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Bubble Ticket Trip: Exploring the Mechanism of miRNA Sorting into Exosomes and Maintaining the Stability of Tumor Microenvironment

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Abstract: Exosomes are vesicles ranging from 30 to 100 nanometers in size that show great potential as carriers for therapeutic uses and drug delivery. Enriching a specific set of miRNAs in exosomes emphasizes the existence of particular sorting mechanisms that manage the targeted cargo packaging. The molecular mechanism for miRNA sorting has not been understood. It is crucial to understand the mechanism of exosome encapsulation to develop its therapeutic potential. In this review, we will explore the particular processes through which exosomes naturally encapsulate miRNA, as well as discuss the effect on tumors after encapsulation of miRNAs. We also summarize the effects of targeted drug delivery using genetic engineering and chemical methods to modify exosome-encapsulated miRNA. Finally, gaining insight into how exosome cargo is sorted could be applied in clinical settings for precise drug delivery and to hinder the progression of diseases.

Keywords: exosomes miRNA, sorting mechanism, tumor microenvironment, cancer

Introduction

Extracellular vesicle (EVs) can be divided into ectosomes and exosomes.[1,2] Ectosomes are created through the process of budding from the outer membrane. Exosomes are a type of membrane vesicles that range from 30 to 100 nm.[3] Exosomes can carry a range of cellular elements, including DNA, RNA, lipids, metabolites, and cytoplasmic, as well as cell surface proteins. To date, the Exo Carta exosome database (http://www.exocarta.org) has collected 9769 proteins, 3408 mRNAs, 2838 miRNAs, and 1116 lipid entries that have been identified in exosomes from different types of cells and multiple organisms. The protein components of exosomes including membrane transport and fusion-related proteins (such as Rab, Annexins, GTPases), heat shock proteins (such as HSP70, HSP90), tetraspanins (such as CD63, CD9, and CD81), ESCRT complex-related protein (such as ALIX, TSG101), and from antigen-presenting cells (such as CD45, MHC-II).[4,5]

At first, the first scientific question asked about exosomes is: how are exosomes formed? It is believed that the physiological function of exosome production by cells is to help eliminate surplus and unneeded materials, thereby supporting intracellular balance. With advances in the field of EVs, increasing data support the notion that tumor-derived EVs not only play a role in promoting changes in the microenvironment, thereby affecting tumor progression but also may lead to malignant transformation by inducing changes in cells outside the primary tumor.[6,7] The process of synthesizing exosomes consists of three key stages: (1) The extracellular components such as proteins, lipids, metabolites, small molecules etc can enter cells together with cell surface proteins through endocytosis, forming the early endosomes. The early endosome is the first vesicle formed when the plasma membrane invaginates and pinches off to enter the cell. (2) Early endosomes can transfer materials to other organelles to further form intracellular multivesicular bodies (MVBs). (3) MVBs can be broken down through fusion with autophagosomes or lysosomes, or by merging with the plasma membrane that eventually forms exosomes[8] (Figure 1).

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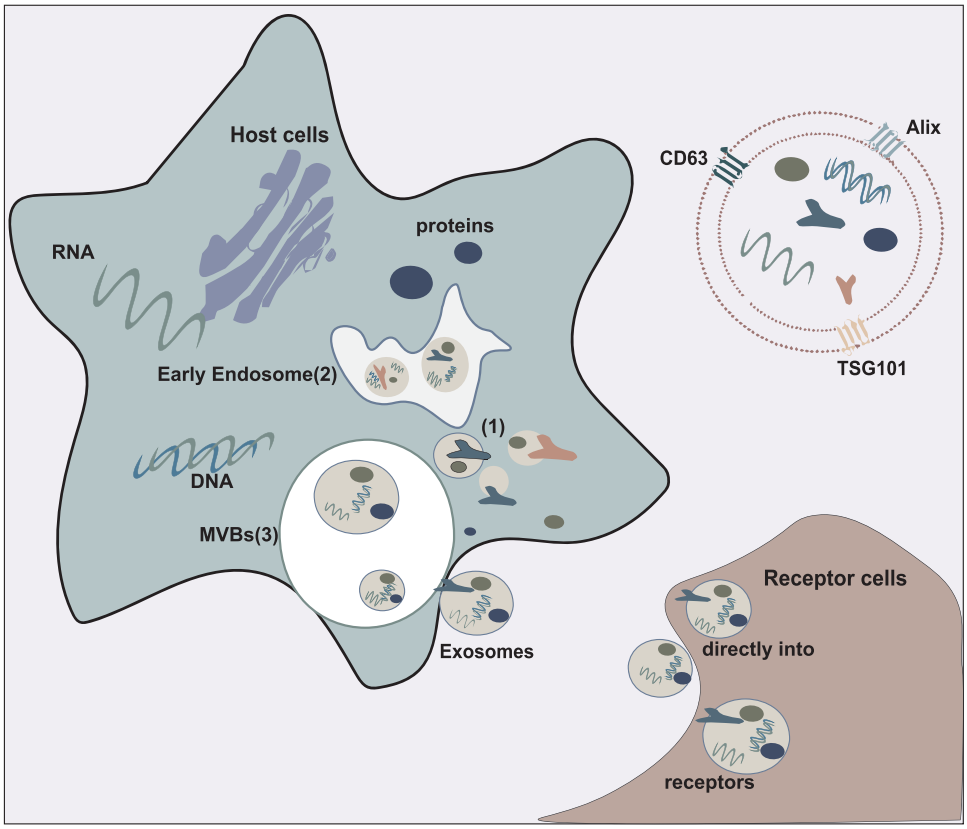

Figure 1 Graphic depiction of subtypes of extracellular vesicle (EVs) secreted by a cell.

With the discovery of exosomes, discoveries lead to the second scientific question: How to identify exosomes? The International Society of Extracellular Vesicles primarily identifies exosomes based on three key criteria.[8] 1) Morphology of exosomes: recognizing exosomes by transmission electron microscopy (TEM). Negative staining under high magnification to observe the exosome.[9] 2) The size of exosomes: particle size analysis is a versatile method for measuring exosomes.[10] 3) Biomarkers of exosome: the marker protein of the exosome was detected, and the exosome was identified from protein level. Commonly used exosome marker proteins include CD63, TSG101, and Alix.[11,12] 4) Exosomes membrane proteins CD9 and CD63 were identified by nanoflow cytometry[13] (Figure 1).

MicroRNAs (miRNAs) are small RNA molecules that are in the length range from 18 to 25 nucleotides. They can regulate gene expression after transcription by attaching to the 3′ UTR of target mRNA. They play a crucial regulatory role in cell growth, differentiation, migration, and progression of diseases. Pri-miRNA, which is the initial transcription of miRNA found in the nucleus, is processed by Drosha into pre-miRNA that has a hairpin configuration. Subsequently, pre-miRNA is moved from the nucleus to the cytoplasm by the transporter known as exportin-5. The mature miRNA is further cleaved by the Dicer enzymes.[9] Mature miRNAs along with other proteins form RNA-induced silencing complexes (RISC) that result in mRNA degradation or the translational inhibition of interest.[10]

With the discovery of microRNAs in exosomes and their transfer to cells, increasing attention has been directed to the important roles of exosomes. Exosomes can be released and transferred into target cells through ligand–receptor interactions, adhesion to the surface of recipient cells, endocytosis by recipient cells, or direct fusion via vesicles and cell membranes. At this point, exosomes complete the transfer from cell to cell.[8] There are two main ways in which exosomes exert their biological effects: one is direct effect. Protein molecules or lipid ligands present on the surface of exosomes directly stimulate receptors on target cells, resulting in the formation of signal complexes and the activation of intracellular signaling pathways. The second is delivery. Exosomes can either fuse with the plasma membrane of a target

cell or directly enter the cell, delivering proteins, nucleic acids, lipids, and other active substances, which then influence the cell's functions.[11]

The expression profiles of miRNA and mRNA in exosomes are distinct from parent cells.[12] Thus, cells may have an active selection mechanism for exosomes and their cargo. In addition, the function of the transferred exosome molecular components in the recipient cells is also under investigation. Therefore, to further the study of exosome biology and comprehend the functions of exosomes miRNAs, this review centers on this article will briefly introduce the sorting mechanism and engineering of exosomes miRNAs, and discuss the effect on tumor after encapsulation of miRNAs.

Exosomes-Derived miRNA at the Crossroads of Cancer: Friends or Foes?

Exosomes, which are extracellular vesicles, serve as communication links between cancer cells and various elements of the tumor microenvironment (TME). They play a crucial role in this interaction by encapsulating miRNA. Exosomes become new biomarkers and are transported to the tumor microenvironment for their carcinogenic or anti-carcinogenic effects.

Dark-Side of Exosomes-Derived miRNA in Cancer

Tumor cells and tumor-associated macrophages (TAMs) through exosomes released non-coding RNAs, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), play a wide range of regulatory roles in the TME. They can affect the proliferation, migration, and invasion of tumor cells, while regulating the activity and function of immune cells, thereby altering the immune status of the TME. In addition, they are also involved in tumor angiogenesis and the formation of drug resistance, profoundly the development and progression of tumors.[13] During tumor progression, miRNAs derived from primary tumor exosomes can be transferred to non-malignant cells in the tumor microenvironment to induce heterogeneity.[14,15] The heterogeneity of TME by exosomes-derived miRNAs is mainly manifested in that exosome miRNAs that can activate cancer-associated fibroblasts, thereby remodeling ECM, which facilitates the spread of cancer cells. Exosomes-derived miRNAs also mediate inflammatory cell infiltration and immune escape, which is conducive to colonization and proliferation of cancer cells.[16] Exosomes-derived miRNAs can mediate multiple signaling pathways and tumor cells can suppress the maturation and differentiation of immune cells, thereby creating the microenvironment suitable for tumor growth.[17] TAMs in tumors usually exhibit an M2 phenotype, which is generally associated with poor prognosis.[18] TAMs play a big role in the proliferation and migration of tumor cells and counteract the cytotoxic effects of T cells and NK cells promoting cancer cells to escape immune surveillance.[19] Mutant p53 colorectal cancer cell-derived exosomes-derived miR-1246 induces M2 polarization of macrophages and remodel the TME by increasing the expression of IL-10, TGFβ, and MMPs.[20] Exosomes-derived miR-301a-3p from hypoxic pancreatic cancer cells activates the PTEN/PI3Kγ signaling pathway triggering M2 phenotype polarization in macrophages.[21]

Increasing studies have revealed that exosomes are closely associated with tumor immunity and regulate tumor immunity and immunotherapy. Tumor derived miRNAs can be packaged into exosomes that metastasize to tumor-infiltrating lymphocytes (TILs) and form an immunosuppressive microenvironment.[22] The cells involved in TIL mainly including T-lymphocyte (T cells), B lymphocytes (B cells), Natural Killer cells (NK cells), dendritic cells (DC cells), and others.[23] In cancer, exosomes develop new abilities to suppress the immune response that leads to immune cell dysregulation and immune escape, which facilitates the advancement and spread of cancer. Exosomes-derived from tumor cells containing miR-30a-5 regulates the ubiquitination of PD-L1 and suppresses the activity of CD8+ T cells to promote colorectal cancer immune evasion.[24] Exosomes-derived from bladder cancer cells contain miR-221-5p and miR-186-5p induce NK cell dysfunction and evade immune surveillance.[25] M2 macrophage derived exosome-derived miR-155-5 induces up-regulation of ZC3H12B expression and IL-6 promotes immune evasion leading to the occurrence of colon cancer.[26] Exosome-derived miR-3591-3 induces macrophage M2 to promote glioma progression.[13] Exosomes derived miR-690 released from melanoma can directly activate the apoptosis of CD4 T cells, therefore accelerating the growth of mouse melanoma cells.[27] Gastric cancer exosomes-derived miR-27a induces fibroblast reprogramming into cancer-associated fibroblasts (CAFs)[28] (Figure 2).

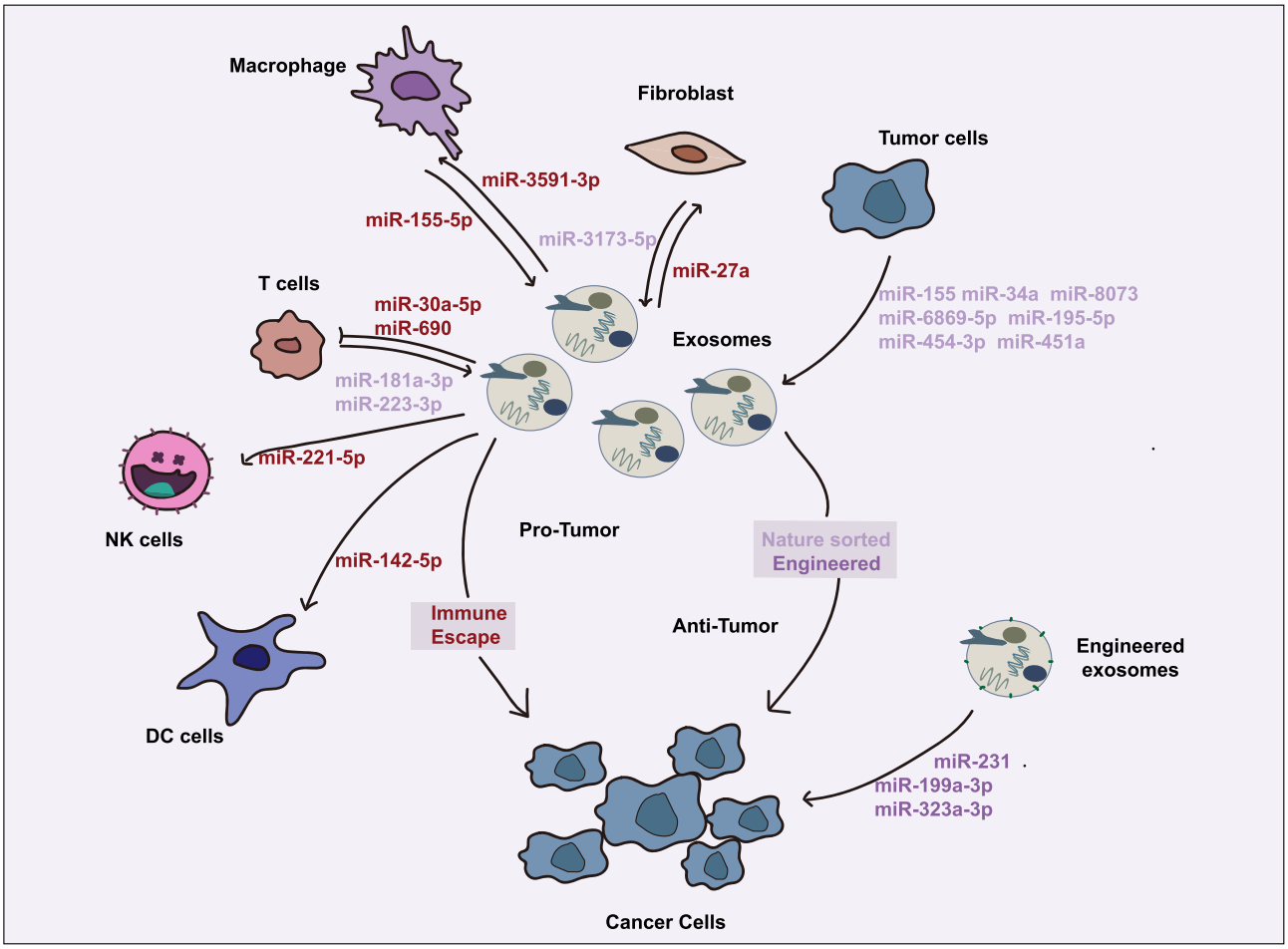

Figure 2 Exosomes-derived miRNAs function in promoting or inhibiting cancer.

The immune modulation triggered by miRNAs in exosomes is complex and versatile. Within the TME, tumor cells interact a diverse array of immune cells, collectively exacerbating the immunosuppressive effect. Although exosome-derived miRNAs play a central role in this process, the mechanisms remain unclear. Therefore, it is of great research value to further explore the functions of exosome-derived miRNAs in the interaction between cancer cells and the host system.

Exosome-Derived miRNA Could Inhibit Tumorigenesis

Exosomes-derived miRNAs can serve as critical tools for intercellular communication, and the exosome-mediated transfer of oncogenic or tumor suppressive miRNAs can impact tumorigenesis.[29] In addition to promoting tumorigenesis, miRNAs carried by exosomes also participate in tumor suppression. miRNAs, when encapsulated within exosomes, facilitate their intercellular communication and transfer, which can exert suppressive effects on cancer development.[30] Since exosome proteins govern vesicle contents, to maintain their oncogenicity and metastasis, tumor cells tend to sequester anti-tumor miRNAs and load them into exosomes.[31]

Exosome-derived miRNAs can exert tumor suppressive effects by directly targeting and inhibiting the expression of oncogenes. Mir-3173-5p derived from CAF exosomes sponges ACSL4 and inhibits iron phosphorylation in pancreatic cancer cells.[32] Tumor-derived exosome miR-34a inhibits associated gene expression with invasion, angiogenesis, and immune evasion in colorectal cancer.[33] Exosome-derived miR-8073 inhibits colorectal tumor volume, suggesting that it has a broad application prospect in tumor treatment.[34] Exosome-derived miR-6869-5p inhibits the growth of colorectal cancer cells and inflammatory cytokine production through targeting TLR4.[35] BEAS-2B cells derived exosome-derived

miR-195-5p significantly inhibited the proliferation, migration, and invasion of lung cancer cells.[36] Exosome-derived miR-454-3p inhibits the proliferation, migration, invasion, and autophagy of glioma ATG12 cells.[37] Exosome-derived miR-451a targets LPIN1 and inhibits tumorigenesis by regulating cell apoptosis and angiogenesis[38] (Figure 2).

Exosome-derived miRNAs can also regulate immune responses and indirectly suppress tumor growth by affecting the function and activity of immune cells. T cell-derived exosomes miR-181a-3p and miR-223-3p significantly reduce the expression of PD-L1 using IL2 surface engineering and inhibit tumor progression in melanoma cells.[39] Exosome-derived miR-1468-5p epigenetically activates the JAK2/STAT3 pathway in LECs by directly targeting homeobox containing HMBOX1 in the SOCS1 promoter, activating an immunosuppressive program that allows cancer cells to escape anti-cancer immunity[40] (Figure 2).

Since exosome-derived miRNAs play an important role in tumorigenesis and progression, they have the potential to be targets for cancer therapy. Regulating the expression and function of exosome-derived miRNAs, the growth and metastasis of tumors can be inhibited, and the effectiveness of treatment can be improved.[41] Breast cancer cell-derived exosomes miRNA-231 suppressed proliferation and migration for lung cancer cells in the blood.[42] MiR-199a-3p inhibits the proliferation and invasion of ovarian cancer cells by electroporation loading into exosomes.[43] Engineered exosomes miR-323a-3p are tumor-suppressive targeting EGFR and TYMS in colorectal cancers[44] (Figure 2).

miRNA Was Not Randomly Packaged into Exosomes

Exosomes and other EVs offer a distinct method of communication between cells, allowing miRNA generated and released by one cell to be absorbed by a distant cell, potentially changing its gene expression. In our previous study, we performed miRNA sequencing on exosomes and cells in esophageal squamous cell carcinoma (ESCC) cell lines. Among the various miRNAs that showed different expression levels, miR-451a was found to be more abundant in exosomes compared to ESCC cells. Additionally, miRNA pull-down experiments and combined exosome proteomic data revealed that miR-451a has a relation with YWHAE. The excessive level of YWHAE results in the accumulation of miR-451a within the exosomes rather than in the donor cells. We discovered that miR-451a was packaged into exosomes via YWHAE[45] (Figure 3). Interestingly, researchers found that miRNAs have specific sequences that determine whether they are secreted into extracellular vesicles, such as exosomes, or retained in the cells. These specific sequences are called exosome motifs (released exosomes) and cell motifs (promoting cell retention). Different cell types, including white and brown fat cells, endothelial cells, liver, and muscle, preferentially use specific sequences. By inserting or deleting these

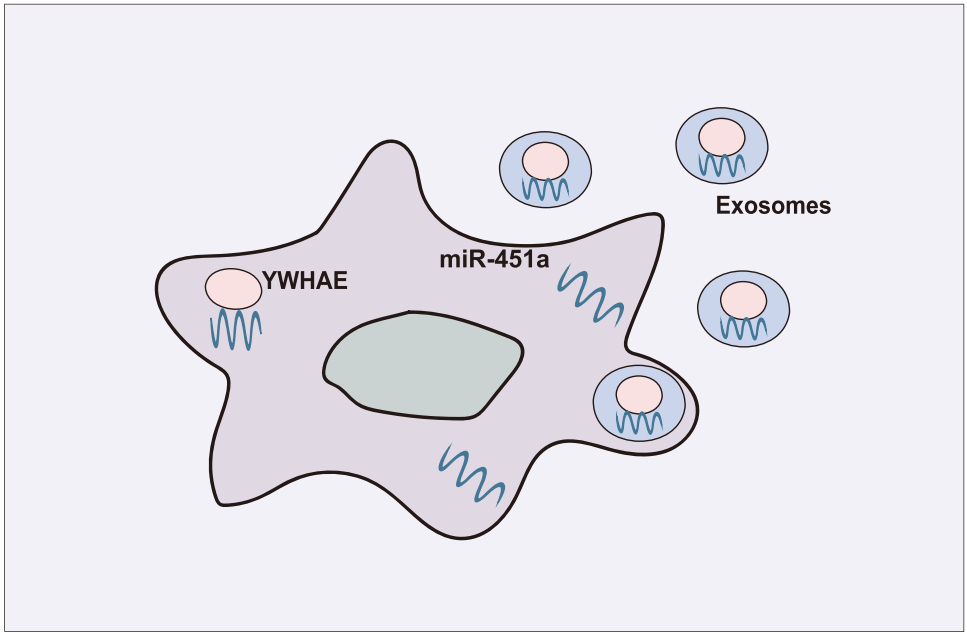

Figure 3 The mechanism of miR-451a sorted into exosomes by YWHAE in ESCC.

specific sequences in miRNAs, the enrichment of miRNAs in cells or extracellular vesicles can be affected, regulating their biological functions.[46] There were significant differences between intracellular and extracellular miRNA profiles. The exosomes from different cell types including T cells, B cells, and dendritic immune cells contain a different miRNA repertoire than their parental cells. The study reveals that T cells influence the function of antigen-presenting cells (APCs) by releasing exosomes loaded with miRNAs, thereby regulating their-presenting ability and immune response. This is of great significance for maintaining the homeostasis of the immune system and regulating immune responses.[47]

According to current research, Zhong et al analyzed miRNA expression levels of the cell lines and their exosomes using microarray. The findings indicated that the expression levels of most miRNAs in exosomes were lower than those in cells. However, some miRNAs were highly enriched in exosomes, indicating that many miRNAs are concentrated in exosomes. Exosomes released from drug-resistant breast cancer cells are also rich in specific miRNAs and may affect the functions of other cells through exosome-mediated delivery, providing new insights into the molecular mechanisms of breast cancer resistance.[48] It has been proposed that specific miRNAs might be selectively exported. By comparing the miRNA profiles released by different cell types, an overlapping set of miRNAs has been identified, which can be transferred to target cells via extracellular vesicles, thereby affecting the function of target cells and regulating intercellular interactions and biological behavior.[49]

All these studies suggest that specific sorting mechanisms exist for miRNAs into exosomes. As some profiling studies have shown, miRNAs are not randomly incorporated into exosomes. There is an active sorting mechanism for miRNA into exosomes. However, the mechanism by which miRNAs are sorted into exosomes remains unknown.

The Sorting Mechanisms of miRNA in Exosomes

Based on the current research, there are various possible ways that miRNAs may be sorted into exosomes, but the mechanisms are still not well understood. The summary is as follows.

ESCRT-Dependent Mechanism

ESCRT is essential for controlling the development of MVBs. It includes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, Vps4-Vta1, and Alix. It is mainly involved in two repair pathways: budding and MVB.[50] There is recent evidence that ESCRT-0 and inclusion proteins form microdomains on endosomal membranes and enrich ubiquitinated cargo proteins. ESCRT-I and ESCRT-II induced MVB vesicle budding promoted vesicle formation and sorted cargo proteins into vesicles. During the process of membrane budding and vesicle formation, ubiquitinated cargo proteins (such as miRNA-protein complexes) are selectively sorted into exosomes.[51] ESCRT-III shrinks and shears the bud neck to complete the final membrane-shedding process. Vps4 dissociates ESCRT for recycling.[52,53]

In addition to this mechanism, ESCRT-independent pathways can still guide exosomes sorting miRNAs. It has a different cargo composition and properties than the ESCRT-dependent mechanism.

ESCRT-Independent Mechanism

RNA Binding Proteins Related Mechanism

Almost all the RNA in the cell is in the form of Ribonucleoprotein complex (RNP).[54] Some studies have shown that specific proteins may control the sorting of miRNAs by recognizing and binding to specific miRNA sequences.[55]

Y-Box Binding Protein 1 (YBX1), a key RNA-binding protein, can specifically recognize and bind to various microRNAs, thereby allowing them to be packaged into exosomes.[56] It has been considered that YBX1 could recognize and bind specific miRNA motifs and control its sorting into exosomes, such as ACCAGCCU, CAGUGAGC, and UAAUCCCA.[57] It has also been proved that miR-223 specifically binds to YBX1 through the 5′ proximal sequence motif UCAGU, resulting in it sorted into exosomes from mitochondria.[26,58] Furthermore, hnRNPA2B1 specifically recognizes and binds to a particular sequence GGAG, selectively interacts with miR-198, and is regulated by its SUMOylation modification status. It guides miRNAs into exosomes, thereby facilitating the transfer of miRNAs between cells.[59] However, hnRNPA2B1 can specifically bind and inhibit miR-503 sorted into exosomes, thereby regulating its intercellular transmission.[60] Other RNA binding proteins, such as SYNCRIP has been shown to interact with specific microRNA molecules, forming a stable complex by binding to the miRNA sequence. This complex is packaged into

exosomes within hepatocytes and released outside the cells with the secretion of exosomes, thereby affecting intercellular communication and gene expression regulation by controlling delivery of microRNAs.[61] The recent research showed that the RNA-binding protein FMR1, which identifies miRNA sequences through its interaction with elements of the endosomal sorting complex required for transport (ESCRT) pathway, is packaged into exosomes.[62] Besides that, MVP selectively mediated miR-193a sorted into exosomes promotes colon cancer progression[63] (Figure 4).

Consequently, various earlier studies have shown that miRNA motif interactions with RNA-binding proteins (RBP) are involved in the process of sorting exosomes, which has been validated in various cell types.

MiRNA Sequence-Dependent Related Mechanism

Ruben Garcia-Martin et al described that the role of miRNA sequences in regulating exosomes, in terms of their release and formation. The research has found that specific miRNA sequences can affect the formation and release process of exosomes, thereby regulating the transmission of these vesicles between. Inserting or deleting cell-motif (AGAAC) or exosome-motif (CGGGAG) into miRNAs increases or decreases their retention in the cells producing or secreting exosomes.[46] While the structure of the RNA 5′ end, the formation of the 5′ cap, and the mechanisms of RNA processing are well understood, there is limited knowledge regarding 3′ end processing. Recent information indicates that the processing of the 3′ end is facilitated by the exosome.[64] Exosome-derived miRNAs are enriched in 3'UTR sequences and appear to be required for specific mRNA loading into exosomes.[65] The 3′ end of a miRNA can be added either uridylated or adenylation without a template. The modification can occur on the mature miRNA, and its effects can affect the biosynthesis of miRNA, stability, and the efficiency of targeting mRNAs, increasing the scope of miRNA or more granular role in gene expression regulation.[66] miR-1289 can recognize and bind to these CUGCC core sequences, thereby promoting the recruitment of mRNAs into exosomes[67] (Figure 4).

miRISC Related Mechanism

As is well known, mature miRNAs can engage with proteins to create microRNA-induced silencing complex (miRISC). The main components of miRISC including miRNA, miRNA-repressed mRNA, GW182, and Argonaute 2 (AGO2). The AGO2 protein is likely to which prefers to bind to U or A at the 5′ end of miRNAs, playing a critical

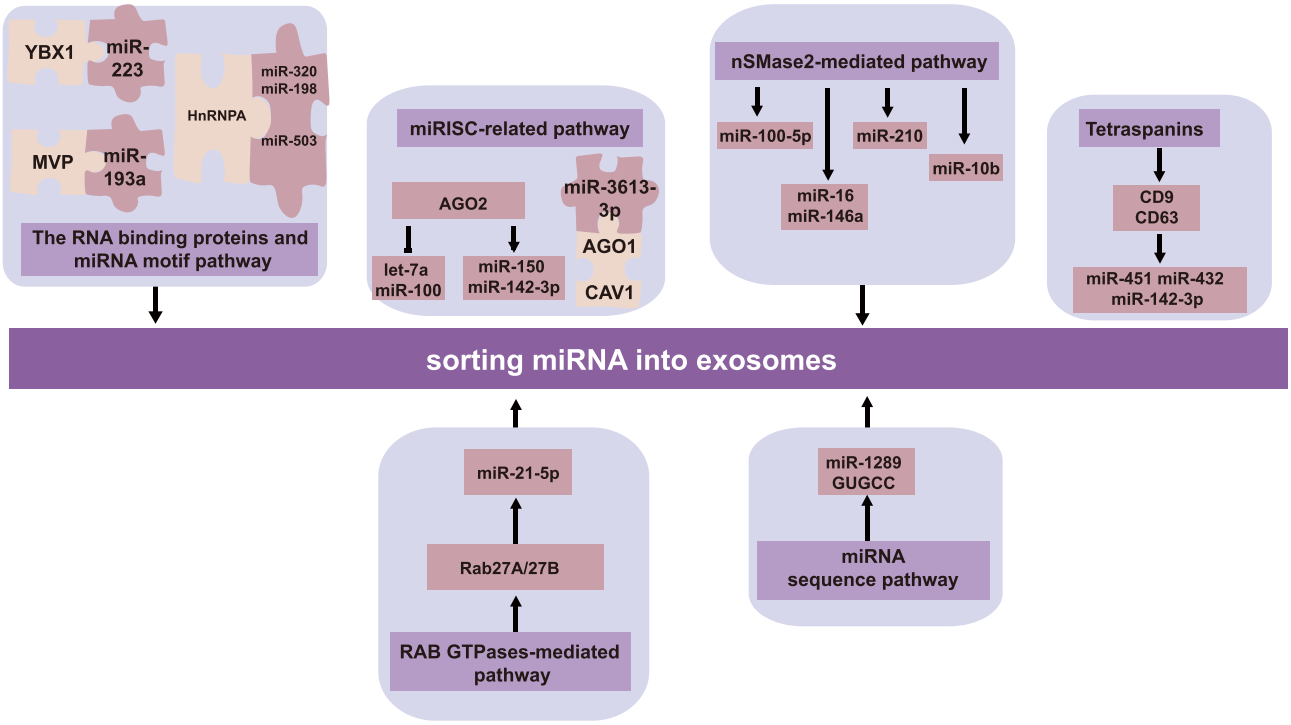

Figure 4 The sorting mechanism of miRNAs into exosomes.

role in a crucial role in miRISC.[68] AGO2 first binds to miRNA to form the AGO2-miRNA complex. This process requires the assistance of trans-activation response TAR RNA binding protein (TRBP) and Dicer enzyme, where TRBP can recruit the Dicer complex to AGO2, promoting the loading of miRNA.[69] Once the AGO2-miRNA complex is formed, it can recognize and bind to target mRNA through the of base complementary pairing.[70] Another important role of AGO2 in miRISC is to cleave target mRNAs. Upon binding of the AGO2-miRNA complex to target mRNAs, the endonuclease activity of AGO2 is activated, leading to cleavage of target mRNAs at specific sites subsequent degradation of the target mRNAs.[70] The finding showed that the function of AGO2 in miRISC is also regulated by other proteins. For example, proteins such as GW82 can interact with AGO2 to enhance the silencing activity of miRISC.[71] AGO2, through the miRISC complex, participates in miRNA-mediated gene silencing, thereby affecting the expression of tumor-related genes. The regulatory mechanism plays a key role in the occurrence, development, and progression of tumors.[72]

It has been recently reported that AGO2 was first recognized as a protein associated with membranes, and certain miRNAs have been discovered in exosomes.[71] Many studies have shown that different types cells are able to secrete exosomes containing a specific combination of miRNA. These miRNAs are detected in the exosomes of various cells, forming a set of overlapping miRNAs. AGO2, as a core component of the miRISC, not only participates in the cleavage of miRNA and target recognition but also affects the process of miRNAs being packaged into exosomes. By deep sequencing and RT-qPCR, it was found that there is a close interaction between AGO2 and these selectively transported miRNAs. Knockdown of AGO2 can reduce the levels of miR-150 and miR-142-3p in exosomes.[49] Recent evidences point that AGO2 did not affect the number of exosomes but affected the content of miRNA in exosomes. The KRAS-MEK signaling pathway regulates the sorting process of AGO2 protein, affecting its efficiency of entering exosomes, thereby changing the abundance of and it associated the specific miRNAs such as let-7a and miR-100 into exosomes.[73] When AGO2 interacts with CAV1, AGO2 is recruited to the plasma membrane, which may facilitate its binding to miR-3613-3p and promote the release and transfer of miRNAs through exosomes, thereby promoting cancer metastasis[74] (Figure 4).

Tetraspanins Related Mechanism

Tetraspanins are the major exosome marker proteins, located on endosomes, MVB, and exosomes, and are thought to be the way to transport intracellular cargo into these vesicles. This is a class of membrane proteins highly enriched in exosomes, which by altering the physical structure and microdomains of the membrane, and through their cytoplasmic domains, regulate the entry of proteins into exosomes.[75] They may be involved in the formation of specific membrane microenvironments that favor the sorting and of specific miRNAs. The finding suggests that the transmembrane 4 superfamily is a part of the membranes of exosomes and is crucial for the process of membrane fusion, cell transport, and membrane recognition.[76] Several transmembrane 4 superfamilies, such as CD63, CD81 and CD9 have been used as markers for exosomes.[77]

The recent research showed that the presence of endometrial epithelial cells via the menstrual cycle is periodically regulated by the Transmembrane 4 superfamily, CD9, and CD63: CD63, which act as markers on the surface of exosomes. Two hundred and fourteen miRNAs were exosomes and cell shared, whereas 13 miRNAs were exosomes including miR-451, miR-432, miR-142-3p, and so on and 5 of miRNAs were cell-specific. These data suggest that some specific miRNAs are sorted into exosomes[78] (Figure 4). Tetraspanins, as key components of the exosome membrane, not only participate in the formation and release of exosomes but also play critical role in the interaction between exosomes and target cells. Sanyukta Rana et al described that tetraspanins on the surface of exosomes can recognize and bind to specific cell receptors, thereby mediating the selective delivery of exosome-derived miRNAs.[79] This finding showed that CD63 not only participates in membrane vesicle formation and sorting mediated by the ESCRT-dependent pathway, also plays a key role in ESCRT-independent pathways. CD63 is involved in two different endosomal sorting processes for a single cargo during the formation of LROs.[80] In addition, tspan6 supports the lysosomal degradation of Syndecan4 and Syntenin inhibits the shedding of the Syndecan4 extracellular domain.[81] The results showed that Tspan8 contributed to the selective recruitment of proteins and miRNA into exosomes.[82]

nSMase2 Related Mechanism

The Neutral sphingomyelinase 2 (nSMase2) related mechanism is one of the potential mechanisms for miRNA sorting into exosomes. Aude et al found lipid raft microregions on exosome membranes, suggesting that they may be involved in vesicle formation and structure.[83] Exosomes contain high levels of cholesterol, sphingolipids, phosphatidylserine, and ceramide, resembling the composition of membrane lipid rafts.[40] nSMase2 plays a key role in the formation of exosomes and the packaging of miRNA in cells. nSMase2 hydrolyzes sphingomyelin (SM) to produce ceramide, which is due to its cone-shaped structure. It is essential for the budding and release of exosomes. These studies describe that the expression level of nSMase2 is positively correlated with the miRNA content of exosomes.[84–86] There is research indicating that the nSMase2 ceramide pathway can control the sorting of multiple substances by exosomes by blocking nSMase2 and knocking down nSMase2 expression with the exosome inhibitor GW4869.[87] Additionally, 17β-estradiol has a significant impact on the selective loading of miRNAs, which may affect the sorting and packaging mechanisms of miRNAs, to the preferential loading of specific miRNAs into exosomes. Physiological levels of 17β-estradiol specifically promote the secretion of EVs in ER BC cells by inhibiting miR-149-5p, blocking its regulatory activity on SP1, a transcription factor for the EV bi factor nSMase2.[88] However, the exact molecular basis of this has not been fully elucidated and requires further investigation.

As mentioned above, reduced α2,6-sialylation impairs nSmase2 activity and nSmase2-dependent exosome-derived miRNA sorting. In addition, α2,6-Sialylation-mediated exosome-derived miR-100-5p sorting promotes migration and invasion of recipient HepG2 through the PI3K/AKT signaling pathway.[89] Furthermore, the secretion and transfer of miRNAs is a complex and tightly regulated process that involves the participation of various molecules and signaling pathways. Moreover, it was reported that nSmase2 has the ability to initiate the release of exosomes. Overexpression of nSmase2 increased the expression of extracellular miR-16 and miR-146a.[90] The studies show that nSmase2 can regulate the secretion of exosomes-miR-210 and the released exosomes-miR-210 can be transported to endothelial cells to promote angiogenesis. [91] Notably, nSMase2 or ceramide promotes exosome-mediated secretion of miR-10b, while ceramide inhibitors suppress this secretion. In addition, miR-10b can suppress its target genes HOXD10 and KLF4 to promote breast cancer cell invasion[92] (Figure 4).

RAB GTPases Related Mechanism

On the other side, growing evidences indicate that the release of exosomes is achieved through a series of well-organized membrane kinetic processes. First, the MVB limiting membrane sprouted inward to the lumen side to form. Certain MVBs are subsequently moved to the plasma membrane, and the secretory MVBs ultimately merge with the plasma membrane, resulting to the release of exosomes from the cell. Therefore, secreted MVBs must be selectively transported to the plasma membrane through certain mechanisms.[93] It has been proved that Rab GTPases were monomeric GTP-binding proteins, containing about 200 amino acids. The human genome encodes more than 60 Rab. Some of these types have been identified as direct regulators of MVB transport to the plasma membrane. Each Rab exhibits specific intracellular localization and regulates different steps of intracellular transport.[94] This protein can directly or indirectly bind the transported molecule into the vesicle.[95]

Notably, it is shown that Schwann cells exosome-derived miR-21-5p enhanced the growth, motility, and invasiveness of human lung cancer cells, which depends on the active Rab small GTPases Rab27A and Rab27B in stem cells for the release of exosomes[96,97] (Figure 4).

Genetically Engineered Exosomes for Targeted Delivery

Compared with natural exosomes, gene-engineered exosomes have enhanced drug loading efficiency, targeting, and resistance clearance by the body. Typically, the size and shape of these exosomes do not change significantly, but depending on different research purposes, their membrane cargo or contents can be significantly different.[98,99] Indeed, gene-engineered exosomes are a type of exosomes obtained through biotechnological processing and optimization. They possess the biocompatibility low immunogenicity of natural exosomes and can also be modified using gene engineering techniques to achieve more efficient drug delivery and targeted therapy.[100] By genetically engineering exosomes, it is possible to achieve precise delivery miRNAs, offering a new strategy for cancer treatment.[101] Gene-engineered exosomes

are modified through methods such as gene editing, endogenous engineering, exogenous engineering, and mixed engineering. These methods exosomes to carry specific miRNAs and deliver them to target cells through intercellular transfer. This approach not only avoids the toxicity of artificially synthesized nanoparticles but increases the bioavailability and biocompatibility of drugs.[102] Gene-engineered exosomes are a type of exosomes obtained through biotechnological processing and optimization. They possess the biocompatibility low immunogenicity of natural exosomes and can also be modified using gene engineering techniques to achieve more efficient drug delivery and targeted therapy. Gene-engineered exosomes show great potential in disease diagnosis and treatment. Due to their unique biological characteristics and targeted delivery ability, they can serve as ideal nanocarriers for carrying and various therapeutic molecules, such as proteins, nucleic acids, small molecule drugs, etc.[40]

The study showed that the gene-engineered M1 macrophage exosomes, by promoting M1 polarization and targeting the IL-4 receptor, can inhibit tumor growth. It significantly inhibited tumor growth by reprogramming tumor-associated macrophages into M1-like macrophages.[103] Targeted inhibition of SIRT6 by gene-engineered blocks prostate cancer tumorigenesis and metastasis.[104] These genetically modified exosomes can significantly improve the function and activity of T cells, thereby enhancing their anti-tumor ability. It reverses the exhaustion of T cells in cancer immunotherapy.[105] In cancer treatment, gene-engineered exosomes can carry chemotherapy drugs to achieve precise targeting of cancer cells. By modifying the targeting peptides on the surface of exosomes, they can better recognize and bind to cancer cells, thereby improving treatment effectiveness and reducing side effects.[106]

Conclusion and Future Perspectives

This article reviews some crucial role of exosome-derived miRNAs in the occurrence, development, and metastasis of tumors. It can regulate the gene expression of cells, affecting the biological behaviors of cells such as proliferation, apoptosis, migration, and invasion. The sorting mechanism of miRNA into exosome is a complex and process, involving the participation of various molecules and mechanisms. Exosome-derived miRNAs have potential application value in tumor treatment, providing new strategies for early diagnosis, prognosis, and precision treatment of tumors. By studying the sorting mechanisms of miRNAs, we can develop exosome-based therapies for specific diseases. For instance, by modifying the composition of exosomes or loading them with specific miRNAs, we can achieve precise treatment of diseases.

As a natural drug delivery carrier, exosomes can enhance the effectiveness of drugs in reaching specific cells or organs through genetic engineering and chemical modification, so as to limit the damage to drugs to normal tissues and cells and reduce the side effects of drugs.[107] Circulating microRNAs contained within exosomes are safeguarded by lipid bilayers, preventing degradation. They have the potential to be used as non-invasive diagnostic and screening methods for early cancer detection and to aid in treatment choices. Moreover, engineered exosomes can act as therapeutic carriers for the precise delivery of RNA interference (RNAi) molecules, allowing them to avoid recognition by the immune system.[108] Despite the many advantages of genetic engineering exosomes, their clinical application is still in its infancy, facing numerous challenges before their antitumor potential can be clinically exploited.

(1) At present, most studies focus on developing exosome systems and demonstrating their potential through in vitro and animal models. However, how to overcome the technical difficulties in the production process and achieve large-scale production and of exosomes remains a pressing issue that needs to be addressed.

(2) In the research of genetically engineered exosomes, how to efficiently encapsulate specific nucleic acid drugs is a key issue. Researchers have developed various to improve the encapsulation efficiency, such as using synthetic biology methods to engineer the chassis cell genome, enhancing the expression of a series of genes involved in exosome and secretion.

(3) The biocompatibility and safety of drugs associated with exosomes are crucial issues that require attention through the help of advanced technological tools at present. On the issue of biosafety of exosomes, in the process of achieving targeted therapies, exosomes may also bring other information from donor cells, such as genes involved in tumorigenesis, and metastasis, into the target cells/organs; therefore, it is necessary to have a full understanding of the mechanism of exosome production and the selection of donor cells used to produce exosomes.

(4) The application of exosomes encapsulating miRNA in disease treatment still faces some challenges. For instance, how to ensure that exosomes can accurately the diseased site and avoid damage to normal tissues; how to monitor and evaluate the therapeutic effect of exosomes encapsulating miRNA, as well as how to its administration method and dosage, etc. These issues all need further research and exploration.

In conclusion, this review provides the role of exosomes-derived miRNA in cancer, an overview of the selective packaging of miRNAs into exosomes, and the applications of engineered exosomes carrying miRNA in therapy while also pointing out some limitations. The preparation process of genetically engineered exosomes can be achieved through two methods: cell engineering and exosome engineering. However, both methods have certain, such as low yield and complex purification process. Therefore, how to optimize the preparation process, improve yield and purity, is one of the current research focuses. Future research should focus on establishing quality standards for all aspects of exosome product development, including production, purification, storage, and transportation, to ensure product stability and safety. Additionally, personalized engineered exosome treatment plans can be developed to improve treatment efficacy and reduce effects. Addressing these issues will aid in the development of fullerene derivatives, which can be clinically applied in tumor treatment.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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