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Extracellular RNAs: A New Awareness of Old Perspectives

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Abstract

Extracellular RNA (exRNA) has recently expanded as a highly important area of study in biomarker discovery and cancer therapeutics. exRNA consists of diverse RNA subpopulations that are normally protected from degradation by incorporation into membranous vesicles or by lipid/protein association. They are found circulating in biofluids, and have proven highly promising for minimally invasive diagnostic and prognostic purposes, particularly in oncology. Recent work has made progress in our understanding of exRNAs—from their biogenesis, compartmentalization, and vesicle packaging to their various applications as biomarkers and therapeutics, as well as the new challenges that arise in isolation and purification for accurate and reproducible analysis. Here we review the most recent advancements in exRNA research.

Keywords

Extracellular vesicles; RNA; Exosomes; Microvesicles; Vesicle biogenesis; Biomarkers; Noncoding RNA

1 Background

exRNA has emerged as an important source of biological information that represents the dynamic processes that occur intra- and intercellularly, in real time. exRNAs are released in a variety of subpopulations that vary amongst cell lines and are isolated via differing protocols. Here we discuss the potential of exRNAs in unraveling a new understanding of information trafficking in cells as well as the challenges in analyzing them in an accurate and reproducible fashion.

2 Biogenesis of exRNA and Role in Cell Biology

Most exRNA is protected from degradation by incorporation into membranous vesicles or association with lipids and/or proteins. Several subtypes of extracellular vesicles (EVs) have been described, including exosomes ($<150\text{ }\mu\text{m}$), microvesicles ($200\text{--}500\text{ }\mu\text{m}$), and oncosomes ($1\text{--}10\text{ }\mu\text{m}$) [1, 2]. Various biogenesis mechanisms are responsible for the formation of EVs. Whereas exosomes are shed through the multivesicular bodies of the endosomal pathway, microvesicles or ectosomes have been shown to bud off from the plasma membrane, and oncosomes can be released directly from tumor cell membranes. It is believed that the mechanism responsible for their formation influences their content, which consists of messenger RNA (mRNA), small noncoding (ncRNAs), DNA, proteins, and lipids. This content can be transferred to different cells and can mediate functional effects in these cells. Indeed, EVs have been shown to play a role in various biological processes, varying from establishing a body plan during development [3], to pathological processes as the formation of metastatic niches in cancer [4]. However, the extent to which RNA contents are responsible for these effects remains to be elucidated. EVs are generally regarded as a powerful source of biomarkers for various diseases, including glioblastoma [5–7], as they can be found in body fluids and their contents reflect the active status of their cells of origin (Fig. 1).

3 Compartments of exRNAs

exRNAs circulate in biofluids (e.g., blood, urine, saliva, cerebrospinal fluid, breast milk, follicular fluid) as part of different compartments, which protect them from degradation by RNases [8]. Namely, exRNAs are either encapsulated in EVs such as exosomes and larger vesicles [5], or bound in complexes with proteins such as the Argonaute 2 (Ago2) and high-density lipoproteins (HDLs) [9, 10]. Recent studies have shown that the profiles of exRNAs by compartment is different. For instance, while EVs are abundant in microRNAs (miRNAs), other types of RNAs are also detected, including small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), long noncoding RNAs (lncRNAs), transfer RNAs (tRNAs) and tRNA fragments, YRNAs, ribosomal RNAs (rRNAs), mitochondrial RNAs, and protein-coding RNAs [11–13]. On the contrary, the cargo of HDL:RNA complexes consist mainly of noncoding RNAs such as miRNAs, tRNA fragments, ribosomal RNAs, snoRNAs, and lncRNAs, but no protein-coding RNAs [14]. Lastly, Ago2 has binding affinity for miRNAs only [9, 14, 15].

4 Proposed Mechanisms of exRNA Packaging into EVs

Packaging of exRNAs in EVs is an active and orchestrated process that favors the sorting and enrichment of certain exRNAs in EVs, whereas it excludes others. To date, two major mechanisms have been characterized to regulate this process, an Ago2- and a chaperone-mediated mechanism. Recent studies have shown that Ago2 is a potent mediator of miRNAs sorting into EVs and that it can be regulated by the KRAS-MEK signaling pathway [16]. Other studies have shown that miRNAs with specific sequence motifs (i.e., GGAG and GGCU) were recognized and selectively sorted into EVs by the chaperone proteins heterogeneous nuclear ribonucleoprotein A2B1 (hnRNP A2B1) and RNA-interacting protein SYNCRIP [17]. In addition, the RNA-binding protein Y-box I (YBX-1) is suggested to be involved in the packaging of miRNAs and other exRNAs into EVs by recognizing secondary rather than primary RNA sequence motifs [18].

5 Overview of Extracellular mRNAs

Of all the subpopulations of exRNA, mRNA is one of the least abundant, accounting for a proportion of about 2% [19]. This low number of mRNA molecules poses the first major challenge in extracellular mRNA sequencing. Extracellular mRNA could be isolated from biological fluids such as blood, urine, saliva, or cerebrospinal fluid, or from in vitro cell culture supernatants. To date, RNA sequencing studies looking at extracellular mRNA focus on EV-associated mRNA. exRNA profiles, however, could vary when using different protocols for cell culture or methods for vesicle isolation [19, 20]. Since different subpopulations of exRNA vary in abundance between fractions after ultracentrifugation [20], EV isolation methods will clearly affect exRNA sequencing profiles. Furthermore, in cell cultures requiring fetal bovine serum (FBS), exRNA isolates are contaminated with FBS-derived exRNA, found in both the EV containing pellet and the supernatant after ultracentrifugation [20]. Up to 13% of FBS RNA reads can be mapped to the human or mouse genome, indicating that FBS-derived RNA significantly contributes to false-positive findings in both human and mouse exRNA sequencing studies [20]. Indeed, after re-analysis of publicly available exRNA sequencing datasets, in exosomes, roughly 2.6–17.2% of exRNA reads corresponded to bovine-specific transcripts [20]. For extracellular miRNA sequencing, the number of unique miRNAs had been shown to plateau at a sequencing depth of ten million reads [19]. For extracellular mRNA, however, optimal sequencing depth is unknown. Quantification of mRNA abundances requires normalization of their expression levels to well-established reference controls. Unfortunately, these controls have not yet been established for any of the exRNA subpopulations, including mRNA [19]. Additionally, for studies involving biomarker discovery for different disease conditions, a healthy control reference is currently lacking. Altogether, the field of extracellular mRNA sequencing faces considerable challenges, including the major concerns of low yield, FBS-derived mRNA contamination, and the lack of standardization of mRNA-seq library preparation.

6 Other Classes of exRNAs: microRNAs

miRNAs are small, noncoding post-transcriptional regulators of gene expression. They play important roles in diverse cellular processes, both in regulatory pathways and by acting as

buffers to stochastic changes in transcription [21, 22]. It is estimated approximately 60% of all mRNAs are targeted by miRNAs [23]. miRNAs are highly processed; they are initially transcribed as components of long precursor transcripts, often many kilobases in length, called pri-miRNAs. These may be co-transcribed along with the mRNAs they regulate, or may be in distant locations of the DNA [24]. These pri-miRNAs form hairpin structures and are trimmed by the RNase III, Drosha, to form a pre-miRNA that is exported from the nucleus in association with Exportin 5. When it enters the cytoplasm, its loop structure is cut by the endoribonuclease, Dicer. The mature miRNA, called the guide strand, forms one half of resulting RNA duplex, and is loaded into the miRNA silencing complex, miRISC, in association with the catalytically active component, Ago2 [25]. miRNAs suppress translation of mRNAs either by direct competitive blocking or by altering the stability of the target mRNA. This destabilization either occurs from shortening of the polyA tail or, if the miRNA has high complementarity with the target mRNA, Ago2 may directly cut the target [26–28]. miRNAs recognize their target mRNAs primarily through their seed sequences, a 6–8 nucleotide sequence near the 5' end of the miRNA [29]. This region typically exhibits high complementarity with targets. In animals, the remainder of the miRNA does not require perfect complementarity to effectively suppress target mRNAs. Consequently, miRNAs are promiscuous regulators of many genes; each may act on dozens to hundreds of mRNAs [30]. Though miRNAs are abundant in the cytosol, they are also released into the extracellular environment either within vesicles or associated with low-density lipoproteins or other lipoproteins. As a consequence of this release, miRNAs are able to act on sites distant from their synthesis, are important mediators of cell-to-cell communication, and have demonstrated utility as biomarkers of both physiologic and pathologic processes [31–33].

6.1 Transfer RNAs

Transfer RNA (tRNA) is a 78–90 nucleotide RNA structure involved in the translation of mRNA to proteins, transporting amino acids to the ribosome where the anticodon of the tRNA binds to the complementary triplet mRNA codon and links the amino acids to form proteins [34]. However, an increasing amount of research shows that tRNA plays an important role in other cellular functions as well, influencing mRNA cleavage, inhibiting translation, and promoting morphological changes [35]. tRNA constitutes a large part of the RNAs found in exosomes, in larger proportions compared to other cellular RNAs [36–38]. This over-representation of tRNA has been observed in EVs shed by breast cells, bone marrow and adipose-mesenchymal stem cells, lung cells, semen, urine, and blood serum [37–40]. In contrast, EVs shed by melanoma cells appear to contain very little tRNA, while the proportion of tRNA in the microvesicles and apoptotic bodies shed by these cells is similar to that of the cellular cytoplasm [41]. The selection of tRNA found within an EV further indicates the discriminatory shedding of tRNA in EVs. One example was observed after the deep sequencing of EVs shed by mouse dendritic- and T cells: roughly seven times more reads of tRNA-Lys-AAA were observed in RNA derived from EVs compared to RNA recovered from intracellular space [36]. Aside from full-length tRNAs, EVs appear to harbor tRNA fragments (tRFs) in high concentrations [36, 37]. The exact mechanism of function of tRFs is unclear to date, but they are suspected to be involved in specific regulatory pathways [42]. For instance, the half of the tRNA-Gly containing the 5' end suppresses protein synthesis, while smaller tRNA fragment inhibit translation nonspecifically [43, 44]. Similar

increases of 5' end tRNA halves in EVs have been recorded in breast, HeLa, and lung cell lines [37]. The reasons for which tRNAs and tRNA halves are concentrated in EVs, and whether those components are functionally transferred to cytosols of other cells remain to be known.

6.2 PIWI-Interacting RNAs

Among the small RNAs that guide gene regulation, piRNAs are the largest class of small ncRNA molecules expressed in animal cells, having prospects of hundreds of thousands of distinct piRNA species [45]. piRNAs are about 22–30-nt-long molecules that protect germ line cells from transposons, mobile genetic elements that threaten an organism's genome. They guide PIWI-clade Ago proteins to complementary RNAs derived from transposable elements, where the PIWI proteins cleave transposon RNA, leading to silencing [46]. piRNAs are generated independently of Dicer from single-stranded precursors with the help of two RNP complexes [47]. Germ granules, specifically, pi-bodies, and a germ line analog of processing bodies, piP-bodies, are the cytoplasmic compartments where PIWI pathway components assemble [48]. Little is known about the extracellular presence of piRNAs. Although they had not been previously known to be widely present in biofluids, a 2016 study by Freedman et al. identified 144 small RNAs in circulation that mapped to piRNAs [45]. To distinguish piRNAs from other exRNAs, the study included two distinct reverse transcriptase experiments showing that piRNA RT-qPCR analyses were specific to piRNA 3' modifications. Abundance of most piRNA species in B cells, neutrophils, peripheral mononuclear cells, platelets, and T cells were found to be nonsignificantly different from their abundances on plasma. However, B cells, neutrophils, platelets, and T cells had significant numbers of upregulated piRNA species as compared with plasma, while a majority of piRNA species were shown to be upregulated in red blood cells as compared with plasma. Interestingly, most piRNAs in exosomes were either not significantly different from downregulated as compared with plasma [45]. We do not yet know why piRNAs end up in EVs and whether they are functionally transferred to other cells.

6.2.1 YRNAs—YRNA is a very conserved class of small noncoding RNA [49] ranging from 84 to 113 nucleotides long in humans. It is highly abundant in cells, having a greater presence than tRNA and U6 snRNA. In extracellular fractions, including MVs, exosomes and RNPs, YRNAs have lower abundances than in cells, but are still more abundant than most miRNAs. Despite their high abundance levels, only four species exist in humans: Y1, Y3, Y4, and Y5. Furthermore, YRNAs have not been studied comprehensively, with less than 100 publications in PubMed. The lower stem domain of YRNAs was found to play a role in RNA quality control and degradation of misfolded RNAs by recruiting chaperone Ro60 and exoribonuclease PNPase [50]. The upper stem domain may participate in the initiation of chromosomal DNA replication [51]. YRNAs can be further processed to YRNA fragments, with the majority of modifications at the 5' end. Although initially mistakenly thought of as miRNAs, these YRNA fragments are now known to be distinct entities. Their biogenesis is independent of Dicer, and they do not bind to Ago2 [52]. Functionally, YRNA fragments do not seem to silence target expression in a miRNA-like manner, as evidenced by a luciferase reporter assay [53]. The biological functions of YRNA fragments have not been clarified yet, but recent studies reported that they might be involved in cell damaging [54]

and histone mRNA processing [55]. Interestingly, although YRNA fragments are less abundant than their full-length versions in cellular RNA, fragment abundance is higher than full-length abundance in extracellular fractions, and especially in RNP fractions where fragments contribute to approximately 20% of non-rRNA small RNA [56]. Further studies focusing on their biogenesis, secretion, and functions offer exciting new directions in RNA biology.

6.3 Other Noncoding RNAs

Apart from mRNAs, miRNAs, piRNAs, tRNAs, and YRNAs that are widely abundant in both cellular and extracellular spaces, the advent of next-generation sequencing revealed a broad spectrum of additional ncRNAs that are present in the acellular portions of biofluids [57, 58]. In the range of newly detectable exRNA, a significant expression of lncRNAs, small nuclear RNAs (snRNAs), snoRNAs, and circular RNAs (circRNAs) were detected [19, 23, 58, 59]. lncRNAs are any nonprotein-coding RNAs with a length >200 nucleotides that lack a long open reading frame and/or do not show codon conservation. Since their relatively low evolutionary preservation and their low level of expression, some posited that they represented only “transcriptional noise” and/or redundant transcripts with no biological significance [60]. However, it is now clear that the lack of primary sequence conservation in lncRNAs does not indicate lack of function [61, 62]. The expression of TUC339, for example, a lncRNA that is selectively released in EV from hepatocellular cancer cells, has a foundational role in modulating tumor cell behavior [63]. snRNAs were found to be localized in EVs. Although their intracellular functions are noted and mainly related to RNA splicing, methylation, and pseudouridylation [64], their role in extracellular spaces is still poorly known. Appaiah, H.N. and colleagues, in 2011, identified U6, small nuclear RNA, that is upregulated in the sera of cancer patients [65]. Sequence analysis showed also that there were diverse collections of snoRNAs in human plasma-derived EV RNAs, among which C1orf213, LINC00324, and LOC388692 were the most abundant [58]. Despite the limited information available regarding their expression and function in human tissues, new examinations using an ion proton system for plasma of 40 individuals revealed that snoRNAs appear to primarily guide chemical modifications of other RNAs, managing alternative splicing and gene silencing [45]. In addition to linear RNA molecules described above, a specific type of ncRNA, circRNA, is generated from pre-mRNA with a back-splice mechanism, that connects the 3′ end and 5′ end of a transcript’s precursor to form a circle [66]. A circular structure makes circRNA more resistant to exonucleases than other types of RNAs, preventing from the characteristic degradation and digestion of molecules in extracellular spaces [66, 67]. Its hypothetical function involves downregulation of miRNAs by sequestering complementary miRNAs like a sponge [59].

7 Challenges in Isolating exRNA Subpopulations

Optimization of methods to isolate high-purity exRNA subpopulations and measuring quantity and integrity is a current technical challenge in exRNA research. The most studied exRNA subpopulation comprises the RNAs found in EVs. However, many biological effects associated with EV-RNA could also be caused by the presence of other RNA-containing components [15, 68], such as ribonucleoprotein complexes, viral particles, and lipoproteins

(e.g., HDL and LDL). The source of these non-EV RNA-carriers could be the EV's biological sample of origin or the fetal bovine serum in cell culture media [69, 70]. The yield and purity of EVs depend on the EVs isolation method, which consequently, defines the quantity and quality of EV-RNA [71–73]. Because current highly sensitive molecular techniques can detect small amounts of components in EV preparations, co-purification of non-EV contaminants generates a significant artifact for further RNA analysis [74–76].

EV isolation techniques are based on different biophysical properties of EVs, such as density, shape, size, and surface proteins and therefore enrich for different subpopulations of vesicles, although none of the methods recover a pure material containing only EVs [77]. These methods include differential ultracentrifugation, density gradient centrifugation, chromatography, filtration, polymer-based precipitation, and immunoaffinity.

Ultracentrifugation is the most widely used method; however, the EV pellet can be contaminated with protein aggregates and viruses [78]. Density gradient centrifugation can be used in combination with ultracentrifugation to isolate highly purified EVs from other extracellular source of RNAs; however, this results in low yields. Lipoprotein may be co-isolated when the starting sample is plasma or serum [79]. Size exclusion chromatography consists of a size-based separation in a column. It promotes high EV recovery and removes most soluble contaminants, but other particles with similar pore-cutoff size may co-elute with the EVs [80]. Filtration can concentrate EVs and may remove soluble components, but similarly sized particles can contaminate the sample [81]. Precipitation techniques have high EV recovery rates, but also precipitate non-EV components [82]. Immunoaffinity uses antibodies against proteins found in EVs; thus, it can be used to isolate EV subpopulations. However, non-EV proteins might also be recovered. Indeed, the specific details of these purification procedures can differ significantly between different groups causing variability in the recovery of EVs, resulting in weaker detection of RNA in EVs and detection of RNA-carrying contaminants [79]. Several alternative techniques, many as commercially available kits, have been developed to improve EV purification, such as antibody-coated magnetic beads, affinity beads, novel precipitation (e.g., ExoQuick and Total Exosome Isolation) and filtration kits (e.g., PureExo), membrane affinity spin columns (e.g., ExoEasy), and microfluidics-based techniques (e.g., ExoChip) [83–88]. Particularly, ExoEasy kit allows recovering of both EVs and other soluble RNA-carriers separately [85]. Moreover, a combination of techniques has been described for purification of non-EV exRNA sources [9, 14, 89]. Despite the rapid growth of the exRNA research in the last few years, the field still needs standardized methods, both for isolation and characterization of exRNA subpopulations to enable successful exRNA applications as biomarkers and therapeutics.

8 exRNA as Biomarkers

exRNAs are contained and relatively stable within circulating vesicles in most biological fluids [90]. Among exRNAs, both miRNAs and piRNAs are the most highly enriched RNA species within extracellular vesicles, with a lower representation of long noncoding RNA, fragments of tRNA, YRNA and mRNA, among others [19]. exRNA-containing vesicles are present in most biofluids, consisting of a mixture of vesicles of different origin; either cells present in body fluids (e.g., blood [91], breast milk [92], and follicular fluid [93]) or cells contouring the space irrigated by body fluids (e.g., saliva, urine) [94]. Considering the

advantage of easily collecting exRNA from biofluids in a noninvasive manner, many studies indicate that, in addition to having an important function in intercellular communication, exRNA could also be used as potential biomarkers as indicators of normal biological processes, as candidates for early stage diagnostic in patients at risk, and also as predictors of pathology recurrence after treatment. There is substantial evidence indicating that exRNA can be used both for diagnostic and prognostic purposes in the oncology field [95]. For instance, tumor-derived exRNA (e.g., hTERT mRNA and miR-141) can be detected in blood of prostate cancer patients, correlating with tumor size and malignancy [70, 96]. Among the tumors in the digestive system, a variety of serum miRNA is found expressed differentially in hepatocellular carcinoma and chronic hepatitis. Additionally, exRNA in saliva is reported to be associated with pancreatic [97] and esophageal cancer [98]. As an additional example of exRNA used as biomarkers in the oncology field, miR-21, which is elevated in glioblastoma patients, has been shown to discriminate this from healthy patients [99]. Similarly, in cardiovascular disease, myocardial specific circulating miRNAs are significantly elevated in advanced heart failure, correlating with a concomitant increase in classical peptide biomarkers of myocardial damage like cardiac troponin I [100]. Furthermore, lncRNA could be considered as independent predictors of pathological cardiac remodeling and diastolic dysfunction in patients diagnosed with diabetes type 2 [101]. In addition to the oncology and cardiovascular fields indicated above, exRNA has also been described as potential biomarkers for pathological conditions in the nephrology [102] and neurology [95] fields, as well as in pregnancy [103, 104].

9 exRNA as Therapeutics for Cancer

To facilitate therapeutic delivery, exRNA can be packaged in stable natural carriers. Most studies have focused on using EVs as carriers for miRNA, siRNA, or other nucleic acids. Packaging anticancer nucleic acids in tumor-targeting EVs offers a novel method for cancer therapy. For example, Ohno et al. demonstrated that expressing GE11 peptide on the surface of EVs targets EGFR-positive tumor cells and that the delivery of a tumor suppressor miRNA, let-7a was able to inhibit breast cancer development in vivo [105]. In another study, microvesicle-encapsulated TGF β 1 siRNA was shown to inhibit murine sarcoma growth in vitro and in vivo [106]. The authors demonstrated that siRNA was associated with argonaute complexes in MVs, suggesting that MV packaged exRNA is stable and readily assembled for downstream gene silencing, as argonaute proteins are part of the RISC complex [107]. Mesenchymal stem cells (MSC) are being increasingly utilized as the source of EVs due to accessibility and therapeutic potency [108]. MSC-derived EVs were shown to be able to deliver anticancer small RNA in several cancer models, including osteosarcoma [110], hepatocellular carcinoma [110], bladder cancer [111], and glioblastoma multiforme. In these studies, miRNA or anti-miRNA-loaded EVs were able to inhibit tumor growth and migration, induce apoptosis, and render tumor cells more sensitive to chemotherapeutic agents. Although EVs are robust carriers of small RNA species, larger RNA species (such as mRNA) were also shown to be incorporated into these structures. The mRNA of a suicide gene was packaged in EVs that were able to inhibit tumor growth in a schwannoma model after the addition of the suicide drug [112]. However, it can be challenging to differentiate between the effects of mRNA or protein made from this mRNA in the producer cells. Taken

together, the above examples suggest that exRNA packaged in EVs has therapeutic potential and once scalability and manufacturing of these complex drug carriers are established, they have an enormous potential in anticancer drug therapy.

10 Conclusion

As our knowledge of exRNA continues to rapidly expand, we are bound to learn more about vaguely understood exRNA subpopulations and their potential applications in medical diagnostics and novel therapeutic pipelines. As a lack of optimized and standardized methods to isolate high-purity exRNA subpopulations still challenges the field, future efforts must be made for the unification of these processes. Nevertheless, the promising field of exRNA research may soon revolutionize the way we look at cancer and related diagnostics in terms of quality, cost, efficiency, uniformity, and real-time analytic capabilities, as well as the future of targeted medicine in cancer therapy.

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Fig. 1. Sorting, release, and isolation/application of exRNAs. As shown in the central panel, several types of RNAs and fragments of RNAs have been detected extracellularly associated with EVs, RBP, and/or lipoproteins (e.g., HDL). (1) *Sorting*: RNAs sorting mechanisms into EVs. RBPs can bind to RNAs by recognizing specific motifs, sequence or structure and target them into EVs. RNA modification such as uridylation (U) has shown to be enriched in EVs when compared to the intracellular content. Argonaute proteins (AGO) are canonical miRNAs binding partners and are related to miRNAs sorting into EVs by mechanism that may involve Alix, an ESCRT member. hnRNPA2B1, an RNP, when modified by SUMO (Su) recognizes certain motifs in miRNAs and regulates their sorting into EVs. (2) *Release*: ExRNAs are released by the cells combined to RBPs (e.g., AGO), lipoproteins (e.g., HDL),

or EVs. (3) *Isolation/Application*: Extracellular RBPs, lipoproteins, and vesicles can be isolated by using different techniques, such as differential ultracentrifugation, density gradient or immunocapture, and the RNAs can be purified for further sequencing or functional assays. exRNAs sources (EVs, RBP, lipoproteins) and exRNAs detection could be used as biomarkers or potential therapies. *AGO*: argonaute, *EVs*: extracellular vesicles, *ESCRT*: endosomal sorting complex required for transport, *ILV*: intraluminal vesicle, *MVB*: multivesicular bodies, *RBP*: RNA-binding protein, *RNP*: ribonucleoprotein