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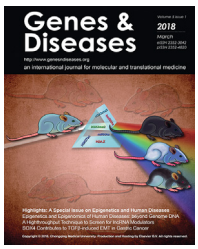

REVIEW ARTICLE

# Recent advances in extracellular vesicles enriched with non-coding RNAs related to cancers

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**Abstract** As membrane-bound structures that could be shedded by a parental cell, and fuse with others after shedding, and then release its contents, extracellular vesicles (EVs) are considered as an indispensable part of intercellular communication system. The EV contents might be all kinds of bioactive molecules including non-coding RNAs (ncRNAs), a large and complex group of RNAs with various subtypes that function to regulate biological events but classically do not code for proteins. In this review we covered the recently published works that validated the underlying molecular mechanisms regulating EV-associated ncRNAs' biogenesis, signaling, and particularly the systemic bio-effects related mostly to any stage of cancer progression, and the clinical potential of ncRNA-carrying EVs as diagnostic biomarkers and drug-delivery system that is being engineered for better loading and targeting capacity. Our views on the future direction of basic research and applications of EVs containing ncRNAs have also been shared.
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## Introduction

Extracellular vesicles (EVs) are small bilayer lipid membrane compartments of different sizes shedded by various cell types into most cell culture media and body fluids such as blood, milk and urine.[1] EVs may form by budding and shedding from the plasma membrane of the parental cells, these have been generally referred to as microvesicles, microparticles or ectosomes with a size range of 100–1000 nm in diameter.[2] The exosomes, another type of EVs with the sizes of 30–150 nm range, are generated inside endosomes or multivesicular bodies (MVBs) and

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released through fusion of these exosome-enriched late endosomes with the plasma membrane.[3] As considered to be one of the basic mediators of paracrine and endocrine cellular communication, EVs bear membrane-bound biomarkers for cell targeting, such as tetraspanin family, of which the CD63, CD9 and CD81 are widely recognized as exosomal immuno-detection surface marker.[2,4] Other EV membrane molecules include MHC for immune recognition,[5] annexins and FLOT1 for transport and fusion, integrins as cell adhesion molecules, MVB proteins, (Alix and TSG101) and membrane-bound receptors and signaling molecules such as G proteins.[3,6,7] The differences of the lipid components between EV membrane and their origin cell plasma membrane have been documented. Compared to cell plasma membrane, exosomal membrane is rich in cholesterol, phosphoglycerides, ceramide and saturated long fatty-acyl chains.[7] EV cargoes include proteins, lipids, RNAs (mRNAs and non-coding RNAs) and other biomolecules from parental cell cytosol. The composition of these bio-molecules were observed to closely reflect the physiological and environmental conditions of the parental cells *in vitro* and *in vivo*.[1,2,7] And the signal molecules of EV cargoes might serve for intracellular signal transduction for recipient cells after internalization of the EVs through plasma membrane fusion or endocytosis.

Non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) and so on, form a large part of eukaryotic transcriptome, participating critical regulatory roles in almost every studied patho/physiological pathways, and are attracting more and more academic interests.[8,9] Large amount of ncRNAs have been identified inside EVs by next generation sequencing.[10] Many EV miRNAs,[11–15] lncRNAs[16,17] and other types of ncRNAs[18,19] have been observed to play significant roles in a variety of cell types and biological systems.

In this review, we summarize the up-to-date knowledge about biogenesis (sorting of RNAs to load into EVs) and patho/physiological functions of EV-associated ncRNAs, review the molecular mechanisms of EV ncRNAs for potential clinical drug targets and diagnostic markers, mainly focusing on EVs derived from cancer cell, stromal cell, and other non-malignant cells which are involved in cancer progression, metastasis or inhibition. In addition, the current state and prospective of EVs as delivering vehicle of ncRNAs for cancer treatment are discussed as well.

## Sorting of EVs' ncRNA cargoes

In order to be transported by EVs, ncRNAs need to be sorted and loaded into EVs first. The detail of such sorting and loading process is still poorly understood. As reported, the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) is a RNA-binding protein that controls the exosomal loading of miRNAs through binding to specific "EXOmotifs" on these miRNAs.[20] And the occurrence of such binding depends on SUMOylation of hnRNPA2B1.[20] It has been observed that the gene KRAS is associated with miRNA exportation to exosomes in colorectal cancer cells (CRC) because exosomal RNA profiles differ significantly from CRC cell lines with or without KRAS mutation,[21] and such effect is attributed to the KRAS-MEK signaling regulated control of exosomal secretion of RNA-induced silencing complex (RISC) component Argonaute 2 (Ago2), which is a RNA-binding protein and a key effector of miRNA-guided RNA silencing process.[22] Other proteins are also found to function in this process. The sorting of miR-223 depends on its binding to the RNA-binding protein, Y-box protein 1 (YBX1), in a devised cell-free reaction, and YBX1 is needed for miRNA-secretion in exosomes by HEK293T cells.[23] Another RNA-binding protein SYNCRIP (also known as hnRNP-Q or NSAP1) is necessary for exosomal miRNA sorting in hepatocyte.[24] This sorting machinery demands direct binding of the SYNCRIP to miRNAs with a specific "hEXO" motif, which would then be exported into exosomes.[24] Another example is Vps4A, which mediates the efflux and influx of miRNAs by utilizing exosomes and, as a result, might function as a tumor suppressor in hepatocellular carcinoma cells.[25] In addition to proteins, the cell-activation-dependent changes of endogenous miRNA target levels in macrophages might also regulate miRNA sorting to exosomes.[26]

Since the sorting and loading machinery of EVs' RNA cargoes not only helps to understand the EV biogenesis and EV-mediated intercellular communication, but also lays the theoretical foundation of EV-based therapeutic approaches, Hung and Leonard devised a platform to actively load RNA cargoes into EVs in order to investigate the impact of general biophysical properties on EVs' RNA loading and delivery.[27] They fused the MS2 bacteriophage coat protein and the corresponding MS2 stem loop respectively to known EV-enveloped proteins and RNAs that were designed to load into EVs, and then found a substantial increase of the labeled RNAs inside EVs.[27]

## EV-associated ncRNAs derived from cancer cells

### MiRNAs

The research field on cancer is of crucial importance for human health. The EVs derived from cancer cells or non-cancer cells residing in tumor microenvironment or distant normal tissue cells that involved in cancer progression and metastasis are under the most intensive explorations.[1,2] EVs are shedded from parental cells to extracellular environment after maturation and can be detected in body fluids like blood, urine and saliva,[1] and EVs' RNA profiles are thought to be closely related to the patho/physiological status of their original cell types.[28,29] RNA expression patterns (especially differentially expressed ncRNAs) of EVs isolated from blood could render considerable convenience to clinical diagnosis and monitoring of cancer diseases. Given that miRNAs are the most comprehensively studied subset of ncRNAs, plenty of research efforts have been attempted to screen for cancer specific EV-associated miRNA biomarkers with next-generation sequencing from blood samples collected from patients and normal subjects, leading to the discovery of many circulating exosomal miRNA candidates that may serve as cancer diagnostic biomarkers as well as therapeutic targets.[30–34]

However, RNA profiling and verification experiments are far from enough to fully reveal the elaborate functions and implications of cancer-related EV miRNAs. Recent

development includes the deciphering of the working mechanisms of these encapsulated miRNAs with systematic studies. Specifically, EV-enveloped miRNAs secreted by cancer cells are able to promote the tumor progression and metastasis. Such promoting effect might be directly acted on cancer cells (as the direct EV receivers) as exemplified by chemoresistance and proliferation of melanoma cells induced by EV-encapsulated miR-211-5p derived from melanoma cells.[29] For example, the BRAF inhibitor vemurafenib increased miR-211-5p level in the melanoma cells and EVs via upregulation of MITF and subsequent regulation of TRPM1 resulting in survival activation, thereupon the miR-211-5p inside EVs could spread out and promote drug resistance and cell proliferation in neighboring melanoma cells.[29] Another case was observed in the dissemination of gemcitabine resistance in non-small cell lung cancer (NSCLC) cells.[14] As carriers of miR-222-3p, exosomes derived from gemcitabine resistant NSCLC cells were internalized by recipient gemcitabine sensitive cells through caveolin and lipid raft mediated endocytosis. These exogenous miR-222-3p then directly targeted suppressor of cytokine signaling 3 (SOCS3) to activate JAK2/Stat3 signaling pathway and to promote the gemcitabine resistance and other malignant phenotypes in recipient NSCLC cells.[14] Similarly, exosomal miR-196b-5p was reported to promote malignancy, stemness, and chemoresistance to 5-fluorouracil of colorectal cancer cells via targeting SOCS1 and SOCS3 of Stat3 signaling pathway.[35] Other reported findings suggest miR-200 family transferring in extracellular vesicles from metastatic breast cancer cells to non-metastatic breast cancer cells altered the gene expression and facilitated the mesenchymal-to-epithelial transition, as a result propagated the metastatic capacity.[36,37]

Other than direct act on cancer cells, EV-enclosed miRNAs from cancer cells also exert their cancer promoting effects on adjacent non-malignant cells of the same tissue type. For instance, miR-146b-5p from BCR-ABL1-positive microvesicles secreted by chronic myelogenous leukemia (CML) cells induced the transformation of normal hematopoietic cells into leukemia-like cells largely by inhibiting the cancer suppressor NUMB, which facilitated progression and relapse of CML.[38] Under other situation, in contrast to taking up malignant phenotype, normal exosome receiving cells experienced metabolic shift toward aerobic glycolysis, which is known as the "reverse Warburg Effect".[39] Specifically, as the etiological agent of Kaposi's sarcoma (KS), Kaposi's sarcoma-associated herpesvirus (KSHV) could latently infect lymphatic endothelial cells (LEC) and induce the Warburg effect in infected cells.[12] The viral miRNAs responsible for the metabolic changes in infected cells were able to transfer to surrounding non-infected cells via exosomes and induced similar aerobic glycolytic effect there, and these metabolically reprogrammed neighboring cells, while staying uninfected, in turn supported the cell proliferation of KSHV-infected cells.[12]

Furthermore, EV-enclosed miRNAs derived from cancer cells also directly target on cells of types other than the original malignant cells, especially immune cells due to their frequent interaction with cancer cells in the tumor microenvironments.[40] The liposarcoma (LPS) cells secrete miR-25-3p and miR-92a-3p through extracellular vesicles that are endocytosed by tumor-associated macrophages (TAM). After releasing inside TAM, these miRNAs bound TLR7/8 receptor and triggered pro-metastatic inflammatory response by secreting IL-6, which in turn promoted LPS development.[13] Another evidence involves TAM as well. The TAMs in CRC microenvironment were polarized into M2-like phenotype by uptake of cancer cell-derived EV-encapsulated miR-145 that inhibits expression of *histone deacetylase 11* (*HDAC11*) in the TAMs.[41] In addition T cells make another target for exosome-associated miRNAs originated from tumor cells. Exosome enriched with miR-24-3p isolated from nasopharyngeal carcinoma (NPC) patient sera exhibited T cell suppression effects such as inhibition of T cell proliferation, Th1 and Th17 differentiation and induction of regulatory T cells (Treg). Upregulation of P-ERK, P-STAT1 and P-STAT3 together with downregulation of P-STAT5 of T cells were observed *in vitro* to be involved in these T cell impeding effects, and *FGF11* was verified as a direct target for miR-24-3p by both *in vivo* and *in vitro* experiments.[15] Other observations found EV-encapsulated miR-9 and miR-210 regulate SOCS5/JAK/STAT pathway and neutral sphingomyelinase 2 (nSMase2) respectively in endothelial cells to promote angiogenesis.[1]

## Non-miRNA ncRNAs

Besides miRNAs, the most thoroughly studied subset of ncRNAs, other subsets, such as lncRNAs associated with EVs from cancer cells were also studied. Some of which are found important on cancer development control. lncARSR (lncRNA Activated in RCC with Sunitinib Resistance) was found to correlate with dissemination of sunitinib resistance in renal cell carcinoma (RCC), which was due to competing with AXL/c-MET for their miRNA inhibitor the miR-34/miR-449 in RCC cells, and consequently the elevated level of AXL/c-MET. Furthermore, lncARSR could be packed into exosomes and transmitted to local sunitinib-sensitive renal cells, resulting in dissemination of the drug-resistance phenotype.[16] In other cases, lncRNA-UCA1 was discovered to be specifically loaded in exosomes secreted by hypoxic bladder cancer cells, which would subsequently transfer to other tumor cells and promote cell growth and metastasis through activation of epithelial—mesenchymal transition.[17]

Because of the fast advancing of research on ncRNAs, previously less-known ncRNA types are now attracting more interests than ever before. Ample reports on such types like small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), circular RNAs (circRNAs), Piwi-interacting RNAs (piRNAs), etc,[42] as well as their association with EVs, have been published in last several years. A delicate example is that the primary tumor cells may educate the pre-metastatic niche into a tumor-cell-welcoming style with secreted exosome-enveloped snRNAs.[43] In this case, alveolar type II epithelial cells (AT-II) were transformed into pre-metastatic niche stromal cells via Toll-like receptor 3 (TLR3), activated by tumor derived exosomal snRNAs. Subsequently the chemokines secreted by transformed AT-II cells induced the recruitment of neutrophils in the lung. Together all these factors led to the rising of lung metastatic niche.[43] According to this report, impaired sensing of

tumor exosomal snRNAs caused by TLR3 deficiency ameliorated lung-tropism metastasis in spontaneously metastatic animal models, while improvement of such sensing by TLR3 overexpression closely correlated with high level neutrophil infiltration, and poor prognosis.[43] The next instance is about pro-malignancy transforming of monocytes activated by leukemia cells.[19] Exosomes derived from chronic lymphocytic leukemia (CLL) cells enriched with ncRNA hY4 could transfer to monocytes and induce CLL-supporting reactions via stimulation of TLR7/8 signaling pathway. These activated monocytes released tumor-supportive cytokines including C—C motif chemokine ligand 2 (CCL2), CCL4 and IL-6, and expressed immunosuppressive surface protein programmed cell death 1 ligand (PD-L1), contributing to pro-cancer immune responses, PD-L1 dependent immune escape and the overall cancer development.[19]

## EV-associated ncRNAs derived from non-cancer cells

Nowadays emerging evidences support the idea that cancer progression depends on intercellular communication and interaction with the cancer microenvironment, which is sometimes referred to as the cancer niche.[44,45] Other than cancer cells, tumor growth and metastasis are also determined by tumor stromal cells that contain multiple types of cells such as activated cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), endothelial cells and immune cells like macrophages, which constitute a large part of tumor microenvironment.[46,47] Cancer stromal cells are generated under the education of cancer cells, which in turn contribute to cancer development through secreting not only cytokines[19] but also EVs containing bioactive ncRNAs.[18,48—52] Considering that omental adipose tissue has been frequently taken as metastatic location for ovarian cancer, researchers have identified miR21-enriched exosomes from cancer-associated adipocytes (CAAs) and fibroblasts (CAFs) instead of ovarian cancer cells. MiR21 may then be transmitted to ovarian cancer cells via exosomes to suppress their apoptosis through direct binding to its target APAF1, providing cancer cells with paclitaxel chemoresistance.[48] Another study found that miR-140-enriched exosomes derived from mouse preadipocytes (3T3-L1) could regulate cell cycle, differentiation, stemness, migration of cancer stem cells, and tumor growth on MCF10DCIS cell model through targeting SOX2/SOX9 signaling.[52] Similar effect occurs in glioblastoma.[49] As the stromal cells of glioblastoma, glioma associated-human mesenchymal stem cells (GA-hMSCs) release exosomes enriched with miR-1587. These exosomes act to enhance the cell growth and tumorigenicity of glioma stem cells (GSCs), leading to increased tumor sizes and decreased host survival rates in orthotopic xenografts. And such pro-cancer effects are partly due to downregulation of the anti-tumor nuclear receptor corepressor NCOR1 in GSCs by miR-1587 contained in GA-hMSCs secreted exosomes.[49] Besides promoting tumor progression, miRNAs concentrated in EVs from cancer stromal cells might also function as tumor suppressors.[50] In the case of hepatocellular carcinoma (HCC), cancer-associated fibroblasts (CAFs) serve as cancer stromal cells through secreting exosomes transmitting miR-320a to HCC cells with lower level of miR-320a. The miR-320a, which was identified as an antitumor agent, directly binds to *PBX3* and inhibits the activity of MAPK signaling pathway since lack of miR-320a results in activated epithelial—mesenchymal transition, upregulation of CDK2 and MMP2, and consequently enhanced cell proliferation and metastasis of HCC.[50]

To maintain the healthy state of tissues, non-malignant tissue cells could communicate with each other and with the latent tumor cells to dampen the initiation of primary tumor with EVs conveying miRNAs.[1] Transferring from normal prostate cells to prostate cancer cells, miR-143 delivered through EVs was observed to implement the growth-inhibition at least in part by interfering with ERK5 signaling.[1,53]

Similar to cancer cells, stromal cells may also regulate cancer development with EV-encapsulated non-miRNA ncRNAs.[54] In a recent publication, breast cancer cells motivate the stromal cells by juxtacrine interaction via stromal NOTCH1/MYC signaling, thereby the *RN7SL1* transcription is elevated for MYC-driven enhanced activity of RNA polymerase III (POL3). Endogenous RNA *RN7SL1* normally has equivalent amount with RNA binding proteins SRP9 and SRP14, thus was bound and prevented by them from binding and stimulating pattern recognition receptor (PRR) retinoic acid-inducible gene I (RIG-I). However when highly expressed in breast cancer stromal cells, excessive *RN7SL1* is no longer shielded by SRP9/14. Then the unshielded 5′-triphosphate RNA molecule transfers to breast cancer cells by exosomes to act as damage-associated molecular pattern (DAMP) to irritate RIG-I. And, for this reason, there comes inflammation, induction of interferon-stimulated genes and breast cancer progression, metastasis and therapy resistance.[18]

Moreover, platelets that are not usually recognized as typical cancer-stromal cells nor canonical immune cells (however they are blood components and are cytoplasmic fragments derived from the megakaryocytes[55]) might as well influence tumor growth with EV-enveloped miRNAs.[11] It has been reported that platelet-synthesized miRNAs shipped in microparticles are capable of reaching cancer cells and causing apoptosis *in vivo* and *in vitro* upon the infiltration of platelet-derived microparticles (PMPs) into tumors in humans and mice, thanks to the high permeability of solid tumor vasculature. Upon uptake by tumor cells, PMP-conveying miR-24 causes mitochondrial failure and apoptosis by directly targeting and repressing, but may not limited to, mitochondrial RNA *mt-Nd2* (an mRNA) and *Snora75* (a snoRNA).[11]

## Engineering EV-associated ncRNAs for anti-cancer therapy

The common endocrine features of EVs: exporting out of the parental cells, delivering along the circulation system and uptake by specific target cells, as well as the selective property of cargo carrying, make them a promising drug carrier.[1,2] Researchers are trying diverse strategies to modify EVs, especially exosomes to meet clinical therapeutic demands.[5,7,56] One of these approaches is directly

modifying the content of isolated eukaryotic exosomes. Synthesized small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) targeting the *KRAS*$^{G12D}$ mutation of pancreatic cancer were introduced into exosomes isolated from culturing supernatant of normal fibroblast-like mesenchymal stem cells by electroporation.[57] Naturally generated exosomes afford several advantages for therapeutic purposes when compared with manmade liposomes. For example, CD47 presented on the exosome membrane offers protection of exosomes against phagocytosis by circulating monocytes and macrophages, and some native exosomal surface proteins (yet unknown to the authors) may have facilitated the accumulation and uptake in tumors. As a result, these "chimeric" exosomes efficiently impeded metastasis and improved survival in xenograft models.[57] Another way is to engineer the parental cells to give modified exosomes. Synthetic miR-143 was introduced to MSCs by Lipofectamine mediated transfection and was found in released exosomes, which were then able to deliver the artificial miRNA to osteosarcoma cells to restrict their migration *in vitro*.[58]

## Conclusions and future directions

Although EVs have long been discovered to be yielded from most cell types and considered as an essential player in signaling among different types of cells, only until recently we are starting to understand the complicated machinery underlying the biogenesis, releasing, uptake and functioning of EVs.[59] Series of remarkable studies have revealed fundamental molecular knowledge of EV under many circumstances.[2,3,60] Particularly, EVs carrying ncRNAs have now been well-acknowledged to extensively take parts in signaling between almost all types of cells involved in literally every steps in cancer progression as discussed above.[1,2,45] However, a lot more details is still needed to know about ncRNAs' sorting, loading to, transportation in EVs and uptake within EVs and functioning inside receiving cells, so as to offer theoretical basis for designing corresponding cancer diagnostic and therapeutic approaches.

So far it is widely believed that RNA binding proteins and other associated RNAs are involved in most ncRNAs behaviors. When it comes to EVs, as we speculated, RNA binding proteins and other accessories would comprehensively participate in processes like recognizing specific RNA cargoes, binding and protecting them from intracellular degradation, escorting them to the EVs, convoying them within EVs all the way to the receiving cells, activating the cargo RNAs inside the receivers, and facilitating the functioning. Nevertheless, knowledge on this aspect, which might be of essential importance and closely linked to cancer progression, are now rare and in urgent need of more exploration.

Currently, ncRNA residual modifications such as acylation, glycosylation and SUMOylation and their functional implications are in the focus of intensive study.[61–64] These are reactions catalyzed by protein enzymes. And the modified ncRNA products exert regulatory impacts on downstream effectors. These enzymes and products may well reflect the corresponding developmental status of cancer cells. In our opinion, for ncRNAs transported in EVs, learning these information would help understanding the relevant cancer molecular processes and designing targeting approaches.

EVs are presently classified by their subcellular organelle origins (briefly, exosomes are derived from MVBs while others are not).[3,60,65] A more informative classification system incorporating EV surface markers and RNA/protein profiles might be necessary to benefit EV studies.[2]

Plus, according to several very recent discoveries, some endogenous ncRNAs are capable of encoding proteins/peptides.[66–69] And some of the protein products were proven to be functional in cancer[69,70] or other conditions.[66,67] It is foreseeable that more translatable ncRNAs relating to tumor growth, metastasis and therapy will be revealed in the near future. The future study on EV-associated ncRNA translation should at least be focused on sensing of the translational start site in a non-classical way, recruiting the ribosomes, regulating the translational activity, and determining the exact position where these processes take place (in parental cells, EVs and/or recipients), and explaining the physiological roles of product proteins in receiving cells. Each of these aspects will depend on RNA-protein interactions, hence the RNA binding protein study and other relevant cellular molecular biology studies should also be in focus.

Due to the impact from fundamental researches, EVs, especially exosomes' potential as a programmable "precision-guided" drug-delivery system is attracting more attentions. For this purpose, in addition to exosomes of native origins, researchers are trying to engineer exosomes for particular contents[27,57,58,71–76] and for arming with special membrane proteins/peptides so as to target specific cell types including cancers for research and therapeutic purposes.[77–79]

## Conflict of interest

None declared.

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