Placenta-specific microRNAs in exosomes – good things come in nano-packages

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Abstract

MicroRNAs (miRNAs) are small noncoding RNA gene products that commonly regulate mRNA expression by repression of translation and/or transcript decay. Whereas common and unique types of miRNAs are expressed by the placenta during pregnancy, the functions of most placental miRNA species are unknown. In addition to their intracellular silencing function, miRNAs are also released to the extracellular space and systemic circulation, where they can potentially target cells to regulate mRNA and protein expression, providing a non-hormonal means of intercellular communication that contributes to tissue homeostasis and disease pathophysiology. This review centers on extracellular miRNAs that originate in trophoblasts and that could mediate crosstalk between the feto-placental unit and the mother during pregnancy. We specifically detail the function of miRNAs from the primate-specific chromosome 19 miRNA cluster. These miRNAs are highly expressed in human placentas and in the serum of pregnant women. They are also packaged into extracellular vesicles of diverse sizes, including exosomes, and endow non-trophoblastic cells with resistance to a variety of viruses.

Keywords

trophoblast; miRNA; C19MC; trophomiR; exosomes

1. Introduction

MicroRNAs (miRNAs) are short, noncoding, single-stranded RNAs that average 22 nucleotides in length. Their main function is to repress target mRNAs via translation inhibition and/or transcript degradation and to silence gene expression [1]. Primary (pri-) miRNAs are transcribed in the nucleus from miRNA genes, where they are processed by the

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endonuclease Drosha to form 70 nt double-stranded precursor (pre-) miRNA [2]. Pre-miRNAs are exported by exportin 5 into the cytoplasm, where the stem-loop miRNAs are further processed by the endonuclease Dicer to form mature short (19–24 nucleotide) miRNA duplexes [3]. These double-stranded sequences are loaded into the Argonaute2 (Ago2)-containing, RNA-induced silencing complex (RISC) [4], where the miRNAs repress mRNAs in a sequence-specific manner.

The process of miRNA-dependent mRNA silencing is exquisitely regulated at several steps, including Dicer processing and Ago2 protein complex formation, unwinding of the miRNAs within the RISC, loop size-dependent recognition and processing by Dicer, sequence complementarity between the miRNA and the target mRNA at the seed region (nucleotides 2–8 of the miRNA), duplex stability in regions outside the seed region, and the presence of DNA Alu elements (see below) in the vicinity [5–10]. Each of the miRNA biogenesis steps is also regulated by common or cell-specific signals, such as epidermal growth factor receptor (EGFR) signaling. EGFR phosphorylation of Ago2, which is enhanced by hypoxia, attenuates its interaction with Dicer, thereby reducing miRNA processing to mature miRNAs [11]. Functionally, there are more than 1,500 human miRNAs, with many of them implicated in important developmental, physiological and pathological functions.

MiRNAs seem to have an important regulatory function in the placenta of eutherian organisms, as they do in other tissues in the body. In light of the critical function of the placenta at the feto-maternal interface and the deleterious consequences of placental dysfunction on fetal survival and growth and on maternal health, it is imperative to profile the landscape of miRNAs expressed in the placenta and decipher their function and regulation. Several recent reviews highlight current knowledge in this field, summarize associations between placental diseases and miRNAs, and suggest pertinent questions for future pursuit [12–15]. Here we focus on progress in understanding the expression and function of extracellular miRNAs that originate in the placenta, centering mainly on the placenta-specific cluster of miRNAs termed the chromosome 19 miRNA cluster (C19MC). We also discuss our own recent findings indicating that members of the C19MC family, packaged in nano-vesicles called exosomes, are able to endow non-trophoblast cells with antiviral activity.

2. C19MC miRNAs

The C19MC is the largest cluster of miRNAs in the human genome. This cluster of miRNAs was originally identified by Bentwich et al. of Rosetta Genomics [16]. The C19MC locus spans about 100 kb of genomic DNA and contains 46 pri-miRNA genes, yielding 59 mature miRNAs that are found exclusively in primates, attesting to their recent evolution [17]. C19MC miRNAs are expressed predominantly in the placenta, although these miRNAs have been detected in the testis, embryonic stem cells, and specific tumors [16–24]. Several groups, including ours, have shown that the C19MC miRNAs is highly expressed in trophoblasts during pregnancy, and are eliminated from the maternal blood after delivery [25, 26]. Notably, the placenta expresses other miRNA clusters, including the large chromosome 14 miRNA cluster (C14MC), which is located at the imprinted, maternally expressed DLK-DIO3 domain on the human chromosome 14q32 site. C14MC miRNAs are not placental specific, and are conserved in mammals, including the mouse [14, 27]. The expression level of the C19MC cluster markedly increases in placental trophoblasts from the first to the third trimester. In contrast, the expression level of the chromosome 14 miRNA cluster (C14MC) decreases during the course of pregnancy [28]. C19MC miRNAs are expressed in placental cell lines derived from trophoblasts, including the choriocarcinoma lines JEG3, JAr, and BeWo, with the notable exception of HTR8/SVneo (JFM and YS, personal communication). Other cell types in placental villi may also express C19MC

miRNAs [29], or these miRNAs might have originated from trophoblasts, as discussed below. Studies by Noguer-Dance et al. showed that the C19MC cluster is imprinted, and exclusively expressed in the placenta from the paternally inherited allele [30]. Indeed, a CpG-rich region located ~17 kb upstream of the first miRNA gene is hypermethylated in cells that do not express these miRNAs, and the expression in C19MC miRNAs can be reestablished in non-placental cell lines upon treatment with 5-aza-2'-deoxycytidine, a DNA methylation inhibitor [31, 32], supporting the notion that epigenetic mechanisms involving CpG island methylation regulate C19MC expression.

The genomic region spanning C19MC presents a remarkable structural organization, harboring many short, interspersed elements (SINE), each approximately 300 nucleotides long, called Alu elements (as they were characterized by the action of the Alu restriction enzyme) [17, 18, 33]. Analysis of the nucleotide sequence of the genomic DNA strongly suggests that these Alu elements have contributed to the evolution and expansion of the miRNA genes within the cluster [17, 33]. The roles of Alu elements are largely unknown, though they have been postulated to serve as miRNA targets or to regulate miRNA activity [10, 34].

The mechanisms underlying C19MC miRNA transcription remain poorly understood, and its analysis has been hampered by the complex and highly repetitive nature of DNA sequences [18]. Although C19MC miRNAs were originally thought to be transcribed by RNA polymerase III, recent studies strongly suggest that they are processed from RNA polymerase II transcripts [18]. In addition, it is not clear whether C19MC miRNAs are processed from a single large transcript or from multiple transcripts. Cells that actively transcribe the C19MC were shown to attract many of the constituents of the microprocessor complex, including Drosha and DGCR8 [35], which is not seen at other loci of highly expressed miRNA clusters. However, the functional significance of the recruitment of the microprocessor complex to the C19MC locus remains to be established.

While the full repertoire of the biological action of C19MCs remains to be established, data from the expression of C19MCs in discrete types of aggressive cancers, in embryonic stem cells, and by ectopic expression of C19MCS imply a role for them in cell proliferation and self-renewal [23]. Separately, we have recently shown (see below) that trophoblastic C19MC miRNAs confer resistance to viral infection on non-placental cell types through a mechanism that promotes autophagy [36].

3. Extracellular miRNAs

Observations made by Valadi et al. and by others, showing that miRNAs are packaged within exosomal nanoparticles and released into the blood or extracellular compartment, have suggested a new mechanism for transmitting miRNA-based mRNA silencing to diverse types of neighboring or distant cells [37–39]. In general, packaged extracellular miRNAs are classified as either lipid vesicle-encapsulated miRNAs, which are released into extracellular space as the cargo of different types of microparticles, including microvesicles, exosomes, and apoptotic bodies [40, 41], or as protein-bound miRNAs, in which the naked miRNAs are complexed with proteins such as Ago2, nucleophosmin1, or high-density lipoproteins (Fig. 1) [42–44]. Either form of extracellular miRNA is relatively well protected from digestion by RNase, conferring relative stability on the circulating miRNAs [37, 42]. Microparticles that package miRNAs are defined not only by their size, but also by their protein composition, density, and cells of origin. The larger microvesicles and apoptotic bodies can be separated from the smaller exosomes (mean diameter ~100 nm) by differential ultracentrifugation, density-gradient centrifugation, gel chromatography or fluid-based field flow fractionation, with purity verified by nanoparticle tracking instruments (Fig. 2) [45].

Such analytical tools have advanced the field, allowing efficient isolation and tracking of the particles and their miRNA cargo.

The biogenesis of microvesicles is distinct from that of exosomes. In general, microvesicles originate from direct shedding of the plasma membrane, thus enveloping cargo near the underlying membranes (Fig. 1). In contrast, exosomes undergo multiple endocytic steps to sort selected cargos and form intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) prior to release into the extracellular matrix. The sorting and loading of cargo into exosomes involves several protein complexes known as Endosomal Sorting Complexes Required for Transport (ESCRT-0, -I, -II, and -III). These complexes and the unique proteins associated with them, such as the cytosolic scaffolding protein Syntenin-1, determine the cargo that is packaged into the ILVs [46]: depletion of Syntenin-1 diminishes exosome production whereas increased expression of Syntenin-1 has an opposite effect [46]. Other proteins directly involved in intracellular ESCRT complexes include Rab7, a critical component of late endosomes; Rab27, which signals the docking of MVBs to the plasma membrane; and sphingomyelinase, which generates ceramide, a sphingolipid necessary for the budding of exosomes into MVBs [47, 48]. Even less is known about the release of miRNAs from cells in nonvesicular form. This process may be ATP-dependent and involve cell necrosis [44].

Recent evidence suggests that nano-sized exosomes are taken up by recipient cells via the endocytic pathway [49–51]. Both phagocytosis and macropinocytosis have been implicated in exosome capture, a process associated with transient ruffling of the plasma membrane [52]. The roles of cell- and context-specific influences, particularly those operating at the cell membrane, remain unknown. In addition, because a subset of extracellular miRNAs are directly associated with proteins rather than encapsulated into lipid vesicles, specific ligand-receptor interactions have been postulated to be involved in the targeting of these miRNAs to recipient cells. For example, extracellular miRNAs that are bound to high-density lipoproteins can be delivered into recipient cells via the HDL receptor scavenger receptor B type I [43]. The interaction among other miRNA-binding proteins and cell surface receptors remains to be identified.

4. The function of extracellular placental miRNAs

The physiological and pathological functions of non-hormonal, vesicle- or protein-bound circulating miRNAs depend on the nature of individual miRNAs and their specific target cells. As noted earlier, trophoblast-specific and nonspecific miRNAs are released to the extracellular fluid and are detectable in the maternal circulation during pregnancy [25, 26, 53]. The rapid decline of these miRNAs after delivery suggests a placental-fetal origin or a maternal origin, with miRNAs selectively induced during pregnancy. Interestingly, using RNA-seq of plasma from pregnant women, Williams et al. discovered that miR-127, miR-134, and C19MC miRNAs are specifically associated with pregnancy [54]. Moreover, certain miRNA members of the C19MC are relatively more abundant in the plasma than in the corresponding placenta, suggesting a selective mechanism to regulate their C19MC release [54]. Naturally, researchers have sought to use these circulating placental miRNAs as biomarkers for pregnancy-related disorders such as preeclampsia, fetal growth restriction, and preterm birth [14]. While conclusive evidence in that regard awaits further research, it seems that the search for biomarkers is unlikely to shed light on the mechanism of action of circulating C19MC miRNAs.

Clear evidence for the biological function of C19MCs was delineated in our recent work [36], in which we sought to define placental mechanisms used to protect the developing embryo against viral infections. In our initial observations, we found that when compared to

other, non-placental cell lines or to several types of primary cells, primary human trophoblasts (PHT) were resistant to infection by a number of RNA viruses (e.g., coxsackievirus B3, poliovirus, vesicular stomatitis virus) or DNA viruses (e.g., vaccinia virus, herpes simplex virus-1, and human cytomegalovirus). Our experiments also revealed that PHT cells can confer an antiviral effect on other, non-placental cell types, and that exosomes, released from PHT cells into the cell culture medium, mediate this effect. Thus, the transfer of either trophoblast-conditioned medium or of exosomes isolated from PHT cell cultures, but not PHT medium that was depleted of exosomes, bestowed the antiviral response upon non-placental recipient cells including primary fibroblasts, primary endothelial cells, and diverse cell lines. Exosomes from other sources, such as the JEG3 cell line or primary murine dendritic cells, had no effect on viral infection. In light of the data on C19MC miRNAs in exosomes [25, 26], we investigated whether ectopic expression of C19MC miRNAs or groups of highly expressed miRNA mimics might confer viral resistance on recipient cells. We found that ectopically expressing the entire C19MC fragment in cells or transfecting a high level of certain C19MC members—such as miR517-3p, miR-512-3p, or 516b-5p—conferred viral resistance to the recipient non-placental cells [36].

Although the mechanism of action of PHT conditioned medium, exosomes, and C19MC is not entirely clear, autophagy seems to play an important role [55]. This evolutionarily conserved process is known to degrade intracellular microbial invaders (a process known as xenophagy or virophagy), and thereby, to suppress infections. Our data show that either PHT conditioned medium, purified PHT exosomes, or C19MC miRNA mimics markedly stimulated autophagy in non-placental recipient cells. Not surprisingly, PHT cells, which naturally express C19MC miRNA, also exhibit a relatively high level of baseline autophagy. Importantly, pharmacological (using the autophagy inhibitor 3-methyladenine) or genomic (using siRNA-mediated silencing of Beclin1) approaches to repress autophagy also attenuated the antiviral response in non- placental cells when induced by conditioned PHT medium. Taken together, our data establish an extraordinary function of C19MC miRNAs in resistance to viral infections. C19MC miRNAs achieve this effect when expressed within PHT cells or packaged within trophoblastic exosomes for delivery to other cell types. While our data point to a prominent role for autophagy in the antiviral effects of conditioned PHT medium, PHT-derived exosomes, and C19MC-associated miRNAs, they do not exclude other possible cellular pathways which may be affected by, or converge on, the autophagic pathway, such as innate immune responses and/or other pathways that may synergize with the action of placental C19MC miRNAs [56–58].

The evolutionary entrance of C19MC miRNAs into the primate genome suggests a late adaptation to viral infections of the developing embryo. This mechanism is unique to pregnancy, as members of the cluster are expressed at a relatively high level in maternal blood throughout pregnancy and rapidly decline in the first 24 hours postpartum [25, 26, 59]. The cellular targets for C19MC miRNAs carried as exosomal cargo remain to be identified, and may include non- trophoblastic placental cells (e.g., fibroblasts, Hofbauer cells), maternal organs, and the maternal blood system. We cannot rule out the possibility that placental C19MCs also target fetal cells, although a mechanism for the transport of exosomes or miRNAs through the villous basal membranes and endothelial cells and into the fetal circulation remains to be established. Similarly, the autophagic targets that might be regulated, directly or indirectly, by C19MCs remain to be identified, and may involve a non-classical entry point into the autophagic cascade, triggered prior to the initiation of viral replication.

5. Conclusions and Perspectives

An emerging concept is that miRNAs are packaged within cells and are released into the extracellular space, where they are found in biological fluids such as blood and breast milk. Extracellular miRNAs exist in vesicle-enveloped and/or protein-bound forms. These miRNAs can exert their silencing activities in nearby or distant cells, thus providing non-hormonal intercellular communication during diverse biological processes. Thus, these circulating miRNAs may have a promising application for physiological or pathological investigations *in vivo*. Future work will focus on mechanisms by which exosomes are selectively packaged, delivered, and taken up by recipient cells. Since current findings are mainly based on studies using cultured cells, it is imperative to develop new technologies for tracking the trafficking of exosomes and miRNA cargo *in vivo* in model organisms. These advances will also be instructive for optimization of tools for therapeutic targeting of miRNA in selected diseases.

Acknowledgments

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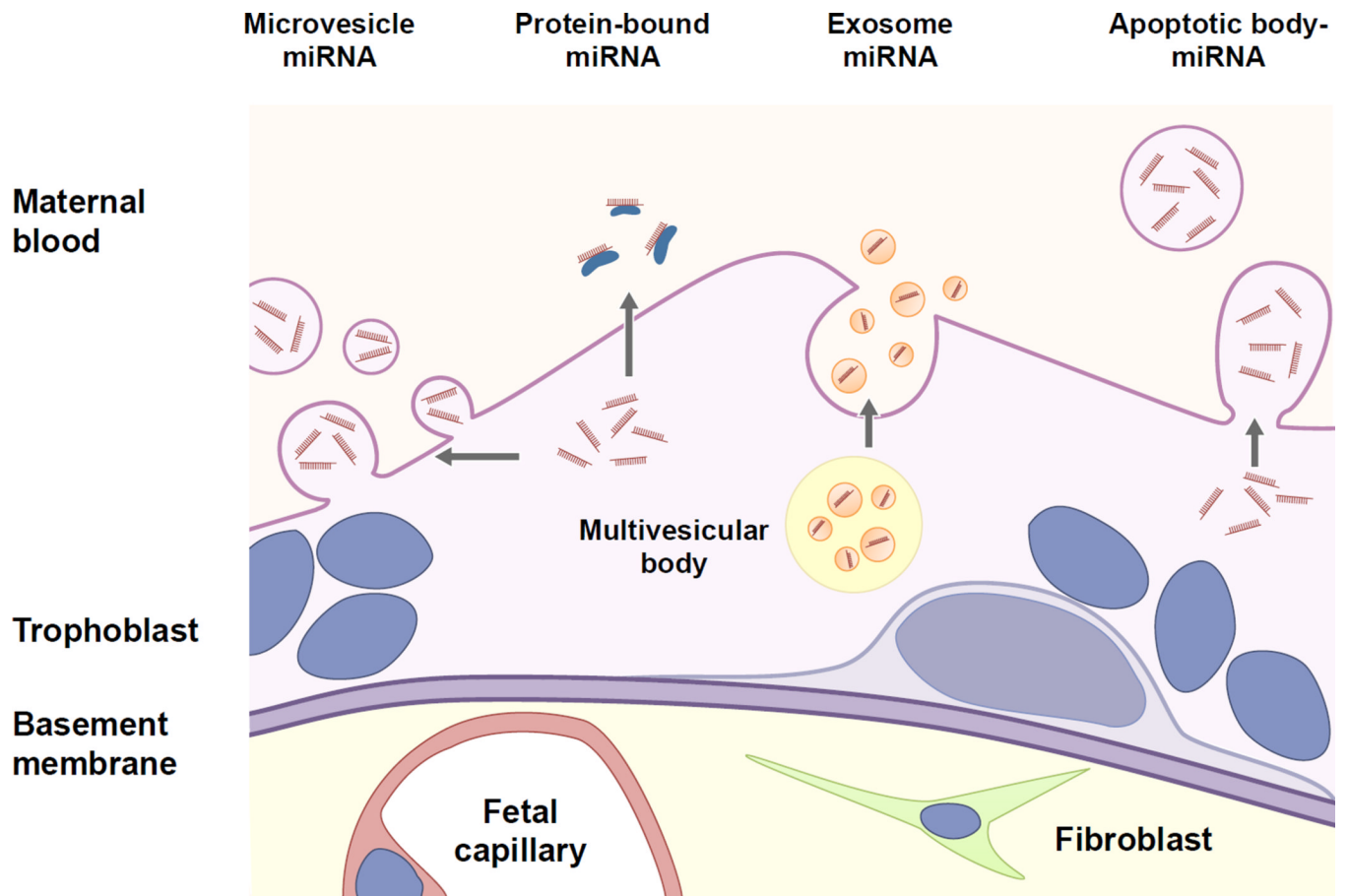
Fig. 1. A schematic of extracellular miRNAs derived from human trophoblasts
MiRNAs can be released from the trophoblast layer in different forms: microvesicle-enveloped form, apoptotic body-enveloped form, nano-sized exosome-encapsulated form, and RNA-binding protein-bound form. Exosome-associated miRNAs undergo multivesicular body processing prior to release into maternal blood. Details of the respective biogenesis pathways are described in the text.
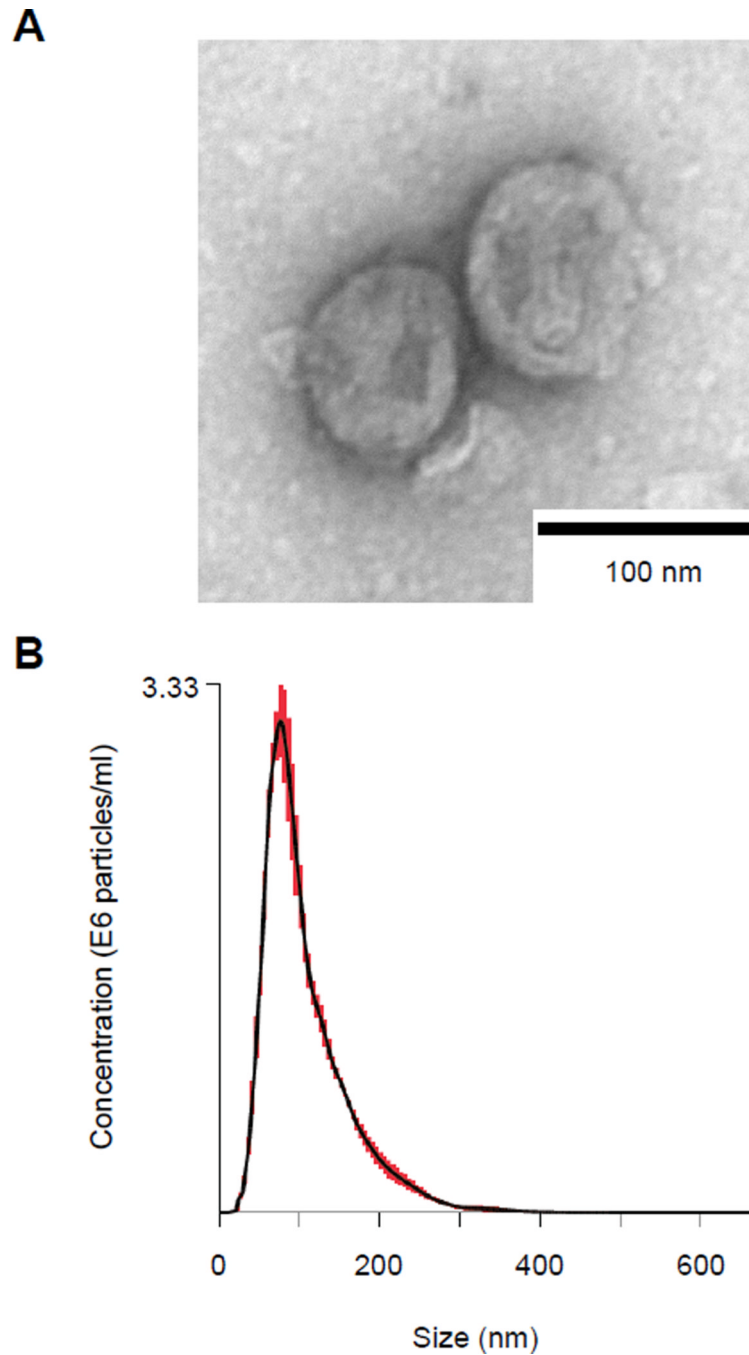
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B

Fig. 2. Characterization of exosomes isolated from primary human trophoblasts
(PHT cells). (A) Purified exosomes commonly have a cup-shaped morphology, as shown in electron microscopy. This is more noticeable for the exosome on the right side. (B) NanoSight tracking instrument profile, showing the characteristic size distribution (mean 100 nm) of purified PHT exosomes.