.[22-24] EVs are found in a number of body fluids including blood, urine, saliva, and amniotic fluid, as well as breastmilk.[25] Over 4,400 different proteins, thought to be implicated in intracellular communication, have been identified in EVs.[24,26] In addition, EVs contain notable amounts of microRNAs (miRNAs), other small non-coding RNAs, and messenger RNAs (mRNAs).[26,27]

The RNA cargo in EVs does not simply reflect the RNA composition of the cell of origin, indicating a selective loading of RNAs into EVs.[28] Because of this, and the observation that transmitted RNAs can function in the recipient cell, it has been suggested that EV-encapsulated RNAs might act as gene expression regulators in the target cell.[27-30] There is emerging evidence showing that EV-encapsulated RNAs can regulate pathways related to cellular growth, division, survival, differentiation, stress responses, and apoptosis.[24] In comparison to other body fluids, breastmilk was recently found to contain the highest concentration of total RNAs.[31] Studies suggest that bovine and human milk transfer substantial amounts of miRNA of functional importance for infant development by EV transport,[32-34] and that immune-related

CONTACT Oskar Karlsson Oskar.Karlsson@ki.se
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*R.J.W. and A.B. contributed equally to this study.

miRNAs are enriched in breastmilk EVs.[35,36] This, together with studies demonstrating that the EVs lipid membrane helps to protect milk-derived RNAs against degradation, suggest an important function of EV-encapsulated RNA in cell-cell communication from mother to child.[34,37] Although EVs contain large amounts of RNAs, miRNAs and mRNAs have so far been the primary focus of research,[27,38,39] recent reports show that long non-coding RNAs (lncRNAs) could also be present in EVs.[39-42]

LncRNAs belong to a novel, heterogeneous class of non-coding RNAs, defined as transcribed RNA molecules greater than 200 nucleotides in length with little or no protein-coding capability.[43] They are often expressed at low levels and generally found to be more cell type specific than the expression of protein-coding genes.[44-46] Although lncRNAs are implicated in gene expression regulation during developmental and differentiation processes,[46-48] the exact mechanisms are not yet fully understood.[43,49-51] To date, only a few functional lncRNAs have been well characterized, and they have been demonstrated to control every level of gene regulation.[52] For example, lncRNAs are involved in post-transcriptional gene regulation through controlling processes like protein synthesis, RNA maturation and transport, and transcriptional gene silencing via regulation of the chromatin structure and DNA methylation.[49,53-55] However, it is currently unknown whether human breastmilk EVs contain lncRNAs.

The aim of this study was to determine whether EVs isolated from human breastmilk contain lncRNAs previously reported to be important for developmental processes, using a custom real time PCR array focused on 87 developmentally related lncRNAs. We consistently detected 5 of the 87 screened lncRNAs—CRNDE, DANCR GAS5, SRA1, and ZFAS1— in the breastmilk EVs of the 30 participating women, while several of the other measured lncRNAs were detected in some of the samples.

Results

Preparations of intact EVs isolated from breastmilk using the exoEasy Maxi Kit exhibited the characteristic morphology and diameter size under transmission electron microscopy (TEM), and were labeled positive for 2 common surface markers, CD63 and CD9 (Fig. 1A–B). Additional nanoparticle tracking analysis revealed a size distribution consistent with that of EVs with mean diameter size of ~100 nm and no contamination from particles with >500 nm diameter, such as apoptotic bodies and larger aggregates (Fig. 1C). Following total RNA extraction from the isolated EVs, the Bioanalyzer's electropherograms consistently demonstrated the presence of both short (20–35 nucleotides) and long RNAs (>200 nucleotides) and the absence of any cellular RNA contamination (Fig. 1D).

The real-time qRT-PCR analysis revealed the presence of 55 of the 87 screened lncRNAs in EVs from at least one of the analyzed individual breastmilk samples (n = 30). The expression heat map shows all detected lncRNAs in the individual samples (Fig. 2), and 11 lncRNAs—CRNDE, DANCR, GAS5, HOTAIRM1, NCBP2-AS2, OIP5-AS1, PRKCQ-AS1, SNHG8, SRA1, TUG1, and ZFAS1—were present in more than 50% of the samples. Of these, 5—CRNDE, DANCR, GAS5, SRA1, and ZFAS1—were detected in 90–100% of the breastmilk samples (Fig. 2). The cycle threshold (Ct) values ± standard deviation (SD) are shown in Supplemental Table 1. Other notable lncRNAs, such as JPX and NEAT1, were also detected, but in lower frequency (Fig. 2). Fig. 3 displays the expression correlations between the 11 most consistently detected lncRNAs. We found that GAS5, SNHG8, and ZFAS1 were highly correlated (Spearman's rho > 0.9; $P < 0.0001$), and that others such as CRNDE, DANCR, and SRA1 were relatively highly correlated (0.75 < Spearman's rho < 0.79; $P < 0.0001$). Overall, the technical variability was low, and the coefficient of variance (CV) for the 5 most consistently detected lncRNAs—CRNDE, DANCR, GAS5, SRA1, and ZFAS1—was between 0.65 and 1.53%.

Discussion

To the best of our knowledge, this is the first report demonstrating the presence of lncRNAs in EVs isolated from human breastmilk. This finding raises the exciting possibility that maternal EV-encapsulated lncRNAs might provide signals that regulate gene expression events in the developing infant. Notably, several of the detected lncRNAs have previously been related to diseases that are relevant to children's development and health including allergic disorders, asthma, obesity and autoimmune disease (Supplemental Table 2).

Essentially, all cells release EVs and it is currently unknown whether breastmilk EVs originate from cells present in the milk, epithelial mammary gland cells, or cells present elsewhere in the body. Previous studies have indicated a selective loading of RNA into EVs.[28] Therefore, the high correlation between the GAS5, SNHG8, and ZFAS1 levels may indicate that the packing of these lncRNAs into EVs is regulated by the same pathways. One advantage of EVs as mediators of intercellular communication is that the message can be targeted, through specific surface markers or adhesion molecules, to specific, multiple locations that might be widely separated locations within a tissue or between different tissues.[24,56, 57] In this regard, EVs are similar to the paracrine and endocrine systems of communication. It has been suggested that milk-derived EVs may pass the intestinal barrier and reach the systemic circulation.[34,37, 58] For example, a recent study has shown that humans absorb miRNAs from cow's milk that are sufficient to alter human gene expression in blood mononuclear cells.[59] Furthermore, studies in mice have demonstrated systemic uptake and distribution to various organs after intraperitoneal injection of labeled EVs.[60] The delivery of lncRNAs through breastmilk EVs could therefore allow for rapid alterations in gene expression in a repertoire of recipient cells and play an important role in infant development (Fig. 4). However, the present study is limited to breastmilk samples from 30 mothers. Further experimental and epidemiological studies are needed to determine the role of the EV-encapsulated lncRNAs in child growth and health.

Research indicates that the lncRNAs GAS5, SRA1, and CRNDE all have important functions in metabolic regulation. GAS5 is induced under conditions of nutrient deprivation and cellular stress, and has pro-apoptotic functions.[61-63] GAS5 acts as a glucocorticoid receptor (GR) decoy and increased levels can efficiently inhibit trans-activation of GR-dependent gene promoters.[61] In addition, GAS5 appears to repress the effects of other steroid hormone receptors and may have a role in saving energy resources as an adaptive response to starvation by restricting the expression of steroid-responsive genes.[61-63]

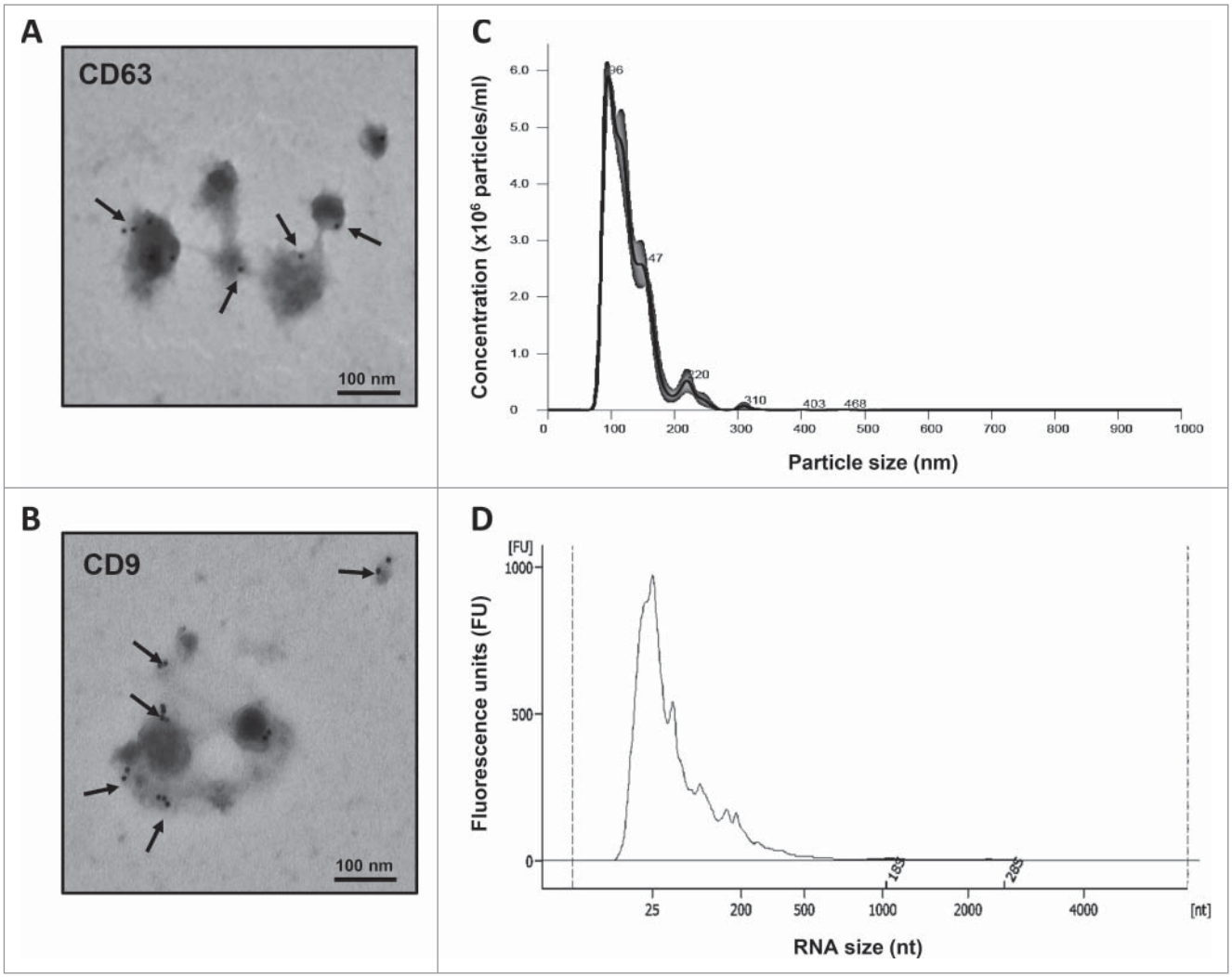

Figure 1. Morphological characterization of breastmilk EVs and size distribution of their RNA cargo. (A) EVs immune-gold labeled by anti-CD63 antibody. (B) EVs immune-gold labeled by anti-CD9 antibody. (C) Concentration and size distribution of EVs in breastmilk by nanoparticle tracking analysis using the NanoSight NS300. (D) Size distribution of EV-encapsulated RNA showing the presence of lncRNAs, as measured by Agilent 2100 Bioanalyzer. Transmission electron microscopy images for (A) and (B) were taken by a JEOL 1200EX microscope. Arrows indicate positive staining.

SRA1 serves as a co-activator for a number of nuclear steroid receptors.[64,65] Additionally, SRA1 is required for full transactivation of the pro-adipogenic transcription factor peroxisome proliferator-associated receptor gamma (PPARγ) and is therefore important for the differentiation of adipose tissue.[66] Microarray analysis reveals hundreds of SRA-responsive genes in adipocytes, including genes involved in the cell cycle, and insulin and TNFα signaling pathways.[66] CRNDE interacts with chromatin-modifying complexes and appears to play an important role in regulation of cell differentiation or pluripotency during development.[67-69] CRDNE is responsive to insulin and insulin-like growth factor signaling, and regulates genes central for cellular metabolism.[67] Hence, the transfer of these 3 lncRNAs, which were detected in EVs from almost all our breastmilk samples, as well as other lncRNAs, from the mother to the infant, could have important implications for the control of infant metabolism and metabolic programming.

Breastmilk also plays an important role in the development of the child's immune system.[70,71] A potential role for milk-derived EVs in immune modulation was first suggested by Admyre et al., who found human breastmilk-derived EVs to facilitate regulatory T-cell induction.[72] The immune modulatory features of EVs have been suggested to be due to their protein and miRNA contents.[36,72,73] Recent publications have shown widespread changes in the expression of lncRNAs during the activation of the innate immune response and T-cell development, differentiation, and activation.[74] Our detection of specific lncRNAs in human breastmilk EVs indicates that lncRNAs could also be important for the programming of the neonatal immune system. For instance, GAS5 plays an essential role in apoptosis and normal growth arrest in T-cells,[75] while ZFAS1 appears to have an important role in cell cycle control.[76] The growth control of lymphocyte populations is crucial to the physiological regulation of the immune system and to the prevention of both leukemia and autoimmune disease. SRA1 may also be important for the infant immune system as it regulates genes in the TNFα signaling pathway.[66] In addition, DANCR has been demonstrated to control the expression of IL6 and TNF-α in blood mononuclear cells.[77]

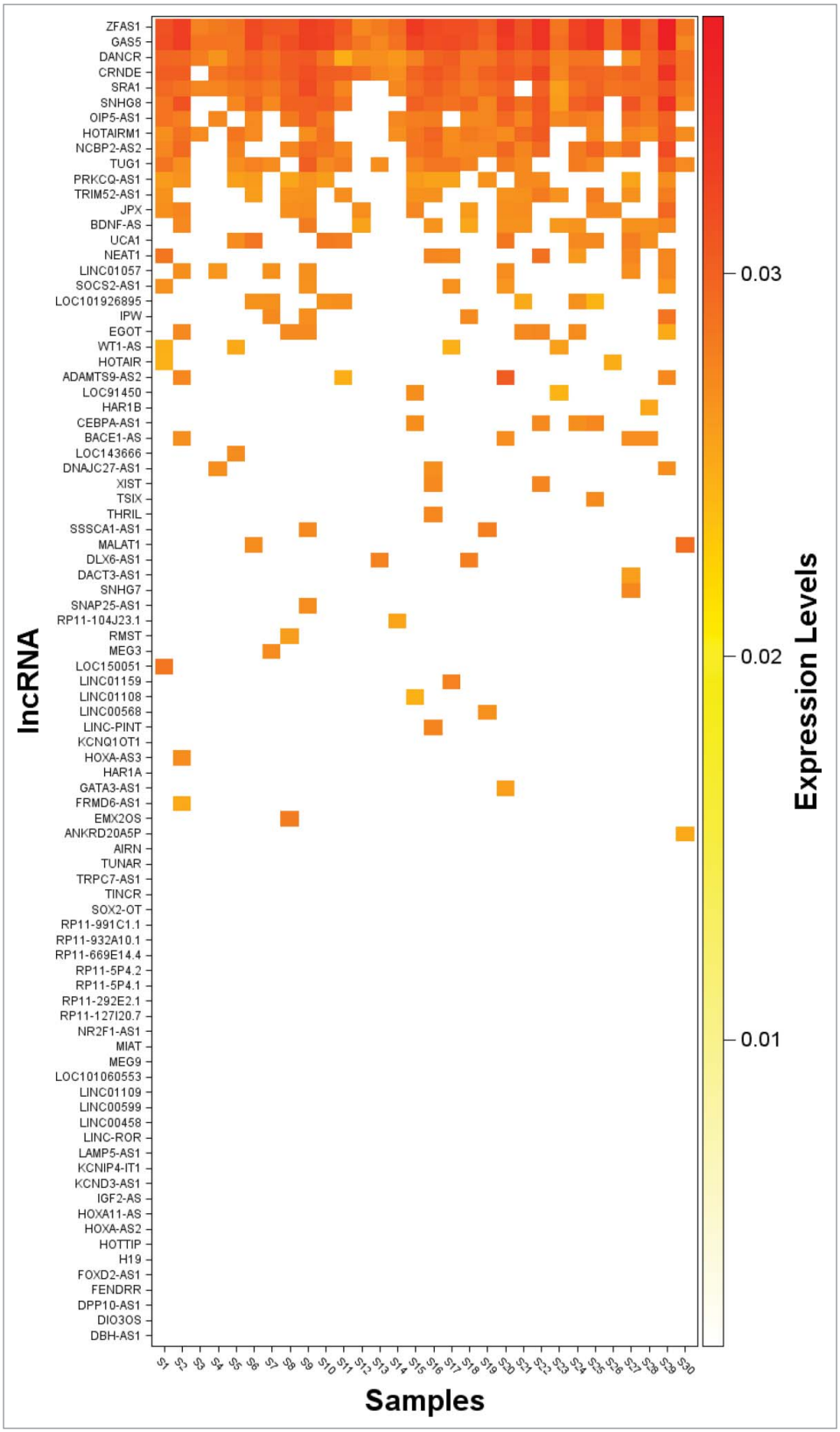

Figure 2. Expression heat map of 87 screened lncRNAs in extracellular vesicles isolated from human breastmilk samples (n = 30).

In summary, lncRNAs are important epigenetic regulators of physiological processes and their presence in human breastmilk EVs provide a novel mechanism to better understand communication between the mother and the child. Our findings may have important implications for early child development, as the identified lncRNAs are known to be involved in processes such as immune cell regulation, adipogenesis, and metabolism. Further experimental and epidemiological studies are warranted to determine factors that influence the lncRNA cargo in EVs, clarify functions

	CRNDE	DANCR	GAS5	HOTAIRM1	NCBP2-AS2	OIP5-AS1	PRKCQ-A1	SNHG8	SRA1	TUG1	ZFAS1
CRNDE	1,00	0,75	0,73	0,38	0,40	0,74	0,36	0,55	0,79	0,68	0,70
DANCR	0,75	1,00	0,59	0,14	0,39	0,61	0,28	0,35	0,77	0,60	0,57
GAS5	0,73	0,59	1,00	0,36	0,80	0,67	0,26	0,92	0,70	0,48	0,97
HOTAIRM1	0,38	0,14	0,36	1,00	0,21	0,19	0,38	0,36	0,25	-0,31	0,42
NCBP2-AS2	0,40	0,39	0,80	0,21	1,00	0,48	0,43	0,76	0,55	0,08	0,84
OIP5-AS1	0,74	0,61	0,67	0,19	0,48	1,00	0,07	0,54	0,71	0,65	0,63
PRKCQ-AS1	0,36	0,28	0,26	0,38	0,43	0,07	1,00	0,22	0,30	0,09	0,28
SNHG8	0,55	0,35	0,92	0,36	0,76	0,54	0,22	1,00	0,53	0,22	0,90
SRA1	0,79	0,77	0,70	0,25	0,55	0,71	0,30	0,53	1,00	0,58	0,70
TUG1	0,68	0,60	0,48	-0,31	0,08	0,65	0,09	0,22	0,58	1,00	0,47
ZFAS1	0,70	0,57	0,97	0,42	0,84	0,63	0,28	0,90	0,70	0,47	1,00

Figure 3. Correlation map (Spearman's Rank Correlation test) of lncRNAs in extracellular vesicles detected in ≥50 % of analyzed human breastmilk samples (n = 30).

and elucidate their impact on infant development and long-term health in children.

Materials and methods

Breastmilk collection

Participants were enrolled as part of the Perinatal Environment & Development Study (PEDS), a prospective pregnancy cohort designed to examine environmental influences on perinatal programming of cardiometabolic, respiratory, and neurodevelopmental outcomes in children. Breastmilk was collected from mothers (n = 30) within the first 2 months postpartum (32.0 ± 14.9 d postpartum, mean ± SD) during their visit at the prenatal clinic at the Mount Sinai Obstetrics & Gynecology Practice in New York City. Almost all the women were from ethnic minorities (16 Hispanic, 13 African American, 1 Caucasian), most had greater than a high school education (n = 20, 66.6%) and were aged 27.3 ± 6.0 y. Research procedures were approved by the human studies committee at the Icahn School of Medicine at Mount Sinai. Written informed consent was obtained from all participants,

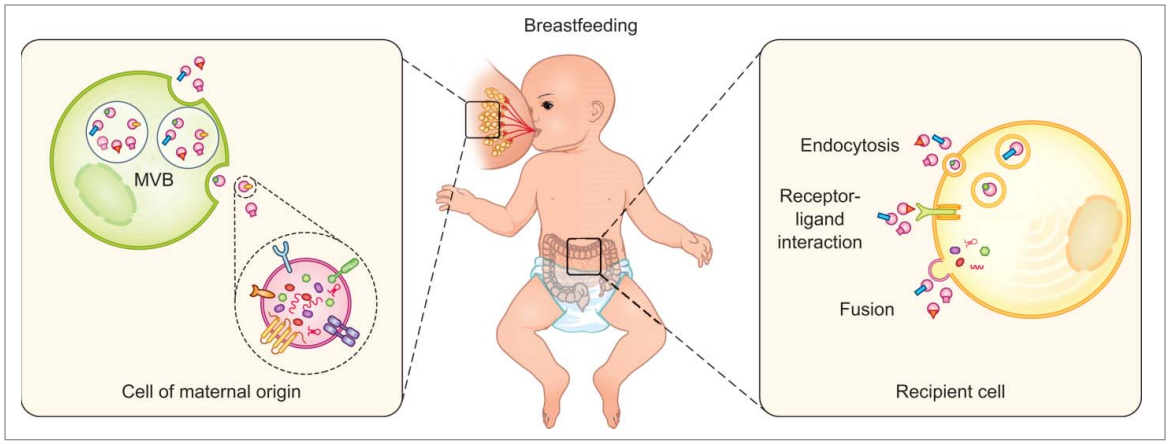

Figure 4. The role of breastmilk extracellular vesicle (EV) long non-coding RNA (lncRNA) in maternal-infant cell-cell communication: A conceptual model. [Left] Human breastmilk contains a mixture of diverse components, including EVs loaded with maternal lncRNAs. [Right] The EV-encapsulated lncRNAs, e.g. CRNDE, DANCR, GAS5, SRA1, and ZFAS1, which are involved in processes such as cell cycle control, apoptosis, immune cell regulation, steroid hormone signaling, adipogenesis, glucose, and lipid metabolism, can be delivered to the infant and have direct effects in the gastrointestinal tract. Absorption through intestinal epithelial cells is also thought to occur, allowing breastmilk-derived lncRNAs to reach various organs and a repertoire of cells via the systemic circulation of the infant and potentially perform functions, such as developmental programming and immunoprotection.

and the experiments were carried out in accordance with the approved guidelines.

For the collection of breastmilk, participants washed their breast and used a manual pump to collect 3–10 ml of breastmilk before the first morning feed from a single breast. Samples were kept at 4°C until transport to the laboratory the same day. Upon arrival, samples were centrifuged (3,000 × g at 4°C for 15 min) to remove fat globules and cells, and the supernatant was stored in 1 ml aliquots at −80°C until further analysis.

EVs isolation and RNA extraction

At time of assay, aliquots were thawed on ice and centrifuged a second time at 3,000 × g for 15 min at 4°C, to remove residual fat globules and any remaining cell debris. The supernatant was carefully aspirated, mixed with 2 volumes of filtered (0.1-μm membrane) phosphate-buffered saline (PBS), and then filtered using a 0.8-μm membrane unit (Millipore Corp., Bedford, MA) to remove any remaining cell debris and large aggregates. The exoRNeasy Serum/Plasma Maxi Kit (Qiagen, Valencia, CA) was then used to extract EV-encapsulated total RNA following the manufacturer's instructions.[78] To evaluate the performance of this kit with breastmilk, we used the exoEasy Maxi Kit (Qiagen, Valencia, CA), which utilizes the same EVs isolation principle, but enables the collection of intact EVs. Upon isolation of intact EVs from 2 replicate aliquots, we used the NanoSight NS300 (Malvern Instruments, Westborough, MA) to measure their size distribution and numbers, and TEM to characterize their morphology. To evaluate the quality, yield, and RNA size distribution of the total RNA extracted from breastmilk EVs, we analyzed all samples by capillary electrophoresis and the Agilent RNA 6000 Nano Kit following manufacturer's protocol (Agilent 2100 Bioanalyzer, Agilent Technologies, Foster City, CA).

Size distribution and concentration of EVs by nanoparticle tracking analysis

The size distribution and concentration of isolated EVs from breastmilk were analyzed using the NanoSight NS300. In brief, EV samples were diluted 1:1000 with PBS and the pump was washed several times with PBS prior to measurement. The settings for the camera were adjusted according to the manufacturer's instructions and samples were analyzed in 5 repeats for 60 seconds.

Transmission electron microscopy

To characterize the morphology of isolated EVs, we used TEM. For negative staining, samples were diluted in water, adsorbed onto formvar/carbon coated grid, and stained with 1% uranyl acetate. Last, samples were dried and immediately examined using TEM. For immune-gold labeling for CD63 and CD9 surface markers, we followed the protocol described by Théry et al.[79] A JEOL 1200EX microscope coupled with an AMT 2 k CCD camera was used for imaging, and all work was performed at the Harvard Medical School Electron Microscopy Core.

Real-time quantitative reverse transcription PCR

We measured the levels of EV-encapsulated lncRNAs using a custom 384-well RT^2 lncRNA PCR Array (Cat. no. CLAH00013; Qiagen). The panel, consisting of 87 lncRNAs, was based on the human cell development and differentiation RT^2 lncRNA PCR array (Cat. no. LAHS-003Z; Qiagen,) that includes 84 differentially expressed lncRNAs during cellular differentiation and organism development, with 3 additional developmentally important lncRNAs: THRIL, PINT, and SRA1. This array allows accurate analysis of the most relevant lncRNAs to cell fate and cell lineage decisions using laboratory-verified SYBR® Green qPCR assays, and includes a set of controls that enable assessment of genomic DNA contamination, reverse transcription performance, and PCR performance. RNA (8.5 ng) was reverse transcribed using the RT^2 First Strand Kit (Qiagen, Valencia, CA) according to the provided protocol that includes a genomic DNA elimination step to remove any residual contamination in the samples. Due to lower RNA extraction yield, we used reduced starting amounts for 3 samples (7.0, 8.2, 6.1 ng). The synthesized cDNA was mixed with RT^2 SYBR Green Mastermix, and automatically aliquoted to the wells of the lncRNA PCR plate using a Microlab STARlet Liquid Handling Workstation (Hamilton, Franklin, MA). Real-time qRT-PCR was performed on a Bio-Rad CFX384 instrument with an initial HotStartTaq DNA polymerase activation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C.

Data analysis

Standard descriptive statistics were used to quantify the expression levels of detected lncRNAs in human breastmilk EVs (reported as mean ± SD, min, and max Ct values). Spearman's Rank Correlation test was used to calculate the correlation between lncRNAs that were detected in ≥50 % of the analyzed samples. For the expression heat map, raw Ct values were transformed using the inverse formula 1/Ct and ranked from high to low, based on their transformed expression levels and frequency of detection among all analyzed samples. Statistical analyses were conducted in SAS 9.4 (SAS Institute Inc., Cary, NC).

Disclosure of potential conflicts of interest

The authors declare no conflict of interest or competing financial interests.

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