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Examining the evidence for exRNAs as communication molecules and emerging cancer biomarkers: a new therapeutic strategy

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Abstract: Extracellular RNAs (exRNAs), a unique form of RNA within the body, serve as carriers of genetic and metabolic information, providing real-time insights into cellular status. Their ability to act as biomarkers makes them valuable for disease diagnosis, treatment, and prognosis. exRNAs can be transported via extracellular vesicles, functioning as signaling mediators in cell-to-cell communication. Tumor cells exhibit heightened vesicle release compared to normal cells, thereby facilitating tumor progression. Leveraging their ease of detection, non-invasive molecular diagnostic technologies can be utilized. This short review presents an overview of exRNAs types, examines the testimony supporting the existence of functional exRNAs in mammals, especially traditional Chinese medicine (TCM)-derived dietary microRNAs with special functions in maintaining health and curing diseases like cancer. In addition, we briefly discuss novel approaches for tumor diagnosis and treatment, and the challenges in this field. We highlight the transformative potential of exRNAs as clinical biomarkers and novel cancer therapeutic strategies.

Keywords: exRNAs; intercellular communication; traditional Chinese medicine; dietary microRNAs; biomarker; cancer therapeutic strategy

1. Introduction

Previously thought to solely exist within cells, RNA is now acknowledged to be involved in a range of intricate cellular processes. Recent studies have unveiled that RNA can be transported out of cells and is crucial in the molecular mechanisms of intercellular communication, which has altered our comprehension of RNA in cell biology and revolutionized the field of extracellular RNAs (exRNAs) biology [1,2]. Multicellular

organisms consist of a variety of types of cells that depend on transmitting or receiving signaling molecules from neighboring cells, either in close proximity or across long distances. The intercellular communication is vital for the functioning of multicellular life and is facilitated by various types of molecules [3]. Cells secrete substances such as cytokines, growth factors, peptide hormones, and neurotransmitters, which initiate corresponding significant physiological processes by interacting with specific receptors [4]. The factual presence of exRNAs in biological fluids gives rise to the idea that exRNAs participate in intercellular communication [5].

ExRNAs are defined as RNAs that are transcribed in the cell, referred to as the 'donor' cell, and are subsequently released into the extracellular environment [6]. Various forms of exRNA encompass both longer mRNA and lncRNA, alongside a range of small ncRNAs. Based on their biological functions, ncRNAs can be classified into two major groups: regulatory ncRNAs and housekeeping ncRNAs. Regulatory ncRNAs include lncRNA, miRNA, piRNA, siRNA, circRNA, tRNA-derived fragments, and Y RNA [7]. The cells that capture exRNAs from outside the cell are defined as 'recipient' cells [8–10]. It is important to note that the recipient cells may release or degrade exRNAs [11]. In fact, establishing the possible biological significance of exRNAs requires stringent experimental approaches. One of the main focuses of exRNA research is the exploration of their ability to enable intercellular communication and act as signaling molecules during normal cellular homeostasis or in the context of disease progression [12]. There is existing literature that substantiates the utilization of exRNA in the diagnosis and prognosis of cancer [13].

To gain a better understanding of RNAs as a means of intercellular communication, an extensive analysis is conducted on the regulatory mechanisms governing the functionality of small RNAs. Subsequently, we examined the endeavors to compile an extensive inventory of exRNAs in mammals. We evaluated the testimony supporting a variety of exRNAs carriers, such as ribonucleoprotein complexes, extracellular vesicles (EVs), and carefully scrutinized the existing evidence regarding the role of exRNAs, especially the TCM-based dietary miRNA in mammalian health and disease. exRNAs are currently under evaluation as biomarkers in various types of cancers, and this review aims to provide insights into the current state of research on exosomes and exRNAs as cancer biomarkers. In addition, we emphasized the importance of understanding the present status of exosome/exRNA-based cancer biomarker research and the demand for further investigation in this field.

2. Discovery of exRNAs

ExRNAs in mammalian cell biology

Evidence dating as far back as the 1970s demonstrated the presence of RNA in culture media derived from human or mouse cells [14,15] (Figure 1). Subsequent high-throughput RNA sequencing studies have provided abundant evidence indicating the presence of exRNAs in biological fluids, including urine, blood, and saliva. Unlike plants and C. elegans, mammals lack the functional proteins required for cell-to-cell RNA signaling, such as RNA-dependent RNA polymerase (RdRP) [16,17]. If exRNAs indeed exert an impact on mammals, it is likely

that they work through fundamentally distinct pathways compared to those observed in plants and C. elegans.

SIDT1 and SIDT2 encode proteins that interact with long double-stranded RNA and appear to be crucial for efficient antiviral response [18]. However, unlike their counterparts in C. elegans, these functional carriers do not facilitate the import of RNA [19]. Intriguingly, overexpressed proteins of SIDT1 and SIDT2 in cells facilitate the transfer of double-stranded RNA analogs from endosomes to cytoplasm. This phenomenon suggests that endocytosed RNAs are very likely to escape endosomes-lysosomes by transporting from endosomes to cytoplasm [18]. However, the experimental data in favor of SIDT1/SIDT2-mediated RNA transportation rely on synthetic chemical analogs with structures similar to double-stranded RNA [20], but the transcription was not in the donor cells and thus did not meet the definition of exRNAs[21].

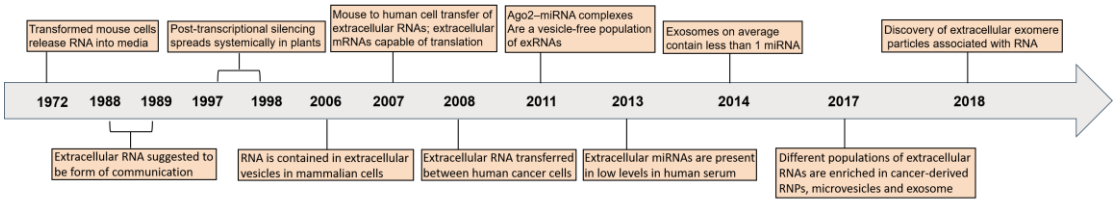

Figure 1. exRNAs discovery timeline. Focus on research affecting the field of exRNAs. RNP, ribonucleoprotein.

3. Carriers of mammalian exRNAs

The journey of exRNAs begins through the process of either secretion or breakdown of a cell (Figure 2). Various possible destinations for exRNAs include liver clearance, kidney clearance, or absorption by recipient cells. In general, extracellular material endocytosis leads to exocytosis or lysosomal degradation [22–24] (Figure 2). Labeling and imaging analyses in immortalized endothelial cells demonstrate that a majority of EVs presented in recipient cells co-localize with markers associated with lysosomes [25]. Accordingly, most exRNAs binding to lipoproteins, ribonucleoproteins and vesicles are likely to be degraded within the lysosomes of the recipient cells.

3.1. Naked exRNAs are degraded and cause immune activation

Viral replication and cell death are abundant sources of RNAs in the extracellular milieu. Following viral infection, cells release double-stranded RNA into the extracellular space and subsequently elicit the immune process in mammals [19]. Because *in vitro* transcribed RNA can activate toll-like receptor signaling, this kind of immune signaling transduction appears to be a common response to extracellular "naked" RNAs [26,27]. By comparison, RNAs within vesicles typically do not elicit the immune process.

In addition, the extracellular milieu is abundant with ribonucleases, which easily break down RNA, indicating that certain exRNAs are resistant to RNases [28,29]. ExRNAs alone do not possess inherent resistance to RNases, since exRNAs are generally degraded by

RNases, whereas exRNAs-protein complex remains stable [30,31]. Therefore, exRNAs may combine with other biological molecules to evade immune activation and enhance the stability of exRNAs.

3.2. The nature of extracellular vesicles harboring exRNAs

Numerous exRNAs, such as mRNA, miRNA, circRNA, and tRNA are presented in EVs. Because the majority of exRNAs research focuses on exRNAs within EVs, the ever-changing findings of EVs have a significant impact on the exRNAs field [32–34]. Indeed, the initial evidence of exRNAs production in mammalian cells emerged from the quantitative PCR amplification of particular mRNAs extracted from extracellular vesicles in human and mouse cells [35,36] (Figure 2).

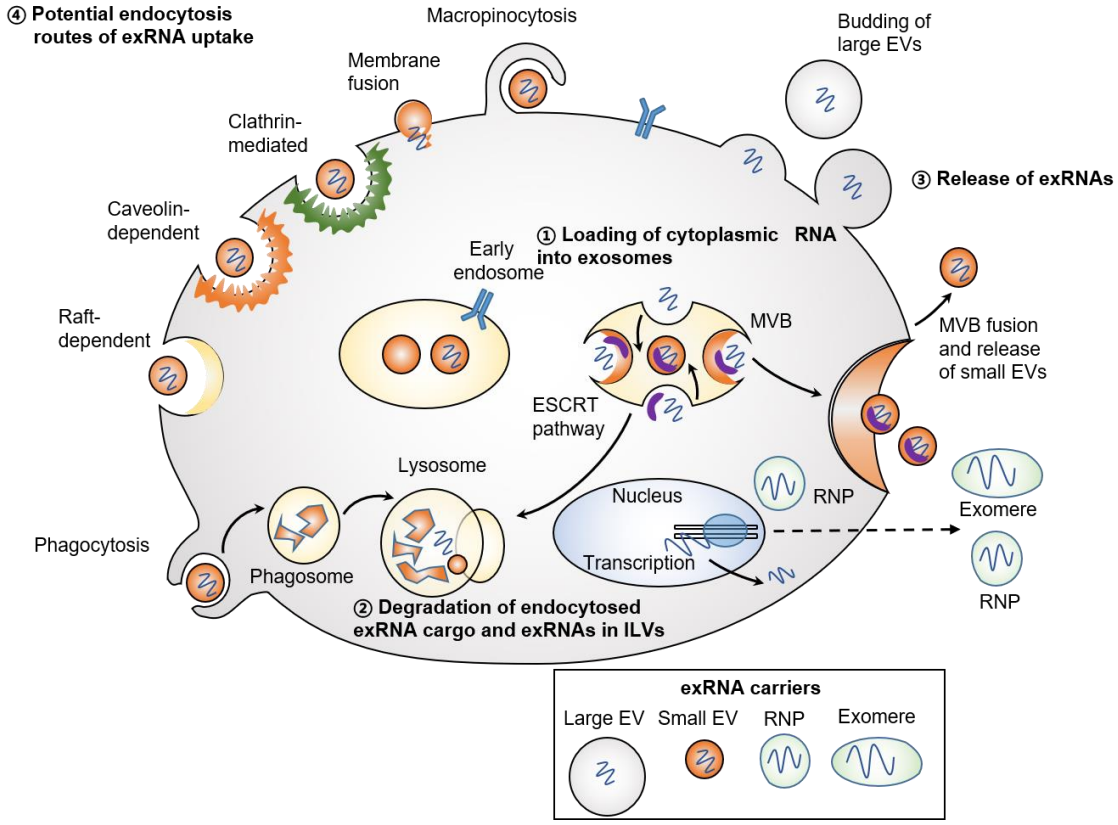

Figure 2. Life cycle model of exRNAs.

There are numerous EVs with distinct characteristics. The nomenclature for different groups of EVs is not uniform, making it challenging to interpret data across studies [32–34]. Certain attributes, including protein marker, size, density, and biogenesis, aid in defining EVs populations [32–34]. Establishing the definition of EVs is a crucial objective for exRNAs research, and characterizing the diverse subpopulations of vesicles remains a challenge for EVs investigation [32–34].

Although initial experimental data from qPCR using mRNA-specific primers indicate the presence of RNAs within Evs [35,36], it is not directly tested whether these are mRNA fragments or full-length transcripts [2]. Generally, EVs only comprise a few molecules of a

specific RNA, which has raised doubts about the ability of exRNAs to serve as robust messengers for cell-to-cell communication [37–39].

For exRNAs exist in small EVs, their transportation relies on the maturation of early endosomes into late endosomes, also known as multivesicular bodies (MVBs). Along with molecules absorbed through endocytosis, the MVB also engulfs certain cytoplasmic cargo, including RNA. These specific RNAs initially attach to the outer surface of the MVB, which subsequently undergoes inward budding, leading to the formation of minuscule vesicles held within the MVB. These vesicles are referred to as intraluminal vesicles (ILVs). (1) The formation of ILVs within the endosome membrane occurs through a series of steps, culminating in the inward scission of the endosome membrane. This process involves the uptake of protein and RNA cargo from the cytoplasm. An alternative pathway, depending on the protein syntenin and involving ceramide or phosphatic acid, can also generate ILVs by influencing the membrane structure of the endosome. (2) Different types of MVBs fuse with either the lysosome or the plasma membrane. When MVBs fuse with the plasma membrane, the ILVs, now referred to as small EVs, are released into the extracellular environment, carrying exRNA cargo. It is noteworthy that exosome release and lysosomal degradation have distinct lipid and protein profiles. (3) The fusion of MVBs is regulated by proteins associated with vesicular fusion. Large EVs are formed by directly budding off the plasma membrane and are believed to encapsulate exRNAs from the cytoplasm. (4) These endocytic routes have the ability to internalize exRNAs linked to EVs, ribonucleoproteins (RNPs), or lipoproteins. Supporting this proposition, exRNAs are frequently detected inside endosomes; however, the mechanism by which exRNAs exit the endosome remains largely unexplored. multivesicular body (MVB); intraluminal vesicles (ILVs); ribonucleoproteins (RNPs).

4. ExRNAs function in mammals

4.1. Evidence for exRNAs function

One of the initial experiments to examine the function of exRNAs demonstrated that RNA obtained from the culture media can promote DNA synthesis [14]. An obstacle in assessing the role of exRNAs is controlling for the confounding impacts of other transporter molecules [14]. In addition to testing the functions performed by exRNAs, it is important to keep in mind that cells may secrete exRNAs to extracellular space as a means of clearance of 'Cellular waste', a concept that has already existed since 1983 as a potential cause of EVs production [40].

After internalization, distinguishing exRNA endocytosed from RNA that has been transcribed in recipient cells is extremely difficult, but it can be resolved through experimental work [41]. Experimental data utilizing the genetic elimination of exRNA loci from the recipient cells authoritatively validate the transportation of extracellular snoRNAs between mice and cultured cells [42]. The *in vivo* experimental data of snoRNAs transportation is derived from parabiosis experimental evidence, in which control mice are linked to circulatory system of mice with deleted snoRNA. Parabiosis is capable of rescuing phenotypes, specifically for the loss of 2'-O-methylation of rRNAs in snoRNA-deficient animals, which provides support for the functional transfer of snoRNA from wild-type to

snoRNA-deletion mice [43,44]. These findings hold potential, however, they are not definitive because the parabiosis technique in animals transfers numerous molecules aside from extracellular snoRNAs, which will contribute to the methylation alterations [45]. Subsequent research has employed experimental designs that specifically confirm the involvement of exRNAs in functional activity, such as utilizing cell-specific genetic deletion of exRNAs from recipient cells [46].

4.2. ExRNAs transfer to recipient cells

Other experimental data of exRNAs transportation between mammalian cells include that exRNAs are unable to transcribed by the recipient cell [2,47,48]. Previous studies have confirmed that mRNAs in EVs derived from mice are capable of translation through an *in vitro* translation method, raising the issue of whether exRNAs can produce functional proteins in recipient cells [2]. Analogously, adding EVs containing luciferase RNA to cells causes luciferase activity in the receiving cells [1]. Multiple studies have examined the transfer of Cre recombinase and mRNA through extracellular vesicles by inducing the expression of Cre protein in specific donor cells and subsequently monitoring recipient cells for changes in levels of a reporter specific to Cre [47]. Injection of EVs containing Cre mRNA into mice leads to the activation of the reporter gene in neural cells, indicating the transportation and coding abilities of Cre mRNA [48]. These *in vivo* experiments are crucial in determining a biologically relevant role for exRNAs, such as the use of EVs derived from serum-containing RNAs [49].

When investigating the transportation of mRNA, it is conceivable that the target proteins are co-packaged into EVs alongside the mRNA, leading to ambiguous outcomes. One strategy is to establish a mechanism of inhibiting the protein synthesis of exRNA solely in the originating cell, thereby guaranteeing that any detected proteins in receiving cells are not sourced from EVs; furthermore, novel techniques for visualizing the protein synthesis of transcripts have emerged [50–53], presenting the intriguing opportunity to track the translation of transferred mRNA. Recently, the CRISPR–Cas9 system has been employed to explore the transfer of exRNA between cells [54,55]. The donor cells with stable expression of single guide RNAs (sgRNAs) are utilized to assess sgRNA-specific activity using the stoplight sensor in recipient cells [54]. Following transwell separation or exposure to high concentrations of concentrated EVs from the donor cells, the recipient cells expressing stoplight displayed GFP fluorescence activity depending on sgRNA [54]. These findings propose a model for the transfer of exRNA activity between cells.

5. Dietary miRNA-a novel functional component of TCM

It has been observed that miRNAs are existed in a variety of sources of food and traditional Chinese medicine (TCM), including plants and animals [56,57]. TCM has been extensively used for thousands of years, however, the pharmacodynamic material basis of TCM is still not undefined [58]. Dietary miRNAs are preserved from digestion and absorbed into the bloodstream, where they regulate gene expressions in target cells and tissues. miRNAs

derived from food and TCM are more challenging to be detected and quantified compared to plant miRNAs for high similarity of the sequences among mammalian miRNAs, which makes it difficult to distinguish dietary and endogenous miRNAs. The compelling evidence that the endogenous production of miRNAs cannot compensate for a deficiency in dietary miRNAs strongly proves animal miRNAs partially from both food and TCM [59,60]. However, accurately assessing the concentrations of animal miRNAs derived from food remains difficult, because distinguishing them from endogenous miRNAs is currently challenging.

Plant miRNAs have 2'-Omethylated 3' ends and are resistant to periodate [61]. The wide application of innovative technologies has promoted the rapid development of exRNAs research and pushed miRNA into the trend of treating diseases [62]. There is growing evidence that miRNAs circulate in a stable cellular form in extracellular biological fluids. The cross-species transfer of miRNAs has a unique position in facilitating cross-talk, communication, and signaling in distant species [63,64]. Recent studies demonstrate that miRNAs derived from TCM have potent therapeutic effects, and miRNAs are detected in stable, cell-free forms in body fluids such as saliva, urine, and breast milk. Dietary miRNAs have important roles in human health [65] and influence various physiological processes, such as immune response, metabolism, and even disease development [66]. Plant-derived miRNAs can regulate gene expressions in the intestine of animals, affecting lipid metabolism and inflammation [67]. Certain dietary miRNAs may have anti-cancer properties by targeting oncogenes or tumor suppressor genes [68]. These new findings take our understanding of inter-species communication to a whole new level and potentially provide a powerful theoretical basis for inter-species coevolution.

The major dietary miRNAs are summarized in Table 1. miR168a is present in high levels in rice that is one of the most enriched exogenous plant miRNAs in the sera of Chinese individuals, which can interact with the mRNA of human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) to decrease LDLRAP1 expression [69]. Additionally, mol-miR168a obtained from Moringa oleifera reduces the protein expression of SIRT1 in the hepatoma cell line G2 (HEPG2) cells [70,71]. Five plant-derived miRNAs (miR166a, miR156a, miR157a, miR172a, and miR168a) in breast milk obtained from healthy participants can be detected in the whole milk samples, which indicates that these evaluated plant miRNAs may potentially influence various vital biological pathways in infants [72,73]. Overexpression of miR156a in human aortic endothelial cells leads to a reduction in the adhesion of monocytes induced by inflammatory cytokines through directly suppressing JAM-A [74]. As an unconventional miRNA encoded by honeysuckle (HS), miR2911 has a wide-ranging ability to directly target influenza A viruses (IAVs), and it exhibits remarkable stability in HS decoction [75]. However, it is reported that miR375 derived from murine milk is rapidly degraded in the intestinal fluid [76]. In addition, miR156a, miR164a, and miR167a derived from corn are found in mice [77]; miR172 and miR166 originated in soy can be absorbed into macaques plasma [78,79]. Zuma-miR164a-5p derived from fresh maize targets some of its predicted target genes in the Argonaute/RISC complex [80,81]. More importantly, our previous study demonstrated that Lycium barbarum L.-encoded miR162a could be

absorbed into the blood to target NcoR and promote osteogenic differentiation to improve osteoporosis [82].

Table 1. TCM derived miRNA studies of cross-kingdom regulation.

miRNA	Source	Target cell or animal	Target gene	Function	Reference
miR168a	Rice	Humans/mice	LDLRAP1	Decreases LDL removal from	[69]
Mol-miR168a	Moringa oleifera	Human hepatoma cell line G2 (HepG2) cells	SIRT1	Absorbed	[70,71]
miR166a, miR156a, miR157a, miR172a	Tomato	Human breast milk	/	Absorbed	[72,73]
miR156a	Cabbage, spinach, and lettuce	Human aortic endothelial cells	JAM-A	Suppresses the progression of Atherosclerosis	[74]
miR2911	Honeysuckle	Mice	/	Targets various influenza A subtypes	[75]
miR375	Murine milk	Mice	/	Rapid degradation of milk miRNAs in the intestinal fluid	[76]
miR156a, miR164a, miR167a	Corn	Mice	/	Absorbed	[77]
miR172, miR166, miR167, miR168	Soy/fruit substance	macaques	/	Absorbed	[78,79]
zma-miR164a-5p	Maize	Pigs	OTX1, PLAGL2, CSPG4	Absorbed	[80,81]
MiR162a	Lycium barbarum L.	Humans/mice	NcoR	Promote osteogenic differentiation	[82]

6. ExRNAs in cancer

6.1. The role of exRNAs in cancer

In recent years, exRNAs including miRNAs, long non-coding RNAs, circular RNAs, and messenger RNAs in cancer have gained significant attention for their potential as diagnostic and therapeutic targets, since they are found to play critical roles in tumorigenesis, tumor progression, metastasis, and drug resistance [55,83,84]. ExRNAs act as signaling molecules, regulating gene expression and cellular functions in both autocrine and paracrine manners,

and they reflect the molecular characteristics of tumors and provide valuable information for cancer diagnosis, prognosis, and treatment response monitoring [38,85].

Different types of exRNAs contribute to cancer development by regulating key signaling pathways involved in cell proliferation, apoptosis, angiogenesis, and immune response [83]. Moreover, exRNAs can be used as non-invasive biomarkers for cancer detection and monitoring, and their presence of altered expression patterns in body fluids makes them attractive candidates for liquid biopsy approaches [1,86]. Valuable information about the presence of cancer, its stage, progression, and potential therapeutic targets are obtained by analyzing exRNAs, and treatment strategies such as exRNAs inhibition or replacement therapy can be employed to modulate their functions and disrupt cancer-related pathways [55,86,87]. Furthermore, exRNAs are used as drug delivery vehicles, enabling targeted delivery of therapeutic agents to cancer cells [55].

6.2. ExRNAs as cancer diagnostic and predictive biomarkers

The rapid development of technology, such as advanced bioinformatics and improved tools, presents a vast range of possibilities for investigating exRNAs in liquid biopsies [88]. A recent study shows the progress of a non-invasive urine exosome-based gene expression assay that can distinguish high-grade and low-grade prostate cancer [89]. It has been discovered that 30 mRNA and 15 miRNA candidates are differentially expressed in patients with gastric cancer by analyzing salivary gland secretions [90]. A new method for using ctDNA and exRNAs to track tumor size and treatment response is developed in multiple myeloma patients [91]. Furthermore, a diagnostics corporation in the United States employed exRNAs as foretelling indicators for prostate cancer and created a urine exosome gene expression test to detect advanced prostate cancer in patients with increased PSA levels [92,93].

However, the isolation of tumor-specific exRNAs and their use as biomarkers in clinical oncology face challenges due to inadequate separation technology and the heterogeneity of exRNAs carriers. The existing methods for isolating EVs from complex biofluids cannot provide clear information about the target cell of exRNAs cargo. As a result, it becomes difficult to determine the tissue of origin with certainty. To address these limitations, there is a need for improving EV separation technology and a better understanding of EV targeting and cargo release [92,93]. The goal is to create liquid biopsy-based analytical tests that utilize circulating exRNAs tailored to the specific tumor type and to discover clinically significant biomarkers.

6.3. Application of exRNAs as a novel cancer therapeutic strategy

Generally, EVs derived from tumor cells are recognized as contributors to the development of cancer, containing a significant proportion of exRNAs [94]. A potential therapeutic approach involves decreasing the secretion of EVs from tumor cells, such as using short interfering RNAs (siRNAs) to downregulate Rab proteins in the release process of MVB [95], or eliminating EVs from circulation through dialysis [93]. EVs derived from immune cells, in contrast, are considered significant contributors to cancer defense, primarily due to their

RNA content. Therefore, various approaches have been employed to include therapeutic RNA in EVs and redirect EVs towards particular tissues.

A conventional approach is to collect EVs from autologous dendritic cells transduced by membrane ligands, load them with siRNA, and then inject them intravenously into mice for further study [96]. Besides, transfecting cells with a DNA expression construct may achieve elevated expression levels of therapeutic mRNA within EVs donor cells *in vitro*, which enables significant amounts of these therapeutic agents to be incorporated into EVs. This approach is utilized to deliver a prodrug activating system (cytosine deaminase-uracil phosphoribosyltransferase) to schwannoma cells [97].

One study demonstrated that introduction of a targeting peptide ligand for EGFR was incorporated into EV membranes to deliver tumor suppressor miRNA let-7a in breast cancer [98]. miR-143 in EVs from normal prostate epithelial cells inhibits the proliferation of prostate cancer cells [99]. EVs specifically targeted to HER2 receptor are loaded with mRNA encoding enzyme HChrR6, which is employed in conjunction with the prodrug to selectively target HER2-positive breast cancer cells and xenografts [100]. To achieve specific targeting of EVs towards cancer cells, RNA aptamer/cholesterol nanostructures have been affixed onto the external membrane of EVs that enable efficient delivery of siRNA cargo to prostate, breast, and colorectal cells [101]. Transducing miR-126 mimic into breast cancer cells increases their susceptibility to fourteen chemotherapy medications like trimetinib and alpelisib, as well as retards the development of drug resistance [102]. Moreover, temozolomide-resistant glioblastoma cells were established through the attenuation of XRCC4-mediated DNA repair subsequent to the introduction of miR-151a mimic into the cells [103]. The study on miR-205 in gemcitabine-resistant pancreatic ductal adenocarcinoma demonstrates that overexpressing miR-205 can impede cancer stem cell proliferation and restore sensitivity in gemcitabine-resistant cells, which offers a viable strategy for treating advanced pancreatic cancer [104]. In summary, exRNAs-based cancer therapies have shown promising prospect.

7. Conclusion

The use of extracellular vesicles for delivering RNA-based therapeutics to treat diseases is gaining increasing attention. Each investigation into the formation process of exRNAs and their intricate roles in disease pathogenesis opens up new avenues in disease diagnosis and treatment. It is worth noting that the role of exRNAs as potential diagnostic markers for diseases including cancers will be validated in the near future. The application of exRNAs has profound transformative potential.

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Conflicts of interests

The authors declare no conflict of interest.

Authors' contribution

Conceptualization, C.G. and Y.Y.; formal analysis, X.T.; investigation, X.T.; writing—original draft preparation, X.T.; writing—review and editing, C.G. and Y.Y.; visualization, X.T.; supervision, Y.Y.; project administration, C.G.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.

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