Review Article

Milk exosomal microRNAs: friend or foe?—a narrative review

Bodo C. Melnik[1], Ralf Weiskirchen[2], Gerd Schmitz[3]

[1]Department of Dermatology, Environmental Medicine and Health Theory, University of Osnabrück, Osnabrück, Germany; [2]Institute of Molecular Pathobiochemistry, Experimental Gene Therapy and Clinical Chemistry (IFMPEGKC), RWTH University Hospital Aachen, Aachen, Germany; [3]Institute for Clinical Chemistry and Laboratory Medicine, University Hospital of Regensburg, University of Regensburg, Regensburg, Germany

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Correspondence to: Bodo C. Melnik. Department of Dermatology, Environmental Medicine and Health Theory, University of Osnabrück, Am Finkenhügel 7a, D-49076 Osnabrück, Germany. Email: melnik@t-online.de.

Background and Objective: Milk exosomes (MEX) and their inherent microRNAs (miRs) were recently promoted as promising effectors and drug carrier systems for the treatment of various chronic human diseases. This review intends to provide a comprehensive view on the potential beneficial and adverse health effects of MEX miRs.

Methods: English literature published between 1990 and 2022 were searched using the PubMed database focusing on publications including human and bovine MEX, milk extracellular vesicles (EVs), miRs, and reported beneficial and adverse effects of MEX miRs.

Key Content and Findings: MEX are regarded as signalosomes produced under control of the lactation genome to support species-specific growth of the offspring during the lactation period. Physiologically, mammals are not exposed to MEX miRs after the lactation period. MEX miRs are important for epigenetic postnatal programming and tissue maturation. Direct and translational evidence indicates that MEX miRs are involved in p53-mediated transcription, DNA methyltransferase-regulated gene silencing as well as Polycomb repressive complex-mediated gene repression and chromatin remodeling. MEX miRs are deficient in artificial formula, but may accidentally modify human gene expression by consumption of pasteurized cow milk. The continuous impact of bovine MEX miRs on the human epigenome and epitranscriptome remains a matter of concern.

Conclusions: MEX miRs are supportive for the growing neonate, whereas continuous exposure of adult humans to bovine MEX miRs may promote obesity, diabetes, cancer and neurodegeneration. Bovine MEX miRs should be removed from the human food chain of adults. The efficacy and long-term safety of MEX miRs should be carefully assessed in future studies before bovine MEX could be introduced for therapeutic purposes.

Keywords: Cancer; diabetes; milk exosomal microRNAs (miRs); transcription; health-risk evaluation

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Introduction

Milk contains abundant large extracellular vesicles (EVs) (1-5) and small nano-sized EVs (30–150 nm) known as milk exosomes (MEX), that transfer microRNAs (miRs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), messenger RNAs (mRNAs) among others (6-22). MEX miRs are highly conserved between mammals (11,23), survive the harsh conditions of the gastrointestinal tract (24-26), are taken up by endocytosis (27,28), are bioavailable (29,30), reach the

Table 1 Potential therapeutic effects of MEX in experimental models

MEX source	Disease—pathological condition	Reference
Bovine	Amelioration of experimental arthritis	(40)
Bovine	Inhibition of catabolic and inflammatory processes in cartilage from osteoarthritis patients	(41)
Bovine	Osteoprotective effect by increasing osteocyte numbers and targeting RANKL/OPG system in experimental models of bone loss	(42)
Bovine	Induction of proliferation and differentiation of osteoblasts and osteogenesis	(43)
Porcine	Protection against intestinal epithelial cell damage	(44)
Porcine	Inhibition LPS-induced intestinal epithelial cell apoptosis	(45)
Bovine	Enhanced goblet cell activity and prevention of the development of experimental necrotizing enterocolitis	(46)
Bovine	Attenuation of experimental ulcerative colitis	(47)
Bovine	Attenuation of experimental colitis	(48)
Human, bovine	Attenuation of experimental colitis	(49)
Bovine	Amelioration of cardiac fibrosis	(50)
Bovine	Potential attenuation of hepatic fibrosis	(51)
Bovine	Regeneration and acceleration of cutaneous wound healing	(52)
Bovine	Modulation of scar-free wound healing	(53)
Bovine	Acceleration of angiogenesis and diabetic wound healing	(54)

MEX, milk exosome; LPS, lipopolysaccharide.

systemic circulation (29,31) and enter cells and peripheral tissues (30,32-36).

Due to the possibility of high yield preparations of bovine MEX (37-39) and their ability to overcome tissue boundaries (30,32-35), MEX are regarded as an ideal nanoplatform for the transfer of either intrinsic miRs (40-55) or as carrier systems for drug or anti-sense RNA transfer to target tissues (34,56-61) (*Table 1*).

Despite these opportunities, the question remains: Is the transfer of MEX miRs beneficial or do they exert adverse health effects? To answer this question, the original biological purpose of MEX miRs and their physiological functions during the breastfeeding period have to be examined in more detail.

Methods

Literature research was performed using the PubMed database between 1990 and 2022 selecting original research papers published in English language including the search items: human and bovine milk exosome, milk extracellular vesicle, microRNA (miRNA, miR), gene expression, gene regulation, beneficial health effects, adverse health effects. Original data of reported biological effects of miRs were extracted and provided in the corresponding disease section. Literature data of human and bovine MEX-derived miRs were inspected and related to their potential health effects (*Table 2*).

Outcome of literature research

Physiological versus artificial MEX miR signaling

The perception of milk as a food for infants (62) has changed to a complex maternal-neonatal signaling system promoting postnatal growth and appropriate metabolic, adipogenic, immunological and neuronal programming of the infant (12,63-72). MEX represent maternal signalosomes relaying gene-regulatory communication between the maternal lactation genome and the infant (69). They have an important impact on epigenetic postnatal programming (73,74) and early-life growth trajectories (75). Notably, miR-148a, the major miR of human and bovine MEX (7,23,76), exhibits an identical nucleotide sequence between *Homo sapiens*

Table 2 The search strategy summary

Items	Specification
Date of search	Jul 2022
Databases and other sources searched	PubMed
Search terms used	Milk exosome, extracellular vesicle, miR, gene regulation, gene expression, health effects
Timeframe	1990–2022
Inclusion and exclusion criteria	Inclusion: original English research articles; Exclusion: articles repeating published data
Selection process	First author conducted paper selection

and *Bos taurus* (11). MEX miRs modify gene expression of MEX-receiving cells and tissues (11,33,50,77-81), although the life period and the magnitude of their gene-regulatory impact is a matter of debate (30,82,83). During the postnatal period, MEX function as epigenetic regulators (82,84). MEX miR-148a targets DNA methyltransferase 1 (*DNMT1*) and p53 (*TP53*), decreasing their expression (11,77,84-88).

In all mammals except humans, MEX miR exposure is restricted to the breastfeeding period and declines after weaning. About 7,000 to 10,000 years ago, Neolithic humans started to exploit milk from other mammalian species (89), but consumed preferentially fermented milk (89,90), which compared to raw milk contains diminished quantities of bioactive MEX miRs (91). Bovine MEX miRs are conserved by pasteurization of cow milk (72–78 ℃, for >15 s), allowing their delivery into the human food chain (92,93).

To evaluate the risk-benefit relation of MEX miRs, five constellations of MEX miR exposure during human life have to be analyzed: (I) the impact of bovine MEX miR exposure during pregnancy and fetal development; (II) the physiological action of human MEX miRs during breastfeeding; (III) the absence of human MEX miRs by artificial formula feeding; (IV) the long-term influence of bovine MEX miRs on consumers of pasteurized cow milk; and (V) the action of intrinsic bovine MEX miRs and MEX miR transfer via drug-loaded MEX for the treatment of human diseases. It is important to be aware that the regulation of gene expression by miRs is highly complex, as a specific miR may regulate or repress the expression of hundreds of different mRNAs, which may act in concert and either stimulate (e.g., repression of an inhibitor) or inhibit (e.g., repression of an activator) a given cellular process.

Bovine MEX miR exposure during pregnancy

Consumption of unfermented milk during pregnancy is associated with fetal weight gain and higher birth weight (94-98), an indicator of fetal growth related to placental weight (99). In contrast to fermented milk with degraded MEX (93), raw and pasteurized milk deliver bioactive MEX including miR-21 and miR-148a (11,29,92,93,100,101). Following oral gavage of bovine MEX to mice, miR-21 and miR-30d accumulated in murine placenta and embryos (35). *MIRNA30D* knockout mice exhibit fetuses with smaller crown-rump length and fetal/placental weight ratio (102). Increased placental expression of miR-21 has been related with placental weight and fetal overgrowth (103,104). MEX miR-21 and miR-148a target phosphatase and tensin homolog (*PTEN*) and promote PI3K-AKT-mTORC1 signaling (77,105). Indeed, target gene prediction of human, bovine and porcine MEX miRs mainly concentrate on the PI3K-AKT-mTORC1 pathway (30,73,106). Increased trophoblast mTORC1 activity determines placental-fetal transfer of amino acids and glucose promoting fetal growth and birth weight (107-112). Thus, oral exposure of pregnant women to bovine MEX miRs may over-stimulate placental and fetal mTORC1 activation enhancing fetal growth.

Breastfeeding: the physiological MEX miR exposure of the infant

Intestinal maturation

Human, bovine and porcine MEX and their miRs improve intestinal maturation and proliferation (113-117), protect

Table 3 Anti-inflammatory targets of miR-148a

Target genes	Regulators of inflammatory responses	References
TLR4	Toll-like receptor 4	(133)
CAMK2A	Calcium/calmodulin-dependent protein kinase IIα	(134,135)
CHUK	Component of nuclear factor κ-B kinase complex	(136)
IKBKB	Inhibitor of nuclear factor κ-B kinase, subunit β	(136-138)
IL6ST	Interleukin 6 signal transducer	(139)

miR, microRNA.

against epithelial damage (44,118-120) stabilize intestinal barrier function (46,121-123), exert anti-inflammatory (47-49,69,124,125) and anti-oxidative activities (126,127), stimulate intestinal and systemic immunity (6,7,64,128-130), antimicrobial defense (46,131), and appropriate conditioning of the gut microbiota (131,132). Notably, miR-148a inhibits TLR4/NF-κB and IL-6/STAT3 signaling (51,69,133-139) (*Table 3*). Experimental colitis models confirm that MEX attenuate TLR4 expression and NF-κB activation (45,118). Thus, MEX are of key importance for postnatal intestinal development and suitable agents for the prevention and treatment of necrotizing enterocolitis (46-49,69,86,140,141).

Immune tolerance

MEX that entered the systemic circulation may stimulate thymic maturation inducing regulatory T-cells (Tregs) (64). Admyre *et al.* (6) showed a dose-dependent expression of Forkhead box P3 (FoxP3) in CD4+ T-cells after addition of human MEX. FoxP3 is the master transcription factor of regulatory T (Treg) cells. Its expression is controlled by a Treg-specific demethylation region (TSDR) (142,143). DNMT1 and DNMT3B are associated with the *FOXP3* locus in CD4+ T-cells. Whereas methylation of CpG residues represses FoxP3 expression, complete demethylation induces stable FoxP3 expression (144). Thus, MEX miR-148a-mediated suppression of DNMT1 and miR-29b-mediated suppression of DNMT3B may enhance Treg cell maturation that maintains immune tolerance, preventing allergy and autoimmunity (64,145,146).

Neuronal development

After oral gavage of bovine MEX to mice, MEX accumulated in the brain (32). In mice, MEX cross the blood-brain-barrier and promote dendritic complexity in the hippocampus, whereas dietary depletion of bovine MEX impaired sensorimotor gating and cognitive performance (147). In early postnatal life, developmental processes are critical for establishing proper neuronal connectivity. One protein functionally important for postnatal synaptic plasticity is α-synuclein (148-150). Hypomethylation of the *SNCA* promoter increases α-synuclein expression, which is regulated by DNMT1 (151). MEX miR-148a-mediated suppression of DNMT1 in neuronal cells may thus promote α-synuclein expression enhancing neuronal connectivity and cognitive function.

Adipose tissue development

Preterm infants receive high amounts of MEX miR-148a and miR-22 (115). Oxytocin, which is released during birth and lactation (152), induces the expression of MEX miR-148a and miR-30 in colostrum (153). Intriguingly, maternal obesity is negatively associated with the content of MEX miR-148a, miR-29a, miR-20b, miR-30b and miR-32 in human milk after one month of lactation (154). MEX miR-148a was negatively associated with infant weight, fat mass, and fat free mass, while miR-30b was positively associated with infant weight, percent body fat, and fat mass at 1 month (154). In mothers with gestational diabetes, levels of MEX miR-148a, miR-30b, let-7a and let-7d were also reduced (155). MEX miR-148a was negatively associated with infant weight, percentage of body fat, and fat mass, whereas MEX miR-30b was positively associated with infant weight and fat mass at 1 month of age. MEX miR-148a was negatively associated with infant weight at 6 months of age (155). Unfortunately, both studies did not differentiate between white adipose tissue (WAT), beige (BET) and brown adipose tissue (BAT), which is a critical limitation because MEX miRs may influence both WAT and BET/BAT development (69). Overexpression of miR-30b/c induces the expression of thermogenic genes such as uncoupling protein 1 (UCP1) in beige/brown adipocytes (156). MiR-30b/c target *NRIP1*, the gene

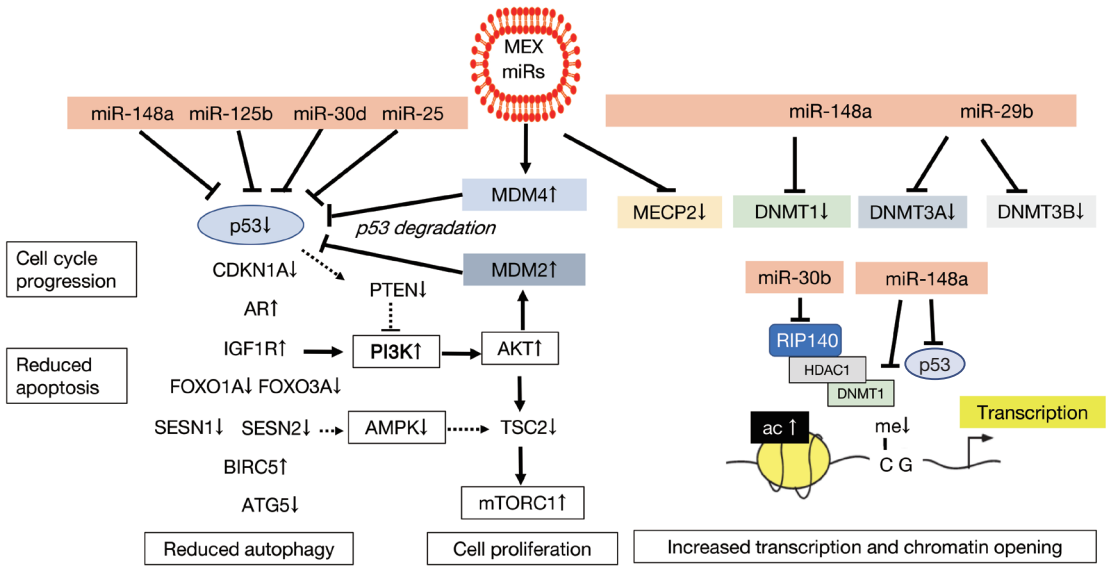

Figure 1 MEX-derived miRs attenuate the expression of p53, DNMT1, DNMT3A and DNMT3B. p53 suppression enhances cell cycle progression, growth factor signaling stimulating mTORC1-dependent cell proliferation, but reduces apoptosis and autophagy. MiR-148a-mediated suppression of p53 and DNMT1 and miR-30b-mediated suppression of RIP140 reduce HDAC activity opening chromatin structure enhancing transcription. MEX, milk exosome; miRs, microRNAs; DNMT1, DNA methyltransferase 1; HDAC, histone deacetylase.

encoding receptor-interacting protein 140 (RIP140) (156). RIP140 is essential for DNA and histone methylation to maintain gene repression and promotes the assembly of DNA and histone methyltransferases on the *UCP1* enhancer (157). RIP140 directly interacts with DNMT1, DNMT3A, and DNMT3B and recruits H3K27me3 for the repression of UCP1. H3K27me3, which contains tri-methylation of lysine 27 on histone H3 protein, is associated with downregulation of nearby genes via the formation of heterochromatic regions. RIP140 represses the "brown-in-white" adipocyte program (158). MEX miR-30b/c-mediated suppression of RIP140 (156), MEX miR-148a-mediated suppression of DNMT1 (11,77), MEX miR-29b-mediated suppression of DNMT3A and DNMT3B (159,160) may thus increase BAT. Among the top 50 miRs abundantly expressed in bovine whey MEX is miR-489 (161), which also targets *NRIP1* (162). Apparently, MEX miRs coordinate thermogenic adipose tissue development, which is important for the infant's energy homeostasis.

Transcriptional activity and chromatin remodeling

The transcription factor p53, the guardian of the genome (163,164), interacts with approximately 1/10th of human gene promoters (165). p53 modifies the expression of target genes involved in cell cycle control (*CDKN1A*) (166), growth factor signaling (*AR*, *IGF1R*), translation and metabolism (*PTEN*, MDM2, mTORC1) (166-168), autophagy (*ATG5*, *BECN1*) (169,170) and apoptosis (*FOXO1A*, *FOXO3A*, *TNFRSF10B*, *BIRC5*) (166,171-173). Importantly, p53 is a direct target of human MEX miR-148a (86). Another highly conserved suppressor of p53 is miR-125b (174), which has been detected in human (7), bovine (24,175) and porcine MEX (9). Further negative regulators of p53 are miR-30d and miR-25 (176), which have been detected in human and porcine MEX (7,8,21,100,175). Thus, a network of MEX miRs synergistically attenuates p53 expression (45,86) (*Figure 1*). Furthermore, MEX miRs activate AKT (11,105,171) via inhibition of PTEN and may enhance p53 degradation via AKT-mediated phosphorylation of mouse double minute 2 (MDM2) (177,178). Mecocci *et al.* (79) identified MDM4 as a central node of transcriptomic regulation of cow, donkey and goat MEX RNAs. MDM4 restricts p53 transcriptional activity and facilitates MDM2's E3 ligase activity toward p53 (179).

MEX miR-148a/miR-21/miR-29b-mediated suppression of DNMT1, DNMT3A and DNMT3B (11,77,159,160) may reduce DNA methylation-dependent silencing of

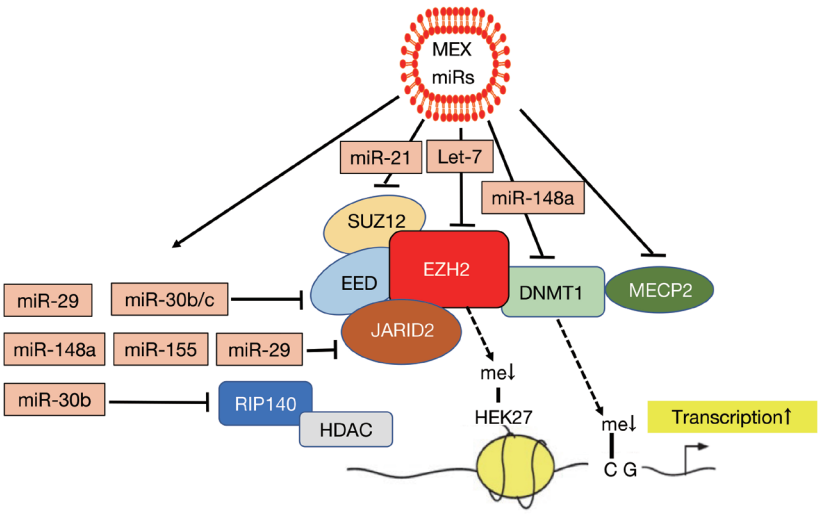

Figure 2 Illustration of predicted MEX miRs-mediated suppression of PRC2. The catalytically active enzyme of PRC2 is EZH2, which is a direct target of the let-7 family of miRs. The PRC2 activating component JARID2, which enhances HEK27me3 formation, is a target of miR-148a. Further suppressive effects of MEX miRs attenuate the interaction of MeCP2-DNMTs and EZH2-DNMTs, thus enhance transcription via reduced DNA and histone methylation. MEX miRs operate under control of the maternal lactation genome programmed to open chromatin structure and enhancing transcription, a reasonable support for cell growth and epigenetic programming of the newborn infant. Continued exposure of adult human cells to lactation signaling via MEX miRs is of critical concern with regard to the initiation and progression of cancer. MEX, milk exosome; miRs, microRNAs; PRC2, polycomb repressive complex 2; JARID2, JUMONJI, AT-rich interactive domain; DNMT, DNA methyltransferase; EZH2, enhancer of zeste homolog 2.

developmental genes (180) that are critically involved in postnatal programming (181). Notably, p53 and DNMT1 cooperate in gene silencing and interact on the promoter of *BIRC5* with histone deacetylase 1 (HDAC1) (182) (*Figure 1*). MEX miR-mediated suppression of p53 and DNMT1 may disrupt the action of HDAC1 (182), thereby opening chromatin structure enhancing transcriptional activity (85,182). Functional analysis for the most abundant mRNAs of bovine MEX showed epigenetic functions such as histone modification, telomere maintenance, and chromatin remodeling (79). Liu *et al.* (106) identified methyl CpG binding protein 2 (MeCP2) as target gene of the most abundant miRs of porcine MEX. MeCP2 induces a repressive state of chromatin by recruiting histone deacetylases (HDACs), methyltransferases and other chromatin-silencing factors, which increase chromatin condensation and prevent transcription (183-186). DNMT1 directly associates with MeCP2 in order to perform maintenance of methylation *in vivo* (187). In addition, the methyl-CpG-binding domain of MeCP2 shows preferential interactions with H3K27me3 (188), which augments transcription repression (189,190). Of note, downregulation of H3K27me3 and upregulation of H3K27ac are involved in the activation of BAT thermogenic program (191,192).

The Polycomb repressive complex 2 (PRC2), composed of enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development protein (EED), suppressor of zeste 12 homolog (SUZ12), and Jumonji and ARID-domain-containing protein 2 (JARID2) catalyzes the deposit of the repressive histone mark trimethyl lysine 27 of histone H3 (H3K27me3) at target gene promoters (193,194) (*Figure 2*). The methyltransferase EZH2 is the catalytic subunit of PRC2 (195). Importantly, the let-7 family, key miRs of human and bovine MEX (15,49), directly targets the 3'UTR of EZH2 and downregulate its expression (196,197). EZH2 directly regulates DNA methylation by serving as a recruitment platform for DNMT1, DNMT3A and DNMT3B through binding of DNMTs to several EZH2-repressed genes (198). In turn, EZH2 regulates the Lin28/let-7 pathway to restrict the activation of fetal gene signature in adult hematopoietic stem cells (199).

MiR-29a/b/c and miR-30b/c target EED (200,201). An inverse relationship between the miR-30b expression and the amount of trimethyl H3K27 has been reported (201). SUZ12

Table 4 Potential inhibitory impact of MEX miRs on formation of repressive H3K27me3

Target genes	MEX miRNAs	References
MECP2	porcine MEX miRs	(106)
EZH2	let-7 family	(196,197)
EED	miR-29s, miR-30b, miR-30c	(200,201)
SUZ12	miR-21	(202)
JARID2	miR-148a, miR-155, miR-29	(204,206,207)
CBX2	let-7, miR-30	(210-212)

MEX, milk exosome; miR, microRNA.

is a predicted target gene of miR-21 (202), another key miR of MEX. PRC2 forms a stable complex with JARID2, which binds to more than 90% of Polycomb group target genes (203). JARID2 methylated by PRC2, triggers allosteric activation of PRC2's enzymatic activity (204). Inhibition of JARID2 expression leads to a major loss of Polycomb group binding and to a reduction of H3K27me3 levels on target genes (203). Remarkably, miR-148a, miR-155 and miR-29, pivotal miRs of MEX, are among the predicted miRs targeting JARID2 (205-207). EZH2-mediated H3K27me3 allows polycomb repressive complex 1 (PRC1) recruitment to chromatin, which establishes a higher repressive state of chromatin (193). Chromobox 2 (CBX2), a major subunit of canonical PRC1 recognizes H3K27me3 (208,209) and is a direct target of let-7 and miR-30 (210-212). The accumulation of let-7 in Lin28a$^{-/-}$ mice resulted in the reduction of PRC1 occupancy at the HOX cluster loci by targeting CBX2 (212). It is thus conceivable, that signature MEX miRs inhibit the activities of both PRC2 and PRC1 attenuating H3K27me3-mediated gene silencing (*Table 4*).

RIP140 is essential for both DNA and histone methylation to maintain gene repression (157,213). RIP140 mediates H3K9, H3K27 and DNA methylation for silencing of Polycomb group pre-marked genes (157). RIP140 directly recruits HDACs for gene silencing (214,215). MEX miR-30b/c mediated suppression of RIP140 may thus relieve RIP140-mediated gene silencing.

MEX miRs synergistically reduce the action of transcriptional repressors (p53, DNMTs, MeCP2, RIP140, EZH2, EED, JARID2, CBX2, H3K27me3) enhancing transcription and opening of chromatin structures, a meaningful epigenetic action of the lactation genome activating transcription for proliferation and infant growth.

Absence of MEX miRs during artificial formula feeding

Compared to raw cow milk, formula powdered milk for infants exhibits significant deficiencies in signature miRs, such as miR-148a, miR-30d and miR-21 (100). Leiferman *et al.* (216) reported that MEX miRs are not detectable in formulas. The levels of miR-148a and miR-125b levels in infant formula were only 1/500th and 1/100th of those in mature human milk, respectively (217). There is substantial concern that the absence of MEX miRs in artificial infant formula negatively affects appropriate postnatal epigenetic programming (84,85).

Pancreatic β-cell mTORC1/AMPK balance

Neonatal β-cells are immature and unable to secrete insulin appropriately in response to a glucose challenge (218). Adult β-cells repress a small set of housekeeping genes—such as those encoding lactate dehydrogenase A (*LDHA*), monocarboxylate transporter 1 (*MCT1*), and hexokinase 1 (*HK1*)—that would otherwise interfere with normal β-cell function. Dhawan *et al.* (219) elucidated a molecular mechanism involved in β-cell-specific repression of LDHA and HK1 that is mediated by induction of DNMT3A during the first weeks after birth. Failure to induce DNMT3A-dependent methylation disrupts normal glucose-induced insulin secretion (GSIS) in adult life. Recent evidence indicates that mTORC1 upregulates β-cell DNMT3A levels via translational control (220). MEX miRs may support appropriate mTORC1 signaling for postnatal β-cell maturation and mass expansion (221). Signature miRs of MEX enhance mTORC1 signaling via targeting *PTEN* [miR-148a, miR-155, miR-21 (77,222,223)], phosphatidylinositol 3-kinase-interacting protein 1 (*PIK3IP1*) [miR-148a (224)], *PRKAA1* [miR-148a (225)], the catalytic subunit α1 of AMP-activated protein kinase (AMPK) and *PRKAG2* [miR-148a (226)], the regulatory subunit γ2 of AMPK. Jaafar *et al.* (227) reported that the control of cellular signaling in β-cells fundamentally changed after weaning and switched from the nutrient sensor mTORC1 to the energy sensor AMPK, which was critical for functional β-cell maturation and GSIS.

Another signature miRNA of human milk is miR-375 (11), which was significantly more abundant in the colon of mice treated with human MEX (49). Bovine miR-375

Table 5 FTO-mediated effects promoting adipogenesis

FTO targets	Adipogenic functions	References
SREBP1c	FTO erases m6A marks on pre-mRNA of SREBP1c enhancing its expression	(239,240)
Pre-adipocytes	Differentiation of preadipocytes to adipocytes	(241)
C/EBPβ	Upregulation of C/EBPβ reducing UCP1 expression and formation of brown adipose tissue	(242)
RUNX1T1	Generation of alternative splice variant of RUNX1T1 stimulating adipogenesis	(243)
MIR130A	Downregulation miR-130a enhancing PPARγ expression augmenting adipogenesis	(244)
ANGPTL4	Reduction of angiopoietin-like protein 4 protein accelerating lipoprotein lipase release and extracellular triacylglycerol hydrolysis promoting triacylglycerol synthesis and formation of lipid droplets in adipocytes	(245,246)
IRX3	Downregulation of hypothalamic IRX3 and inhibition of lipolysis in peripheral adipocytes	(247)
CUX1	Interaction with CUX1 increasing retinitis pigmentosa GTPase regulator-interacting protein-1-like expression, which reduces leptin signal transduction and leptin-induced lipolysis in adipocytes	(248)

FTO, fat mass- and obesity-associated gene.

also belongs to the most highly expressed miRs of bovine whey exosomes (161). Intriguingly, miR-375 is one of the most abundant miRs expressed in pancreatic β-cells and its overexpression suppresses GSIS, whereas its inhibition enhances insulin secretion (228). Importantly, miR-375 regulates the expression of genes involved in the control of β-cell mass and identity (229). Mice with genetic deletion of miR-375 exhibit decreased β-cell mass and function (230,231).

Exosomal miR-155 derived from adipose tissue macrophages of obese mice also exerts profound regulation on β-cells, leading to impaired GSIS and increased β-cell proliferation by repressing MAF bZIP transcription factor B (*MAFB*) (232). MAFB is a direct target of miR-155 (233), miR-148a (234) and miR-29 (235). It is thus conceivable that MEX miR-148a/miR-155/miR-29 via targeting MAFB enhance β-cell proliferation but impair GISIS, a meaningful mechanism during the breastfeeding period characterized by low variations in external glucose challenge and predominant dependence on amino acids for insulin secretion.

Thus, during breastfeeding, MEX miRs apparently maintain β-cells in a functionally immature state with restricted GSIS to use the developmental period of lactation for adequate mTORC1- and MEX miR-dependent β-cell growth and mass extension. The disappearance of MEX during weaning may be the critical signal for switching β-cells to functional maturation and GSIS. Absence of MEX miRs in the setting of artificial formula feeding may thus compromise adequate β-cell growth and mass extension increasing the risk for type 2 diabetes mellitus (T2DM) later in life.

Fat mass and obesity gene-dependent pathologies

Another critical gene involved in the development of adipogenesis and T2DM is the fat mass and obesity-associated gene (*FTO*), which encodes a mRNA N6-methyladenosine (m6A) demethylase (236-238). FTO targets key regulatory checkpoints enhancing adipogenesis (239-248) (*Table 5*). It is of critical concern that the levels of FTO expression in peripheral blood mononuclear cells (PBMCs) of formula fed infants is significantly higher (89.2±19.3) compared to breastfed infants (3.39±1.1) (249). Tews *et al.* (250) showed that FTO-deficient adipocytes exhibit a 4-fold higher mitochondrial expression of UCP1 compared with controls.

MEX miRs appear to be critically involved in balancing appropriate levels of FTO expression. In fact, miR-21, miR-22, miR-30b, miR-150 and miR-155, which represent miR components of milk and MEX (7,9,15,18,100,115,251), directly target FTO mRNA (252-255). Intriguingly, miR-30b targets both RIP140 (156) and FTO (255), thus synergizes with different regulatory checkpoints known to enhance the expression of UCP1 and development of BAT. Suppression of miR-30b upregulates FTO expression resulting in aberrant FTO-mediated m6A methylation levels promoting lipid accumulation (255). Thus, deficient MEX miR signaling during formula feeding may explain the significant exacerbation of FTO expression in blood

mononuclear cells of formula-fed infants compared to breastfed infants (249,256).

Increased expression of FTO is not only involved in the pathogenesis of obesity (236-241) but also of T2DM (257-259). Notably, m6A mRNA methylation plays a key role in human β-cell biology in physiological states and in T2DM (260). Recent transcriptome and m6A methylome analyses provided evidence for m6A-dependent mechanisms in regulating cell identity, insulin secretion, and proliferation in neonatal β-cells (261). Wang *et al.* (261) found an essential role of RNA methyltransferase-like 3/14 in neonatal murine β-cell development and functional maturation, both of which determined functional β-cell mass and glycemic control in adulthood. In addition, increased FTO expression is also associated with hypertension (262), cancer (239,263-265) and osteoporosis (266).

Insufficient MEX miR-mediated inhibition of DNMT1 and FTO via MEX-deficient formula feeding may negatively affect the maturation of Treg cells, as stable FoxP3 expression and T-cell homeostasis is epigenetically and epitranscriptionally controlled at the appropriate methylation level of DNA and mRNA (145,146,267-269). These epigenetic modes of action further support the association between formula feeding and increased risk for obesity and allergy, which is also related to increased mTORC1 activity (270). Recent evidence shows that Mettl3f/f; Foxp3Cre Treg cells lost their suppressive function over T cell proliferation (271). Obviously, m6A mRNA methylation sustains Treg cell suppressive functions (271). Formula-induced FTO overexpression may thus impair m6A-dependent Treg cell suppressive functions enhancing the risk of allergy and autoimmunity. Wood *et al.* (272) recently showed that breastfeeding compared to formula resulted in a twofold higher early neonatal Treg cell expansion.

Health risks associated with persistent bovine MEX miR exposure

Consumers of pasteurized cow milk are persistently exposed to bovine MEX miRs. During the last century, pasteurization of milk has been introduced to improve the microbial safety and quality of milk (273,274). At that time, the presence of MEX and their miRs in pasteurized milk were unknown. Recently, the bioavailability of bovine MEX miRs in pasteurized commercial milk has been confirmed (92,93,275,276). Potential adverse health effects of milk's MEX and their intrinsic miRs are a matter of medical concern (277), because bovine MEX miRs can enter the human body and affect human gene expression (11,30,78-81,277).

Cancer risk

Bovine MEX transfer oncogenic miRs such as miR-21 (29,100), which is identical with human miR-21 (278). Common cancers of industrialized societies such as breast cancer (BCa) and prostate cancer (PCa), which show a risk association with milk consumption (279-282), exhibit exosomal overexpression of miR-21 in the circulation and tumor tissues (283-290). Thus, persistent bovine MEX miR-21 exposure may enhance mTORC1 signaling promoting tumor initiation and progression (291-296). Furthermore, MEX miR-mediated destabilization of p53 (86,88,174,176) may promote cancer development and disturb tumor immunity (297-299).

Intriguingly, bovine MEX orally administered to mice implanted with colorectal and BCa cells reduced the primary tumor burden but accelerated metastasis in BCa and pancreatic cancer mouse models (78). Upon treatment with MEX, epithelial-to-mesenchymal transition (EMT) has been observed in cancer cells (78). Increased expression of miR-148a has been detected in PCa tissue correlating with increased Gleason score (300). The androgen-responsive miR-148a promoted LNCaP prostate cell growth by repressing cullin-associated neddylation-dissociated protein 1 (*CAND1*) (301) and *DNMT1* (11,77). Reduced expression of DNMT1 was associated with EMT induction and cancer stem cell phenotype, enhancing tumorigenesis and metastasis of PCa (302). PCa-derived exosomes, which contain oncogenic miR-21, miR-155 and miR-125b promote PCa progression (303). Notably, exposure of human breast cells with human MEX enhanced EMT-associated proteins related to transfer of MEX-derived TGF-β2 (304), a known inducer of EMT-promoting miR-155 (305-308). Bovine MEX transport TGF-β (288) and miR-155 and increase intracellular levels of miR-155 (126). Exosomal miR-125b was upregulated in highly invasive pancreatic cancer cells with increased migration, invasion, and EMT (309). Melanoma-secreted exosomal miR-155 can induce a proangiogenic switch of cancer-associated fibroblasts (310). Exosomal miR-21 has been shown to promote melanoma development and progression (311). Mastitis, a common chronic health problem of dairy cows, has been associated with increased expression of MEX miR-223 (312), another oncogenic miR associated with human cancers (313). Thus, persistent MEX miR exposure via consumption of pasteurized cow milk may synergize with cancer-derived

exosomes enhancing the total burden of oncogenic exosome-mediated miR signaling.

Adipogenesis and satiety control

In adipose tissues from obese individuals and mice fed a high-fat diet, the expression of miR-148a is increased and silences the endogenous inhibitors of adipogenesis WNT1 and WNT10B (314-316). Increased expression of miR-148a via suppression of DNMT1 enhances adipocyte differentiation and precociously promoted adipocyte-specific gene expression and lipid accumulation (317). Inhibition of DNA methylation at late stage of pre-adipocyte differentiation promotes lipogenesis and adipocyte phenotype in 3T3-L1 cells. This is mediated by promoter demethylation of *SREBF1c*, which is upregulated during adipogenesis (318). *MIR148A* represents a domestication gene of dairy cows increasing milk yield (319,320) but is also an obesity risk gene in humans (321-323). Persistent transfer of bovine MEX miR-148a may thus enhance adipogenesis and SREBF1c-mediated lipid accumulation in adipocytes.

In addition, miR-148a directly inhibits the mRNA of the low density-lipoprotein (LDL) receptor (*LDLR*) (324,325), the pivotal regulator of cholesterol homeostasis and hepatic LDL clearance (326). Importantly, miR-148a also inhibits ATP-binding cassette transporter 1 (*ABCA1*) (324), the key player for HDL-mediated reverse cholesterol transport (326-328), thus promoting hypercholesterolemia and atherosclerosis.

Adipocyte-derived exosomes can regulate proopiomelanocortin (POMC) expression through hypothalamic mTORC1 signaling, thereby affecting body energy intake. Adipocytes of obese mice secreted MALAT1-containing exosomes, which increased appetite and weight when administered to lean mice (329). It is thus conceivable that MEX, which reach the brain (32,147), may also have an impact on hypothalamic appetite and satiety control. One of the gut hormones sending satiety signals to the hypothalamus is cholecystokinin (CCK), which is secreted from intestinal mucosa cells when the duodenum is filled with food (330). CCK binds to the CCK A receptor (CCKAR) and the CCK B receptor (CCKBR). CCKBR knock out mice developed obesity associated with hyperphagia (331). As shown in rodents, hypothalamic CCKBR mediates inhibition of food intake (331,332). CCKBR deletion was associated with increased body weight and hypothalamic neuropeptide Y content, explaining the increased food intake in *CCKBR* knockout mice (333). Notably, the gene expressing *CCKBR* is a direct target gene of miR-148a (334) exhibiting four different target sites for miR-148a on the mRNA of *CCKBR* (335). Further predicted miRs that target *CCKBR* are miR-29b, miR-30a, miR-30d, miR-223 (335), which are all miR components of MEX. Hypothalamic MEX miR signaling may thus attenuate satiety signals. This mode of action may be of advantage for postnatal growth and weight gain of the infant, but could promote obesity in adults persistently exposed to bovine orexigenic MEX miRs.

MiR-21 is another important miR which contributes to adipocyte differentiation (336-339) and promotes human adipose tissue-derived mesenchymal stem cell (MSC) differentiation towards adipocytes (336). Overexpression of miR-21 in MSCs enhances the expression of PPARγ and modulates ERK-MAPK activity by repressing Sprouty 2 (337), a known negative regulator of receptor tyrosine kinase pathways involved in ERK-MAPK signaling during MSC differentiation (337). Notably, miR-21 expression was twofold higher in WAT of patients with T2DM (339).

MiR-29b is another abundant miR of cow milk and MEX (27,275), which is detectable after pasteurization and homogenization of milk (276) and exhibits increased expression in plasma and PBMCs after milk consumption (275). MiR-29b promotes adipogenic differentiation of human adipose tissue-derived stromal cells (340). In bovine mammary epithelial cells, miR-29s enhance lactation performance and lipogenesis via suppression of DNMT3A and DNMT3B, whereas inhibition of miR-29s increased the methylation levels of promoters of lactation-related lipogenic genes, reducing the expression of PPARG and *SREBF1* (341).

Overexpression of miR-155 in mice has been shown to reduce BAT mass (342), whereas thermogenic miR-30b promotes UCP1 expression and BAT formation (156).

Obviously, MEX miRs may synergize with key miRs involved in the regulation of adipogenesis (343).

T2DM

Only few epidemiological studies compared the risk of milk consumption versus fermented milk/products in relation to the risk of T2DM (344). The Lifeline Cohort Study identified positive associations of full-fat dairy products, non-fermented dairy products and milk with newly diagnosed T2DM (345). Unfortunately, no study related pasteurized versus ultraheat-treated (UHT) milk with a T2DM risk analysis. This is of importance because consumers of pasteurized milk are exposed to bioactive MEX, while MEX miRs are reduced by UHT (92,93). Kleinjan *et al.* (92) showed that UHT of

milk resulted in a loss of EVs, whereas EV numbers after pasteurization were not affected. Although pasteurization and homogenization of commercial milk reduced total milk-EV-associated RNAs [from 40.2±3.4 ng/μL in raw milk to (17.7±5.4)–(23.3±10.0) ng/μL in processed milk], miR-148a, miR-21, miR-30d, although reduced, were still detectable in pasteurized and homogenized milk (92). Zhang *et al.* (93) recently confirmed that bioactive miRs in raw milk were lost after ultraheat treatment but not after pasteurization.

Observed exosome crosstalk between pancreatic β-cells and exosomes derived from different cell types (lymphocytes, adipocytes, and muscle cells) supports the concept that exosomal miRs communicate with β-cells (346-350). For instance, human T lymphocyte-derived exosomes, which transfer miR-155, induced apoptosis in β-cells and promoted type 1 diabetes mellitus (T1DM) in mice (348). Exosomes from insulin-resistant muscles influenced gene expression and proliferation in murine β-cells (349). Exosomes from obese adipose tissue were harmful for human β-cells (350). Exosomes of inflammatory adipocytes exhibited a fourfold increased expression of miR-155 (351).

Postnatal β-cell maturation is associated with islet-specific miR changes induced by nutrient shifts at weaning (352). It is thus conceivable that MEX miRs may participate in exosomal β-cell interactions supporting postnatal β-cell proliferation and mass expansion but suppress GSIS, which is activated after weaning (221). Pancreatic β-cells differentiate during fetal life, but only postnatally acquire the capacity for GSIS. Jaafar *et al.* (227) found that the control of cellular signaling in β-cells fundamentally switched from the nutrient sensor mTORC1 to the energy sensor AMPK, which was critical for functional maturation. Notably, T2DM is associated with a remarkable reversion of the normal AMPK-dependent adult β-cell signature to a more neonatal one, characterized by mTORC1 activation (227). Allowing mice to continue assimilating milk fat throughout their entry into adulthood was sufficient to maintain neonatal levels of β-cell mTORC1 activity, which was otherwise completely repressed in control mice (227). Recent evidence confirms that the shift from amino acid- to glucose-stimulated insulin secretion after birth is mediated by a transition in nutrient sensitivity of the mTORC1 pathway, which leads to intermittent mTORC1 activity (353).

The switch from mTORC1 to AMPK signaling is important to shift β-cell mitochondrial biogenesis to oxidative metabolism and functional maturation (227). AMPK is regarded as guardian of metabolism and mitochondrial homeostasis (354). AMPK upregulation inhibits β-cell apoptosis (355). AMPK is activated by the action of metformin (356), which inhibits mTORC1 (357) and suppresses miR-21 (358). The suppression of miR-21 enhances the expression of critical upstream activators of the AMPK including calcium-binding protein 39-like protein and sestrin-1 (358-361) leading to AMPK activation and inhibition of mTORC1 (358). Thus, metformin-induced mTORC1 inhibition and AMPK activation simulate the switch of mTORC1 to AMPK activation, a comparable mechanism found after weaning associated with the physiological termination of MEX miR signaling.

Importantly, human and bovine milk fat as well as human and bovine MEX are a rich source of miR-148a (11,101,362,363), which targets key regulatory components of AMPK (225,226,364), thereby attenuating AMPK's inhibitory function on mTORC1 via AMPK-mediated phosphorylation of TSC2 and Raptor (365,366). Milk fat and MEX miR-148a may stimulate β-cell mTORC1 activity promoting β-cell growth and mass expansion (367). The disappearance of MEX miR-148a after weaning may induce the critical switch to enhanced AMPK activity promoting β-cell mitochondrial function and GSIS to respond to dietary challenges of varying glucose intake. Persistent exposure of humans to bovine MEX miR-148a may dedifferentiate β-cells back to postnatal conditions characterized by overactivated mTORC1, which promotes β-cell proliferation and in the long run induces early β-cell apoptosis (221,341).

This concept is further supported by the regulatory events of MAFB, which is required for the generation of functional β-cell populations by directly activating insulin gene transcription and key regulators of β-cell differentiation and function (368). Importantly, MAFB increases the expression of MAFA, which is important to maintain β-cell function in adults (369). MAFB is a direct target of miR-148a (234). Reduced expression of MAFB in murine and human β-cells has been associated with decreased GSIS (370). Loss of MAFB is associated with β-cell dedifferentiation. Loss of MAFA and/or MAFB represents an early indicator of β-cell inactivity and the subsequent deficit of more impactful NKX6.1 (and/or PDX1) resulting in overt dysfunction associated with T2DM. Notably, MAFA, MAFB, NKX6.1, and PDX1 expression levels are compromised in human β-cell in T2DM (371). The significance of MAFB to primate β-cells is supported by suppressed GSIS in the human EndoC-βH1 β-cell line by MAFB knockdown (372). Both, MAFA and MAFB mediate GSIS in human β-cells (373).

The primary glucose transporter of human β-cells is GLUT1 (*SLC2A1*) (374,375). GLUT1 expression is activated by MAFB (372), which is required for formation of glucose-responsive β-cells (376). Remarkably, GLUT1 (*SLC2A1*) and *MAFB* are targets of miR-148a (234,377).

It has recently been demonstrated that overexpression of miR-21 in β-cells markedly reduced GSIS and led to reductions in mRNA expression of MAFA, NKX6.1, INS1, INS2 and GLUT2 associated with a loss of β-cell identity and increased markers of β-cell dedifferentiation (378). Increased expression of miR-21 reduced the expression of TGFB2 and SMAD2, direct targets involved in β-cell commitment (379). TGF-β signaling plays a key role for adult β-cell function and maturity (380). Induction of miR-21 in human islets was also associated with a dedifferentiated phenotype and reduced expression of miR-21 target mRNAs linked to β-cell identity (378). Furthermore, lentiviral overexpression of miR-21 in the β-cell line INS-1, increased proliferation, but also induced apoptosis, questioning the potential of miR-21 as a therapeutic agent to increase β-cell survival (381).

Metformin, which reduces the expression of miR-21, may thus counteract β-cell dedifferentiation (358). Bai *et al.* (382) reported that miR-21 acts as a bidirectional switch in the formation of insulin-producing cells by regulating the expression of target and downstream genes (*SOX6*, *RPBJ* and *HES1*).

In analogy to miR-21, miR-148a also targets TGFB2 and SMAD2 (383-386). Increased serum levels of miR-148a and miR-21 have been reported in patients with T1DM (387). Of note, SMAD2 deficiency impaired insulin secretion in response to glucose (388,389). SMAD2 disruption in mouse pancreatic β-cells leads to islet hyperplasia and impaired insulin secretion due to an attenuation of K_{ATP} channel activity (389), which plays a critical role in glucose homeostasis by linking glucose metabolism to electrical excitability and insulin secretion (390). TGFBR-SMAD2/3 signaling sustains functional maturation of neonatal β-cells (391). TGFBR–SMAD2/3 inhibition counteracted upregulation of CDKN2A, NEUROD1, UCN3 and ABCC8, the ATP-sensitive component of the K_{ATP} channel (391). Of note, TGFBR–SMAD2/3 signaling repressed aldolase B (*ALDOB*), a disallowed gene in mature β-cells and a marker of functionally immature as well as of diabetic β-cells (391). Inhibition of TGF-β signaling promotes human β-cell replication, whereas TGF-β signaling induces CDKN2A (INK4A) expression leading to replicative decline in β-cells through the recruitment of SMAD3 as a part of the recruitment of histone methyltransferase Mll1 complex (392). Furthermore, TGF-β has been reported to stimulate insulin secretion (393), insulin gene transcription, and islet function (394). TGF-β/SMAD pathway enhances the transcription of miR-375, miR-26a, and NGN3, thereby promoting β-cell differentiation (395). Notably, a fasting-mimicking diet in mice reduced PKA and mTORC1 activity and induced SOX2 and NGN3 expression and insulin production (396). MiR-375 and miR-26a induced insulin-producing cell differentiation from nestin-positive umbilical cord-derived MSCs by suppressing target genes including *MTPN*, *SOX6*, *BHLHE22* and *CCND1* (397).

Let-7 family members, major components of MEX (15,49), also suppress TGF-β signaling and decrease mRNA expression of TGFBR1, TGFBR3 and SMAD2 (398-400). Notably, the lncRNA H19, a sponge of let-7 (401), is profoundly downregulated during the postnatal period (402). It has been shown that let-7 promotes the expression of IRS2 and mTOR in β-cells (403), whereas suppression of let-7 expression in endothelical cells increased the expression of TGF-β and TGF-βR1 (404). Glucose-responsive genes are highly regulated by TGF-β signaling (405).

It is conceivable that MEX miR-148a/miR-21/let-7 signaling during the breastfeeding period via suppressing TGF-β signaling may promote β-cell proliferation and delays β-cell differentiation and GSIS, a physiological mechanism that fades after weaning.

Mice with specific inactivation of *ABCA1* in β-cells exhibited impaired insulin secretion (406,407). Lack of β-cell ABCA1 leads to impaired exocytosis of insulin granules (408). ABCA1 as well is a direct target of miR-148a (324).

Another important target of MEX miR-148a is *TP53* (86). Suppression of p53 activates mTORC1 (167) and inhibits CDKN1A, accelerating cell cycle progression (409). Loss of p53 function decreases lysosomal TSC2 and increases lysosomal Rheb, resulting in hyperactive mTORC1 (410). Survivin (*BIRC5*), which is inhibited by p53 (173), is critically involved in the regulation of β-cell mass after birth (411). Targeted deletion of survivin in the pancreas resulted in a significant decline in β-cell mass throughout the perinatal period, leading to glucose intolerance in the adult. Survivin-deficient islets showed decreased cell proliferation as a result of a delay in cell cycle progression with perturbations in cell cycle proteins (412). MEX miR-mediated suppression of p53 may thus promote survivin-induced β-cell mass expansion (*Figure 3*).

Peroxisome proliferator-activated receptor-γ coactivator

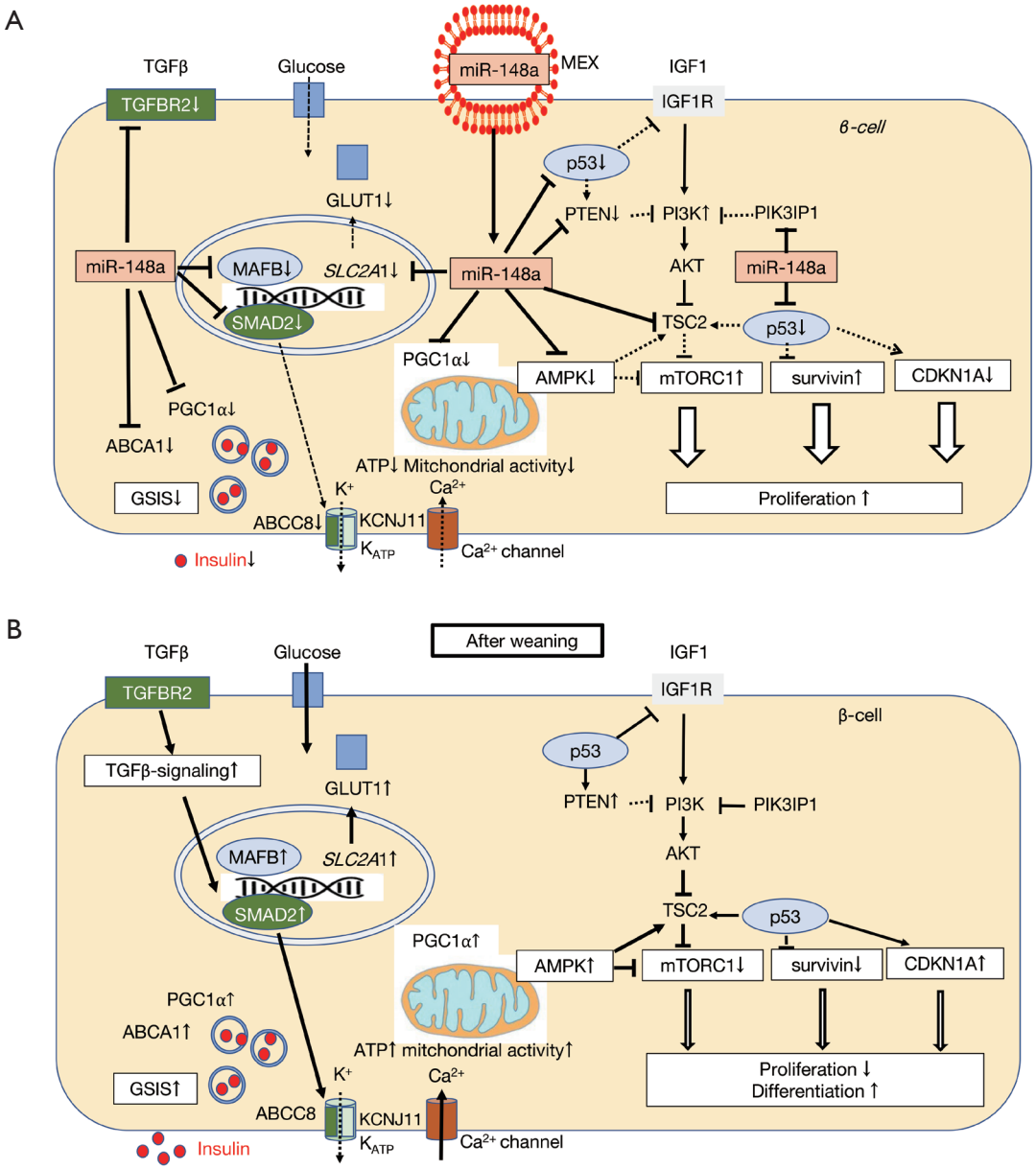

Figure 3 Illustrated β-cell model showing a β-cell exposed to MEX miR-148a signaling (A) during postnatal breastfeeding and (B) absent MEX miR-148a signaling after weaning. (A) MEX miR-148a suppresses TGFB2, SMAD2, p53, MAFB, GLUT1, PTEN, AMPK, PGC-1α and ABCA1 increasing mTORC1- and surviving-dependent β-cell proliferation but suppressing β-cell differentiation and GSIS. (B) After weaning, disappearance of MEX miR-148a enhances TGF-β signaling promoting β-cell differentiation with increased expression of MAFB, GLUT1, AMPK, ABCA1, ABCC8 activating GSIS but attenuating mTORC1-driven β-cell proliferation. Lack of MEX miR-148a in artificial formula compromises miR-148a-mediated β-cell proliferation and mass expansion. However, persistent exposure of humans with MEX-miR-148a/miR-21/let-7 of pasteurized cow milk may dedifferentiate the β-cell back to the postnatal hyperproliferative state with overactivated mTORC1, impaired AMPK and TGF-β signaling promoting ER stress and early β-cell apoptosis, key mechanism in the pathogenesis of type 2 diabetes mellitus. MEX, milk exosome; miR, microRNA; GSIS, glucose-stimulated insulin secretion; TGF, transforming growth factor.

Table 6 Potential regulatory effects of MEX miR-148a on postnatal β-cell maturation

miRNA-148a targets	Predicted regulatory effects of MEX miRNA-148a	References
TP53	Increased expression of survivin (BIRC5) and suppression of p21 (CDKN1A) stimulates β-cell proliferation; decreased expression of TSC2 activates mTORC1; reduced sestrin (SESN1, SESN2) expression attenuates AMPK activity enhancing mTORC1 activation	(86,166,167,173,360,382)
PTEN	Reduced expression of PTEN activates PI3K and thus AKT-mTORC1	(77)
PIK3IP1	Reduced expression of PIK3IP1 activates PI3K and thus AKT-mTORC1	(224)
PRKAA1	Reduced expression of PRKAA1 reduces the catalytic activity of AMPK promoting mTORC1 activation and suppressing mitochondrial activity	(225)
PRKAG2	Reduced expression of PRKAG2 reduces the activity of AMPK promoting mTORC1 activation and suppressing mitochondrial activity	(226)
PPARGC1A	Reduction of mitochondrial biogenesis and GSIS	(364,392)
MAFB	Reduced expression of the GLUT1 attenuates glucose sensing of β-cells; reduced expression of MAFA impairs the MAFA/MAFB-dependent maintenance function of β-cells	(234,235,376)
SLC2A1	Reduced expression of GLUT1 attenuates glucose sensing of β-cells	(377)
ABCA1	Impaired insulin granule function and insulin secretion	(324,378-380)
TGFB2	Increased β-cell proliferation with decreased GSIS, delayed differentiation	(381-384)
SMAD2	Increased β-cell proliferation with decreased GSIS, delayed differentiation	(381-384)

MEX, milk exosome; miR, microRNA.

(PGC)-1α, a transcription coactivator that plays a central role in the regulation of cellular energy metabolism, stimulates mitochondrial biogenesis (413) and controls β-cell homeostasis (414). Of note, the susceptibility of β-cells to environmental programming continues into the neonatal period (415). Overexpression of PGC-1α in β-cells during fetal life in mice is sufficient to induce β-cell dysfunction in adults, leading to glucose intolerance (416,417). Ling *et al.* (418) demonstrated that downregulation of *PPARGC1A* expression in human islets by siRNA, reduced insulin secretion. Remarkably, key signature miRs of milk and MEX including the let-7 family and miR-148a target *PPARGC1A* (364,419,420) and may thus restrain insulin secretion during the breastfeeding period. Increased plasma levels of miR-148a have been associated with T2DM progression, increased HbA1c, HOMA-IR, and hyperinsulinemia (421) (*Table 6*). Melkman-Zehavi *et al.* (422) reported that miR-148 acts as a positive regulator of insulin transcription.

In accordance with miR-148a, miR-375 is overexpressed in T2DM (423-425). MiR-375 reduces GISIS and enhances β-cell proliferation (228-231,426,427) and plays a critical role for the differentiation of human embryonic stem cells into insulin-producing cells (428). Upregulation of miR-375 is associated with T2DM and apoptosis of pancreatic islet β-cells (429,430). Dietary exposure of adult milk consumers with MEX miR-375 may thus stimulate β-cell proliferation promoting early β-cell apoptosis.

MEX deliver miR-29s (27,275), which are considered as diabetogenic miRs promoting insulin resistance (431-435). Elevated plasma levels of branched-chain amino acids (BCAAs), crucial activators of mTORC1 (105), correlate with an increased risk of insulin resistance and T2DM (433-440). Intriguingly, miR-29b is critically involved in the regulation of cellular BCAA homeostasis (441). MiR-29b inhibits branched chain α-ketoacid dehydrogenase (BCKD) via targeting its core protein dihydrolipoamide branched-chain acyltransferase (441) thereby decreasing BCAA catabolism. This is a meaningful metabolic regulation for the newborn mammal protecting BCAAs from catabolism and saving them for the synthesis of functional and structural proteins as well as for BCAA-mediated mTORC1 activation (442,443), which is important for postnatal β-cell proliferation (221,341).

However, persistent overactivation of mTORC1 induces endoplasmic reticulum (ER) stress, which in the long run impairs β-cell autophagy, promotes β-cell apoptosis and β-cell failure (444,445). Notably, mTORC1 activity is markedly increased in islets from patients with T2DM

(227,446).

Recent evidence indicates that β-cells control glucose homeostasis via the secretion of exosomal miR-29 family members (447). Notably, intravenous administration of exosomal miR-29a/b/c attenuated insulin sensitivity (447). In fact, β-cell-specific transgenic miR-29a/b/c mice are predisposed to develop glucose intolerance and insulin resistance when fed a high-fat diet (448). Expression of miR-29 in pancreatic β-cells promotes inflammation and diabetes via targeting tumor necrosis factor (TNF) receptor-associated factor 3 (*TRAF3*) to promote CCXL10 release from β-cells, which then attracts nearby circulating monocytes (448). Furthermore, miR-29 targets *MAFB* (235).

Taken together, key miRs donated via MEX promote β-cell proliferation and reduce GSIS, a meaningful metabolic regulation during the breastfeeding period to support β-cell mass expansion for insulin demands in adulthood. In contrast to the importance of MEX during the lactation period, in adult life MEX miRs may exert diabetogenic effects promoting β-cell dedifferentiation and loss of function (*Figure 3*).

Parkinson's disease (PD)

Epidemiological studies associate consumption of unfermented milk with an increased risk of PD (449-456). PD is an α-synucleinopathy associated with deficient lysosomal clearance and aggregation of misfolded α-synuclein (456-458). Accumulating evidence supports a gut-brain axis in PD pathogenesis involving α-synuclein synthesis in intestinal enteroendocrine cells (EECs) and retrograde traffic of α-synuclein via the vagal nerve to the brain (459-462). Hypomethylation of the *SNCA* promoter increases α-synuclein expression, which is controlled by DNMT1 (151,351). In the chronic MPTP mouse model of PD increased expression of miR-148a reduced the expression of DNMT1, diminished α-synuclein methylation resulting in increased α-synuclein expression (463).

Uptake of MEX miR-148a by intestinal EECs may thus enhance intestinal α-synuclein synthesis increasing its retrograde transneuronal traffic to the brain and pancreatic islets (351). Although the major aggregating peptide in β-cells of T2DM patients is islet amyloid polypeptide (IAPP), α-synuclein in β-cells interacts with IAPP (464). Mucibabic *et al.* (465) showed that α-synuclein is a component of amyloid extracted from the pancreas of transgenic mice overexpressing human IAPP (denoted hIAPPtg mice) and from islets of T2DM individuals. Notably, α-synuclein promotes IAPP fibril formation *in vitro* and enhanced β-cell amyloid formation *in vivo*, whereas β-cell amyloid formation was reduced in mice on a $SNCA^{-/-}$ background (465). As bovine MEX are able to cross the blood-brain barrier and reach the brain (32,147), MEX miR-148a may stimulate neuronal α-synuclein in the *substantia nigra*, whereas MEX miR-21 may suppress lysosome-associated membrane protein type 2A (LAMP2A) (466,467), a critical effector protein of chaperone-mediated autophagy clearing neurotoxic α-synuclein (349).

Limitations

This narrative review focuses on MEX miRs and their potential beneficial and adverse effects on human health. However, it is important to consider that milk contains a large spectrum of EV subtypes whose components may exhibit a differential resistance to digestion and may have differential impacts on health (1,14,24). Furthermore, MEX contain a wide spectrum of very small, small, medium and long non-coding RNAs in addition to miRs (19,468), that may also contribute to biological effects. It has recently been discussed that exosomes provide unappreciated carrier effects that assist transfers of their miRs to targeted cells (130). In addition, MEX deliver other biomolecules such as lipids and proteins that may also determine biological outcomes of MEX-derived signaling (55), which are beyond the scope of this review. As long as risk assessments on a statistical basis are not yet available, statements of the potential hazardous nature of cited miRs based on translational and indirect evidence have to be judged with caution.

Conclusions

To approximate risks and benefits of MEX and their miRs, it is mandatory to appreciate their physiological inherent nature. MEX are signalosomes generated under control of the lactation genome to support growth and tissue maturation during the breastfeeding period. MEX miRs apparently interfere with p53-mediated transcriptional activity, DNMT-regulated gene silencing, histone methylation and chromatin remodeling. MEX miR-dependent effects are not provided by formula feeding potentially resulting in overexpression of FTO. The time period and dose of MEX miR exposure appears to be of critical importance for MEX miR-induced desired and adverse gene-regulatory effects. The persistent transfer of

Table 7 Potential effects of bovine MEX miRs and related adult human pathologies

Target tissue	Potential MEX-mediated effects	Potential mode of action
Intestine	Anti-inflammatory action of miR-148a in inflammatory bowel diseases (Crohn disease, Colitis ulcerosa); reduction of inflammation-induced colon cancer; Stimulation of intestinal α-synuclein synthesis promoting Parkinson disease	miR-148a-mediated suppression of TLR4 and NF-κB signaling; miR-148a/DNMT1-mediated stimulation of α-synuclein synthesis
Liver	Attenuation of liver fibrosis and fibrosis-related liver cancer; stimulation of liver cancer	Transfer of anti-fibrotic miR-148a and miR-29b; Transfer of oncogenic miR-155 and miR-21
Pancreatic islet	Increased β-cell proliferation resulting in early onset of β-cell apoptosis promoting type 2 diabetes mellitus; Impaired TGFB2/SMAD2 signaling promoting β-cell dedifferentiation	miR-148a/miR-21/miR-375-mediated stimulation of β-cell proliferation and mTORC1 activity; MEX miR-mediated suppression of p53, MAFB, GLUT1; AMPK, PGC1α, ABCA1, TGFB2, SMAD2 and GISIS
White adipose tissue	Promotion of white adipogenesis promoting obesity	miR-148a-mediated inhibition of WNT1 and WNT10B; miR-21-mediated stimulation of adipocyte differentiation; miR-148a-mediated suppression of LDLR and ABCA1 compromising cholesterol homeostasis
Brown adipose tissue	Promotion of beige/brown adipogenesis reducing obesity	miR-30b/c-mediated stimulation of UCP1 expression
Hypothalamic centers	Hyperphagia promoting obesity	miR-148a-mediated suppression of CCKBR reducing satiety signaling
Brain	Over-expression of α-synuclein and reduced CMA promoting neurodegenerative diseases	miR-148a-DNMT1-mediated overexpression of α-synuclein, miR-21-mediated suppression of LAMP2A-dependent CMA
Malignant tumors	Promotion of tumor growth and epithelial-mesenchymal transition	miR-148a/miR-125b/miR-30-mediated suppression of p53; transfer of oncogenic miR-155 and miR-21; miR-30b/c-mediated suppression of RIP140; MEX miR-mediated suppression of MeCP2, H3K27me3, opening of chromatin structure; increased permission of transcription

MEX, milk exosome; miR, microRNA; CMA, chaperone-mediated autophagy.

bioactive MEX miRs via consumption of pasteurized cow milk beyond the breastfeeding period is a recent insight (92,93), which has been neglected in all epidemiological studies correlating cow milk intake with human pathologies (90,277). The use of concentrated MEX either native or uploaded with drugs will expose the recipient to increased amounts of intrinsic MEX miRs that may exert desired gene-regulatory effects in the target tissue but may adversely affect other non-target tissues. Bovine MEX showed beneficial effects in inflammatory bowel diseases and states of fibrosis (47-51,469), but may promote hyperphagia, obesity, T2DM, cancer and neurodegeneration (88,90,221,277,296,351) (*Table 7*). Certainly, human MEX are supportive for the newborn infant and contribute to the superiority of breastfeeding compared to formula. However, long-term bovine MEX miR exposure via pasteurized cow milk may enhance the risk for diseases of civilization and should be terminated. MEX miR-mediated suppression of TGF-β signaling may be beneficial for the treatment of fibrosis (50-54), but may be deleterious for adult pancreatic β-cell homeostasis (386-393). Thus, there is no clear-cut answer to judge the benefit/risk evaluation of MEX miRs in general. Before systemic administration of bovine MEX miRs—either native or combined with uploaded drugs or anti-sense RNAs—is employed, long-term safety studies have to be performed, including tissues, which are not direct targets for therapeutic intervention. Future experimental investigations are required that determine bovine MEX miR delivery to cells and tissues of human cow milk consumers and their effects on gene expression

not only in short term but especially in long-term studies to evaluate their physiological and pathological effects.

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Footnote

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