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Review Article

Extracellular vesicles in Inter-Kingdom communication in gastrointestinal cancer

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Abstract: The production and secretion of extracellular vesicles (EVs) are common features of cells (including various normal cells, neoplastic cell lines as well as bacteria) that span all domains of life. Tumor-derived exosomes are enriched with kinds of tumorigenesis mediators which are derived from the cytoplasm of cancer cells and fully reflect the tumor conditions. Indeed, the major topics and challenges on current oncological research are the identification of tumorigenic and metastatic molecules in tumor-cell-derived exosomes as well as elucidating the pathways that guarantee these components to be included in exosomes. The bacterial EVs have also been implicated in the pathogenesis of gastrointestinal (GI) tumors and chronic inflammatory diseases; however, the possible function of outer membrane vesicles (OMVs) in tumorigenesis remains largely underestimated. We suggest that EVs from both eukaryotic cells and different microbes in GI tract act as regulators of intracellular and cross-species communication, thus particularly facilitate tumor cell survival and multi-drug resistance. Therefore, our review introduces comprehensive knowledge on the promising role of EVs (mainly exosomes and OMVs) production of GI cancer development and gut microbiome, as well as its roles in developing novel therapeutic strategies.

Keywords: Extracellular vesicles, exosomes, gastrointestinal cancer, gut microbiome

Introduction

EVs can be divided into three types according to the different molecular sizes and release modes, namely exosomes, microvesicles, and apoptotic bodies [1, 2]. Exosomes are small monolayer secretory organelles, 30-200 nm in diameter, with the same topological structure as cells and rich in selected nucleic acids, proteins, lipids, and glycoconjugates. The molecular diameter of microvesicles is 100-1000 nm, and they are produced directly from the surface of the mother cell membrane by budding. They are heterogeneous in size and highly express phosphatidylserine. They do not have specific surface molecular markers, but like exosomes, they express surface markers derived from the mother cell. Bacteria, on the other hand, release membrane sacs (MVs), with diameters ranging from 20 to 400 nm, that affect a variety of biological processes (including virulence factor transport, DNA transfer, phage interception, antibiotic and eukaryotic host defense factors, cell detoxification, cell metabolite output, and cell-to-cell communication). It was first found that MVs are produced through controlled vesicles of the outer membrane of G- bacteria, so it is often called outer-membrane vesicles (OMV). In addition to OMVs, other types of MVs have been recently discovered, including outer-inner membrane vesicles (OIMV), cytoplasmic membrane vesicles (CMV), and tube-shaped membranous structures (TSMS). MVs can also be formed by cell lysis triggered by lysin in phages. Unfortunately, many studies have confused exosomes with EVs. The EVs discussed in this paper are mainly exosomes and OMVs, which are also the focus of EV research at present.

Ten years ago, due to the availability of next-generation-sequencing (NGS) technologies, we come to realize that we harbor 'another' genome

(namely, the microbiome) [3, 4]. The significance of the gut microbiome is self-evident since an imbalance of microbiota leads healthy individuals to physiological disorders and even tumorigenesis [5, 6].

There are growing evidence of EV-mediated "guest/host dialogue" from gut microbiota, opening the way for other interesting findings. Bacteria, both G+ and G- bacteria, produce EVs in much the similar way that our human cells produce EVs. Now that we have known more about our "other genome", we think it's time to decide how to interact with it.

Characteristics of different extracellular vesicles

Molecular structure and composition of EVs

Evidently, EVs can be found in almost all living cells [1, 2], demonstrating that EVs are highly evolutionarily conserved as a ubiquitous communication pattern among species.

According to the online exosome database (www.exocarta.org), the most recent update lists 3,408 mRNAs, 2,838 miRNAs, 9,769 proteins, and 1,116 lipids [7] (**Figure 1**). Exosomes are heterogeneous in composition and size, and enriched in membrane-associated protein complexes. What exosomes carry depends on the functions and states of the original cell types [8-12]. The distinct heterogeneity of exosomes is due to their limited load capacity, the mechanistic forces that lead to the variant protein distribution and the differential gene expression. The secretion of exosomal DNA may facilitate DNA quality control, regulation of inflammation, and perhaps may play a powerful role in tumor biomarkers or chemotherapeutic resistance. Exosomal RNA contains non-coding RNAs (ncRNAs). However, most studies focus only on the exosomal overall RNA composition, not single-particle levels, and thus may have underestimated the actual complexity of RNAs in exosomes [13].

OMVs are mainly composed of proteins, lipids and various pathogen-associated molecular patterns (PAMPs) [14, 15]. As far as the proteins in OMVs are concerned, most of the proteins are virus-related factors, such as enzymes, molecular chaperones, toxins, etc. [16-18]. The composition of OMVs protein showed great heterogeneity among different strains [19]. Phospholipids and LPS are the main lipids contained in OMVs. Moreover, other PAMPs, including peptidoglycan, lipoprotein, DNA and RNA, are also presented in OMVs. The co-existence of bacterial antigens and abundant PAMPs endows OMVs the potential for superior vaccines.

Biogenesis, secretion, and release of EVs

Exosomes are produced by vesicle budding into the endosomal membrane, with subsequent accumulation and fusion at large multivesicular bodies (MVBs) [20-22]. However, the presence of an endosomal pattern of exosome biogenesis does not imply that all exosomes are produced by endosome budding alone. In fact, multiple evidences suggest that exosomes also germinate from the plasma membrane [23-27] (**Figure 1**). Unfortunately, this pattern is largely overlooked by the majority cartoon models of exosome biogenesis, while the view of the endodermal body of exosome biogenesis is widely accepted [28, 29]. Stephen J. Gould considers this may be due to observational bias [30].

Besides, the exosomal secretion mechanism has also been extensively studied. Ras associated proteins of the Rab family are regarded as important modulators in exosomal secretion pathways [31, 32]. Exosomes can communicate with their recipient cells by sending signals directly through the interaction of receptor molecules or ligand on their respective surfaces.

For bacteria and archaea, EVs are released outward from their membrane, whereas eukaryotes can also produce EVs from the endocytic pathway and release them through the multivesicles [33]. Up to now, EV release in eukaryotes and archaea has been understood to be mediated by ESCRT-related proteins and homologues. However, the biogenic mechanisms of bacterial EVs are largely vague, and many scholars speculate that there may be more diverse mechanisms [34]. It is noteworthy that the number of EVs generated by G+ bacteria is naturally lower than that of G- bacteria. Our understanding of the biological origin and composition of the bacterial EVs is primarily from the research of the OMVs of G- bacterial EVs (**Figure 2**).

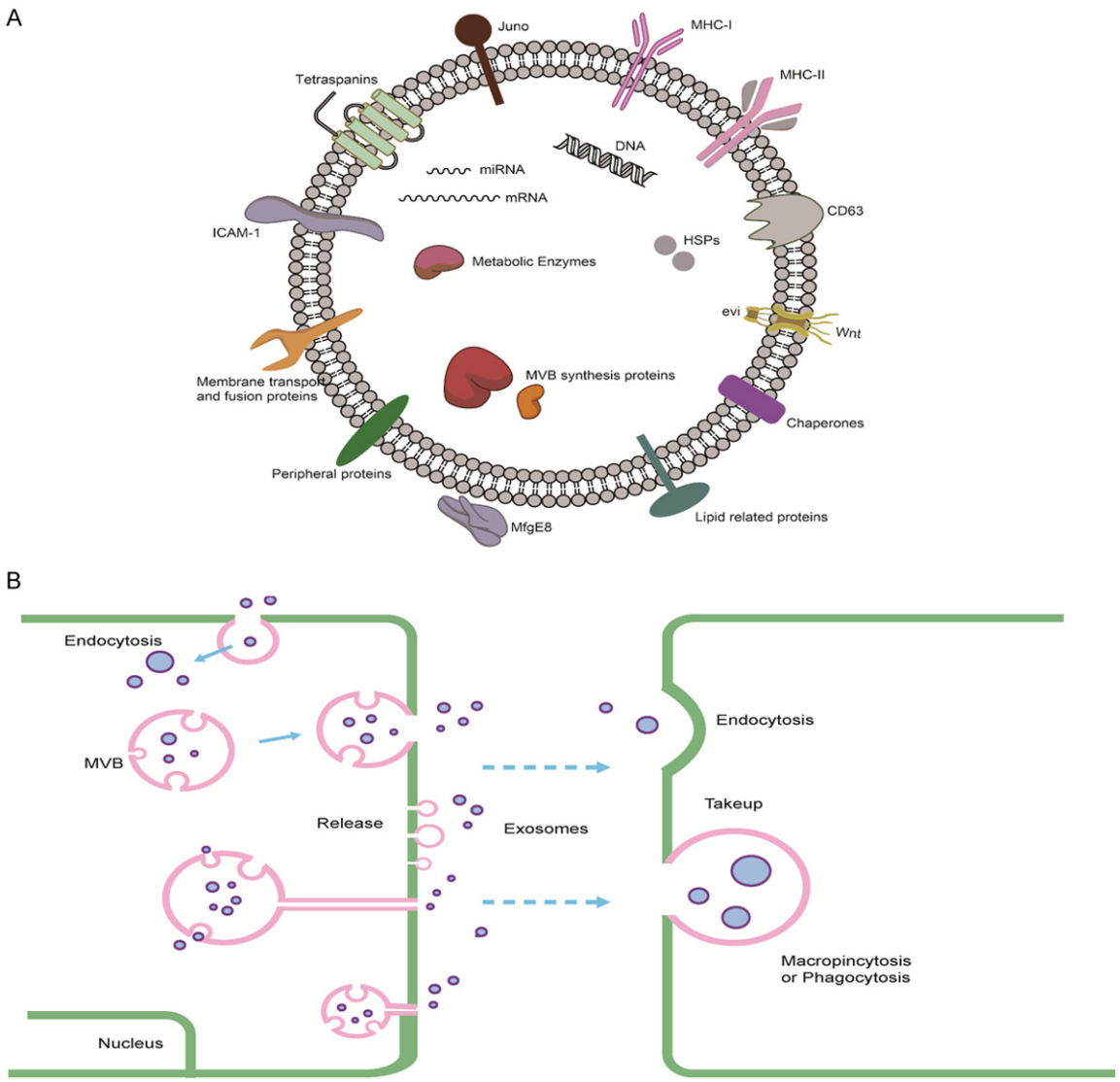

Figure 1. Composition, structure, biogenesis, and uptake of exosomes. A. Exosomes are heterogeneous in size and composition and are rich in protein complexes, DNA, RNA molecules, etc. B. Exosomes are produced in three ways (left): (a) After endocytosis, scattered endosomes develop into mature MVB, which then fuse with the cell membrane and release exosomes; (b) The plasma membrane secretes exosomes directly; (c) The intracellular-plasma-membrane connected compartments (IPMCs) bud out and subsequently release exosomes through the IPMC necks. After exosomes are released, they can interact with recipient cells through ligand/receptor signaling. Exosomes can enter recipient cells in different ways (right), such as endocytosis, direct membrane fusion, micropinocytosis, and even phagocytosis. HSP, heat shock protein; ICAM-1, intracellular, adhesion molecule-1; MfgE8, milk fat globule protein E8; Wnt proteins, wingless proteins.

Purification and identification of EVs

To understand the physiological and pathological functions of EVs, the purification, identification, and quantitative analysis of EVs are the basis of basic research and clinical application. Based on some specific characteristics of EVs, such as their morphology, density, size, or surface protein, there are generally five types of EV isolation methods (**Table 1**), among which the UC technique is the most traditional and widely recognized method [35-37]. However, there is no single protocol that is universally applicable for the analysis of various body fluids, such as saliva, plasma and feces. The isolation method must base on the complexity of the samples (e.g., composition and sample size). Bacterial EVs in human body fluids has rarely been stud-

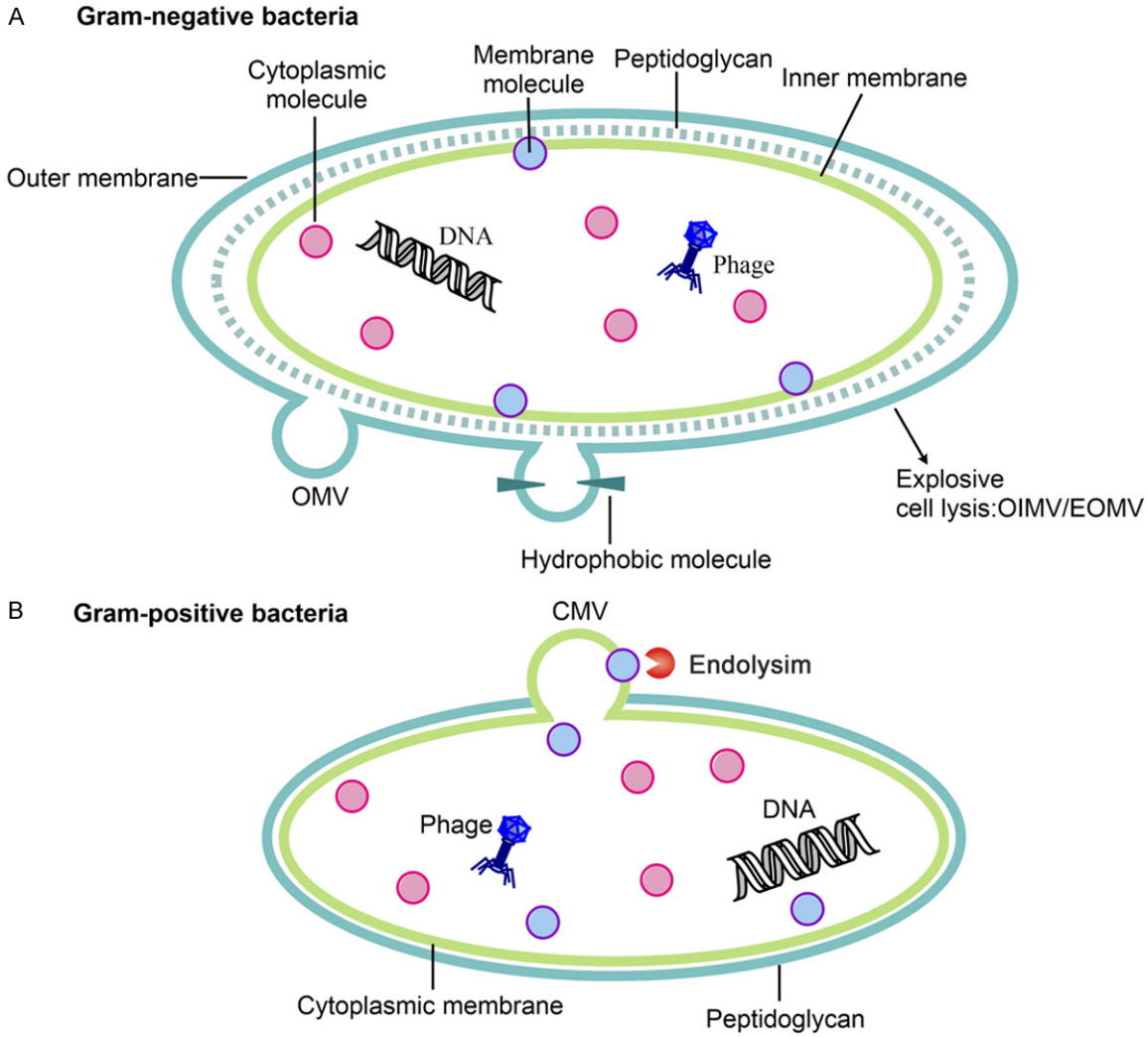

Figure 2. Different routine of bacterial membrane vesicles. A. There are two main ways for G- bacteria to form vesicles: the blebbing and formation of OMVs, or explosive cell lysis, producing OIMVs or EOMVs. B. The bubbling of G+ bacteria leads to the formation of cytoplasmic CMVs. The OMVs of G- bacteria harbor an internal phospholipid lobule and an external LPS lobule, which activates immune cells through TLR-4. At present, our understanding of the composition, molecular structure, and function of OMVs mainly comes from bacteria cultured in the lab. OIMVs: outer-inner membrane vesicles; EOMVs: explosive outer-membrane vesicles; LPS: lipopolysaccharide. TLR-4: toll-like receptor 4.

ied, possibly due to methodological challenges in isolating them from stromal- and host-related eukaryotic EVs, such as human exosomes and micro-vesicles [38, 39].

EVs derived from gastrointestinal tumors and gut microbiota

EVs derived from gastrointestinal tumors

There have been many studies focusing on the role of exosomes in GI cancer tumorigenesis, progress, and metastasis. Zhou et al. [40] studied patients with different types of esophageal diseases, and reported two kinds of exosome miRNAs (miR-223-3p and miR-584), which can be satisfactorily used for the detection of esophageal squamous cell carcinoma. Exosomes loading mutant p53 DNA were separated from the serum of patients with pancreatic cancer, which contribute to the diagnosis, treatment of cancer [41]. Costa Silva b. et al. confirmed that macrophage migration inhibitor (MIF) was elevated in PDAC-derived exosomes from stage I PDAC patients and can block the formation and metastasis of pre-liver niches.

Table 1. Isolation methods of EVs

Isolation method	Principle	Advantages	Disadvantages
Ultracentrifugation	Particle density and size	Simple procedure and good extract-uniformity	Limited efficiency and purity
Ultrafiltration	Molecular size and morphology	Simple extraction process, high uniformity, and tiny effect on the bio-activity of EVs	Limited product loss and purity
Density-gradient centrifugation	Density	High purity	Time-consuming and multi-step process
Protein precipitation	Ammonium sulfate precipitates proteins	Economical and is promising for large sample separation	Time-consuming, low separation efficiency and purity
Immunoaffinity capture	The interaction of the molecule with a specific ligand	High purity	Expensive and not suitable for mass extraction of OMVs

Table 2. Comparison of bacterial and human EVs application

	EVs in human (mainly exosomes)	Bacterial EVs (mainly OMVs)
Application fields	Mediate ECM regulation, signaling and molecular transfer	Vaccine (bacterial OMVs are promising vaccines induce humoral and cellular immune responses humans and animals)
	Mediate signaling and molecular transfer	Adjuvants that can enhance and regulate immune responses to specific antigens
	A noninvasive and efficient approach for tumor diagnosis and prognostic monitoring	Cancer immunotherapy drugs to target tumor tissues
	Activate the immune response to enhance immunity	As a carrier of chemotherapy drugs, OMVs increase the accumulation of chemotherapy drugs in tumors
	Exosomes serves as delivery systems for drug therapy	OMVs is a bacterial mimic that competitively inhibits parental pathogen attacks on host cells

These results suggest that MIF derived from PDAC exosomes may be an effective prognostic marker for the occurrence of PDAC liver metastases [42]. It is worth noting that EVs in quantitative peritoneal lavage fluid (PLF) may have an advantage over blood exosomal levels. Tokuhisa et al. recently identified the expression of miR-21 and miR-1225-5p in PLF exosomes and suggested that they could indicate the metastatic stage of gastric cancer (GC) [43]. In addition, the levels of these two miRNAs in T4 patients were higher than those in T1-T3 patients. GC diagnostic tests will probably include the detection of EVs in gastric fluid in the future [44]. These data deserve further investigation, and the sensitivity and specificity of the research should be validated by large and multicenter studies. The findings of Herrera m. et al. suggest that exosome-loaded ncRNAs are potential biomarkers of CRC, while CAF (cancer-associated fibroblasts)-derived exosomes are specific communication mediators between CAFs and colon cancer cells [45]. Exosomes-derived miR-10b from CRC microenvironment cells, such as CAFs, can also accelerate proliferation and promote tumor progression by influencing stromal cells. These CAFs have been proved to enhance CRC growth in vitro and in vivo [46]. Some studies demonstrated that after the p53 R273H mutation was improved, exosomal miR-21-3p and miR-769-3p can motivate the activation of fibroblasts in lung tissues and its tumor microenvironment. Furthermore, they demonstrate that this process consolidates the formation of CRC pre-metastatic niches and enhances its lung metastases [47]. Cook T. et al. observed that exosomes rich in miR-1246 can be selectively released by CRC cells carrying mutant p53. Derived from unique mutant 53 exosomes, miR-1246 may be suitable for the treatment and diagnosis of CRC. They discovered that the ingestion of mutated p53-derived exosomes activated the formation of a tumor-related macrophage subgroup that is involved in tumor progression and metastasis [48]. Another notable molecule is heat shock protein 60 (HSP60). After surgical resection of CRC tumor, the level of HSP60 in exosomes is reduced, so this assay can be applied to monitor treatment response [49]. It should be emphasized that most of the researchers believe that the expression of exosomal antigens in the blood of GI cancer patients is increased and are associated with patient survival and reflect disease severity. Zhou J. and colleagues proved that miRNA-21 from hepatocellular carcinoma (HCC) cell exosomes was able to significantly transform normal hepatic stellate cells (HSCs) into CAFs [50]. Also, exosomes can regulate Wnt pathway receptors by influencing stem cell-associated signaling pathways [51] to regenerate the phenotypes of stem cells and transform them into tumor stem cells. Recently, the function of both FZD10 and FZD10-mRNA which were reported to be associated with GI cancer cells were investigated. The results demonstrated that FZD10 and exosomal FZD10-mRNA may be potential deliverers of cell transformation in the distant metastasis process [52]. Other studies suggest that exosomes containing FZD10 were involved in controlling cancer progression and cancer cell modification and can be an indicator of the pathological condition [53]. Notably, the panel of exosome molecules and CEA may increase the efficacy of CRC diagnosis. In addition, some research has shown that exosomes have the potential to be biomarkers for the early diagnosis of CRC [54, 55]. The oncogenic miRNA-203 in CRC exosomes may enhance liver metastasis by eliciting the activation of tumor-associated macrophages [55]. Additionally, a report demonstrated that miR-25-3p supports liver and lung metastasis via enhancing vascular permeability and promoting angiogenesis, and is associated with the establishment of pre-metastasis niches in vivo [56]. Importantly, EVs is involved in GI tumor genesis and development as a drug resistance modulator. Exosomal miR-155-5p is associated with paclitaxel resistance in GC [57]. Bhome et al. [58] demonstrated that miR-21 is transferred to CRC cells via fibroblast exosomes, leading to oxaliplatin resistance in these cells.

However, due to the differences in the approaches of separation and purification, sample size, sample population, sample types, and other experimental conditions, the results of different experiments are still lack repeatability and consistency. Stratified analysis indicators such as age, sex, and race may be important for the identification of diagnostic biomarkers, however, few investigations have focused on these possible factors.

EVs derived from gut microbiota

Harboring about 3×10^{13} bacteria, the GI tract is guarded by epithelial cells, which are main-

tained by constant interaction between the GI microbiota, the mucosal barrier, and immune cells [59]. Dysregulation of gut microbiota has been shown to occur in a variety of tumors, particularly in the GI tract [60-64]. In different correlation and mechanism studies, Bacteroides fragilis, Enterococcus faecalis, Escherichia coli and Streptococcus gallolyticus, etc. were reported to be individually associated with CRC [65-68]. For example, the fecal abundance of Enterococcus, Fusobacteria, Escherichia coli, and Streptococcus in CRC patients is abnormal [69, 70]. Viljoen etc. al. [71] found that, compared with healthy individuals, the number of Fusobacterium spp. and Bacteroides fragilis were significantly higher in patients with advanced CRC. Fusobacterium spp. does not have direct carcinogenicity, but it may indirectly lead to tumorigenicity by enhancing inflammatory response and promoting the proliferation of tumor cells [72]. Fusobacterium spps. can activate the adhesion of FadA, trigger the Wnt signaling pathway of colonic epithelial cells, and promote the proliferation of epithelial cells. Another important participant, enterotoxigenic Bacteroides fragilis (ETBF), produces B. fragilis toxin (BFT), which plays a significant role in colorectal cancer, diarrhea, and other diseases [73].

The OMVs/MVs production of G- and G+ bacteria are one of the main mechanisms of the gastrointestinal microbiota [74]. OMVs are about 40-300 nm in size and are part of the bacterial release and transport system that transfer their cargos such as nucleic acids, proteins, etc. [75] to other bacteria, fungi, or host cells. OMVs are also rich in bioactive components such as immune-regulating lipopolysaccharides, lipoproteins, peptidoglycans, etc. These molecules have been reported to exist in a variety of G- bacteria in the human GI tract, including Escherichia coli, Fusobacterium nucleatum, Helicobacter pilori, B. fragilis and others [75-77]. Bacteroides fragilis can secrete OMVs to deliver immune molecules to human immune cells. These secreted OMVs and IBD (Inflammatory bowel disease, IBD)-related genes (ATG16L1 and NOD2) play an important protective role in inflammatory bowel diseases that activate non-classical autophagy pathways [78]. Recently, researchers at New York University have found that pancreatic ductal adenocarcinoma (PDA) tumors contain many fungi that originate in the gut and have shown that these fungi can induce normal cells to turn into PDA. It also suggests that an anti-fungal approach may be a promising treatment for pancreatic cancer [79]. OMVs also play a critical role in the pathogenesis of chronic inflammation of GI tract, including Crohn's disease (CD) [80] and Hp. associated inflammation [81].

In addition to the secretion of carcinogenic toxic components and the direct effect of specific microorganisms on the host, microorganisms may also play a critical role through inflammatory and metabolism-associated pathways. Notably, the occurrence of GI tumors is caused by microorganisms, inflammation, and intestinal immune regulation. Among them, the pathogenicity of microbial EVs cannot be ignored. This intercellular transport medium can induce continuous inflammation and even cancer, leading to the establishment of the original tumor environment.

EVs communicate across boundaries

Interspecies and even inter-boundary communication and interaction occur continuously in the GI tract. EVs of different origins (host eukaryotic cells/pathogenic cells, fungi, viruses, worm and edible plants [82, 83], etc.) meet in the three-dimensional space of the gastrointestinal cavity and interact with intestinal cells. EVs can be secreted by many human parasites, which play a significant role in stimulating and sustaining parasite infections [84]. Bacterial DNA integration and associated mutations through horizontal gene transfer are presented in cancer cells [85]. As a container of nucleic acid molecules, EVs have the potential to be carriers of such transfer. In addition, this transportation enables protein epitopes to be shared between self-antigens and microbial molecules, resulting in a cross-reaction that leads to tissue destruction, apoptosis, and the accompanying expression of self-antigens and microbe antigens [86]. We hypothesized that the EVs released by the GI cancer cells and the gut microbes might have a certain level of matching protein sequences. Barteneva et al. [87]. compared the protein sequences of the colorectal EV protein group [88] and different symbiotic bacteria and viruses studied by Choi and colleagues, and found a large number of matching protein sequences. Recently, the

authors [89] analyzed the gut microbes and related human proteins in the pediatric IBD population and identified the characteristics of new host-microbial interaction (including microbial metabolism). In addition, the intestinal mucosa of patients with IBD can secrete EVs containing host defense proteins which, when ingested by microorganisms, will cause the response of microbial defense stress and functional adaptation, leading to the imbalance of intestinal microbiota and the subsequent development of mucosal inflammation. Moreover, CRC derived EVs can influence symbiotic bacteria in the GI microenvironment. These novel analytical approaches contribute to the understanding of overall composition and similarities between bacteria and human eukaryotic proteins, as well as their functions.

Intriguing, in addition to bacteria, fungus and protozoa, plant-derived exosome-like nanoparticles (ELNs) also play a role in disease development. Recently, Yun Teng and Huang-ge Zhang et al. [90] found that small RNAs in ginger ELNs can affect GI microbiota, thus improving intestinal barrier function and alleviating colitis in mice.

Malignancies include not only tumor cells with genetic and phenotypic heterogeneity, but also heterogeneous healthy cell populations involved in the anti-tumor immune response that forms a specific extracellular matrix and its EVs guarantee tumor evolution and development [91]. Another key aspect of tumor evolution is the epigenetic regulation at the gene transcription level that influences proliferation, differentiation, and the fate of tumor cells. The initial strategy of using abnormal methylation to diagnose tumors is challenging since chronic inflammation can also lead to changes in gene methylation expression levels [92, 93].

Clinical application of EVs

Multiple cell types [94, 95] have been applied as donor cells for EVs production, including autologous ascites, autologous monocytes, bacteria, and worms, among others (**Table 2**). Mesenchymal stem/stromal cells derived EVs (MSC-EVs) is promising and effective in animal models of 30 human diseases. However, several key issues including the efficacy, safety, characteristics, and heterogeneity of MSC-EVs need to be seriously addressed before they can be successfully converted to clinical practice [96]. Some institutions have proposed several indicators to define the potential of MSC-S EVs to treat COVID-19 [97, 98], which clinical trials are urgently needed to assess and confirm the potential. EV from non-MSC cell sources have also been reported and are in ongoing preclinical studies [99]. EVs can be loaded with antisense oligonucleotides (ASOs) or Cas9 mRNA and gRNA and can deliver them to cancer cells [100]. RBC-EVs can be absorbed by leukemia cells and has the advantages of high efficiency and low cytotoxicity. In xenograft mouse models, ASO-loaded RBC-EVs can effectively knock out miR-125b and inhibit the development of leukemia or breast cancer, demonstrating the potential of EV for the treatment of cancers. EVs can be utilized as carriers for anti-tumor drugs, small RNAs, and anti-inflammatory therapeutic agents. A series of studies have shown that EVs from different sources can cross tissue barriers, and carry cargos and deliver them to target cells [101-103]. Professor Robert Blelloch's team found that exosomes secreted by prostate cancer may enter the draining lymph nodes and spleen of tumors and suppress immune cells. They also found that inhibiting the formation and release of tumor exosomes eliminates the resistance of many tumors to PD-L1 inhibitors, and allows the immune system to form a long-term immune memory of the tumor that acts as a tumor vaccine [31]. EVs carrying pathogen-specific antigens can be used as new vaccines for human and animal infectious diseases. Parasite-derived EVs modifies intestinal inflammatory molecular (including cytokines and signaling molecules) in preclinical mouse models [104]. In a preclinical model of inflammatory bowel disease (IBD) in mice, EV secreted by worm parasites is beneficial in the inflammatory response, resulting in a substantial reduction of pro-inflammatory cytokines [105, 106]. However, due to the large number of active macromolecules carried by parasitic EV, the therapeutic use of parasitic EVs needs to be thoroughly evaluated.

However, there is still a lot of room for further development of targeted strategies, we can improve the targeting efficiency by engineering the surface molecules. Adhering to the highest standards of EVs separation and production is critical for clinical applications. The European

Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have published guidelines covering the manufacture and clinical evaluation of novel EVs therapeutic agents. In addition to general requirements such as EVs isolation and storage, safety and efficacy evaluation of EVs-based therapies are also required. Besides, the safety of donors and recipients is also an important issue to consider. Therefore, EVs must be manufactured per Good Practice principles aimed at ensuring product safety, meeting its intended use, and following quality control processes during manufacturing, monitoring, storage, and distribution. In the future, we should provide advice to research institutions and clinical trial sponsors at the national or international level, which will facilitate interdisciplinary collaboration between academia and industry and accelerate the success of preclinical development and clinical transformation (**Figure 3**).

Conclusion and future outlooks

Over the years, the field of EVs has made considerable epoch-making and milestone progress, revealing the immeasurable role of EVs in microbe-host and microbe-microbe cross-border communication, but it is still a daunting task to recognize and translate various research results into clinical practice.

Other critical issues in EVs biogenesis and delivery

Although the basic framework of EVs has been understood over the past decade or so, it is not deep and comprehensive enough. (a) The clear function of ESCRT proteins in EV biogenesis is unclear; (b) Due to the lack of endogenous control to standardize the expression of exosomal nucleic acids and the contradictory results of various studies, its clinical application remains limited. (c) Intensively investigating the physical and chemical properties of EVs, as well as the positive and negative regulatory factors involved in the occurrence of EVs; (d) Explaining the protein-protein interaction networks involved in EV biological processes; (e) Determining the delivery mechanisms that mediate the interactions between bioactive proteins, nucleic acids and lipids in the host and microbial EVs and target cells. (f) Studying the changes of host-microbial EVs composition and function in both health and disease conditions, and mapping their flow through the body-fluids. Meanwhile, we also need new experimental methods and techniques to assist us in studying the composition and biogenesis of EVs at single-vesicle and single-cell levels. These advances will accelerate our comprehension of EVs and facilitate clinical translation of EVs into diagnosis and therapies (**Figure 3**).

Challenges in the application of EVs in tumor therapy

Firstly, since EV can be used as clinical diagnostic and prognostic biomarkers, vaccines, or drug delivery devices, more precise and standardized isolation and purification methods are urgently needed. Secondly, the loading efficiency of exosome antigen should be improved in order to improve the odds of immunotherapy or exosome-based immunization. Thirdly, it is of importance how to massively produce EVs in clinical application. Currently, EVs of good manufacturing practice grade have been reported from certain cells (such as MSCs) [107], and in the future, we will need to extract EVs from more different cell types and identify which cell types are best suited to produce clinically grade EVs. Besides, the combination of EVs and other anti-tumor therapies has a broad and promising application prospect and may contribute to making a breakthrough in the bottleneck of tumor treatment. Recently, EVs have been reported as siRNA delivery carriers to silence oncogenes in cancer cells [108]. The activation of GTPase KRAS is very common in pancreatic cancer [109, 110]. Due to the low stability and uncontrollability of KRAS-targeted nucleic acids, it has become a difficult problem to promote efficacy by targeting KRAS. Delivery of KRAS siRNA using EVs from normal human prepuce fibroblasts has been reported to be highly efficient, significantly inhibiting pancreatic tumor progression and improving overall survival in mice [111]. Shortly, immune-cell-derived EVs will be applied in cancer immunotherapy. For example, NK cell EVs have cytotoxic effects on different human tumor cells. Activated EVs of CD8+ cells can also deplete the mesenchymal cells in the mesenchymal tumors and inhibit cancer progression [112]. At present, liquid biopsy, a non-invasive and simple method, acts as an important biomarker for tumor diagnosis and prognosis evaluation. EVs have sufficient concentration and high stability in human circulation, thus they have obvious advantages over other liquid biopsy methods

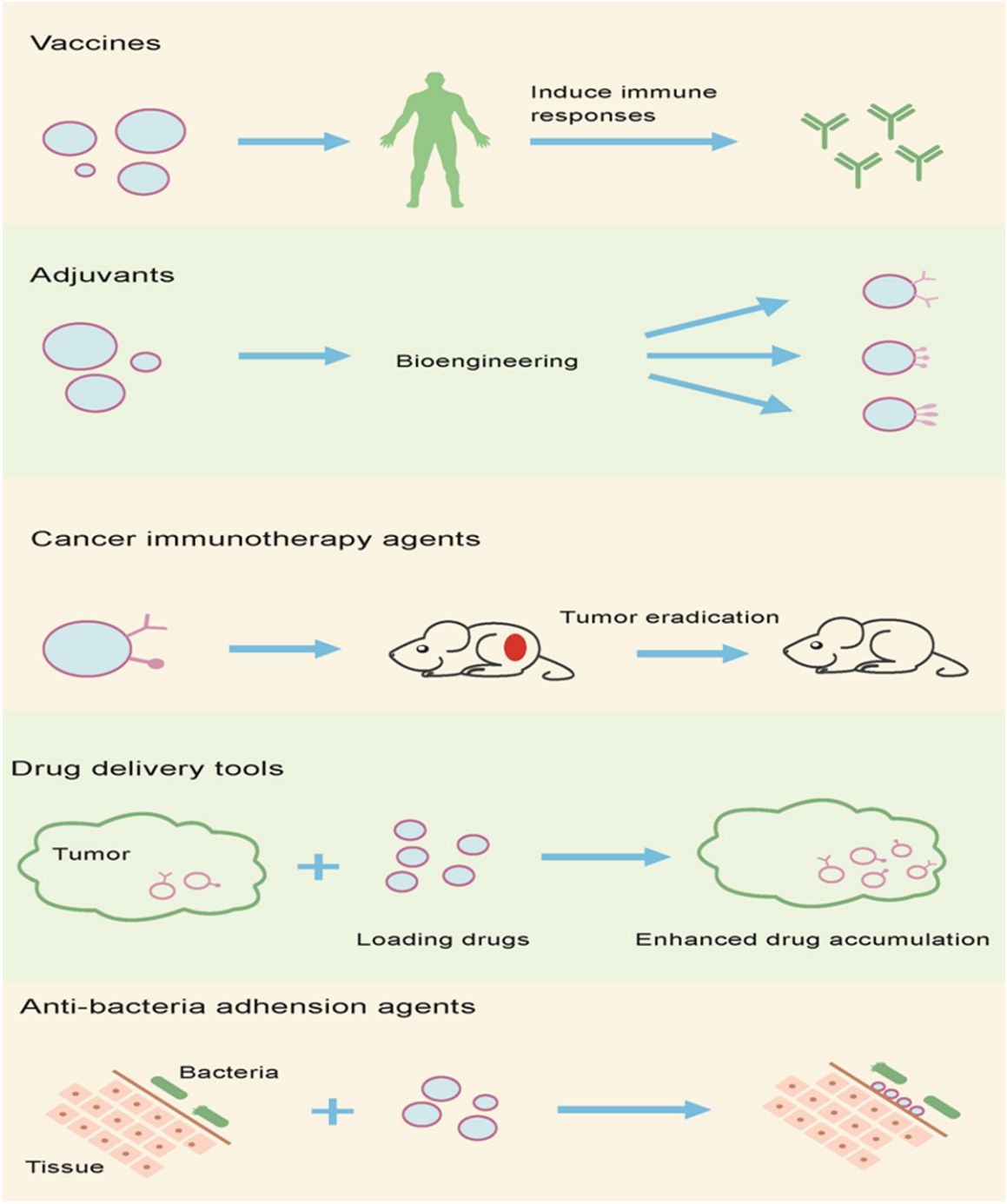

Figure 3. Clinical applications of EVs. The structure and composition of EVs contributes to its clinical applications. Multiple proteins, PAMPs, lipids and other EV molecules make it promising vaccines, cancer immunotherapy agents, adjuvants, drug delivery tools and anti-bacteria agents and endow it broad prospects.

[113]. Also, EV contains a variety of cargos, such as proteins and nucleic acids (miRNAs), which can be used as biomarkers for disease diagnosis [114, 115]. The GPC1+ EVs can be applied as potential non-invasive biomarkers to detect early pancreatic cancer. Overwhelming studies have indicated that miRNAs in EVs contribute to immune regulation, drug resistance, and cancer metastasis of various tumor types [116-118]. In addition to miRNAs, circular RNAs

from cancer cells and patient serum may serve as novel biomarkers [119, 120].

In summarize, EVs from different sources have great potential for early cancer diagnosis, tracking chemotherapy drug resistance and therapeutic responses, and tailoring precision treatment strategies for patients with cancers. Nevertheless, there are many problems in EV field that need to be solved urgently, and it is worth us to further study its mechanism and carry out cross-field and cross-disciplinary research to enrich its application prospects.

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Disclosure of conflict of interest

None.

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