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Review

Extracellular Vesicle Heterogeneity in Colorectal Cancer: From Biological Diversity to Therapeutic Translation and Precision Intervention

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Abstract

Colorectal cancer (CRC) remains a major global health burden, with persistent challenges in disease monitoring, therapeutic resistance, and durable treatment efficacy. Extracellular vesicles (EVs) are key mediators of intercellular communication and have emerged as promising biomarkers, therapeutic delivery platforms, and intervention targets in CRC. However, EVs are highly heterogeneous in biogenesis, cargo composition, cellular origin, biological source, surface properties, and functional state, leading to context-dependent effects on tumor progression, microenvironment remodeling, immune regulation, metastasis, and therapy response. In this review, we integrate EV heterogeneity with CRC biology, biomarker-guided stratification, and therapeutic translation. We first discuss how distinct EV populations shape CRC-associated signaling, tumor-stromal crosstalk, immune remodeling, metastatic dissemination, and treatment resistance. We then summarize emerging analytical strategies, including single-vesicle profiling, multi-omics integration, and AI-assisted biomarker discovery, that may help resolve EV heterogeneity and identify clinically informative EV subpopulations. Finally, we focus on EV-based therapeutic engineering, including therapeutic cargo delivery, vaccine platforms, immune microenvironment reprogramming, targeting of EV biogenesis, secretion and uptake, biomimetic platform development, standardization, and scalable manufacturing. By linking EV-mediated molecular signaling, cellular phenotype remodeling, biomarker discovery, drug response, and therapeutic engineering, this review proposes a mechanism-informed translational framework for exploiting EV heterogeneity in CRC. Rather than viewing EV heterogeneity solely as a barrier to clinical translation, we highlight its potential as a resource for identifying EV-mediated therapeutic vulnerabilities, improving patient stratification, and guiding the development of EV-based precision intervention strategies.

Keywords: colorectal cancer; extracellular vesicle heterogeneity; tumor microenvironment; biomarker-guided stratification; therapeutic engineering

1. Introduction

Colorectal cancer (CRC) remains a major global health burden, ranking as the third most commonly diagnosed malignancy worldwide. Epidemiological projections further indicate that its incidence will continue to rise, with the global burden expected to increase by nearly 60% by 2030 [1]. Despite advances in screening and treatment, the prognosis of advanced CRC remains poor, with a 5-year survival rate of approximately 10% in patients with distant metastasis [2, 3]. These clinical limitations underscore the urgent need to better understand the molecular mechanisms underlying CRC progression and to develop more effective strategies for early diagnosis and therapeutic intervention.

Extracellular vesicles (EVs) have emerged as key mediators of intercellular communication in CRC. By transferring proteins, nucleic acids, lipids, and metabolites between cells, EVs regulate tumor growth, microenvironment remodeling, immune evasion, metastatic dissemination, and therapy response [4]. However, EVs are not a uniform entity. Their biological activities are shaped by substantial heterogeneity in biogenesis pathways, molecular cargo, cellular origin, and species source. This heterogeneity provides the basis for their functional diversity, but also complicates EV isolation, characterization, mechanistic interpretation, and clinical application [5].

From a translational perspective, EV heterogeneity represents both an opportunity and a challenge. EV-derived molecular signatures offer promising minimally invasive biomarkers for CRC detection, prognosis, and longitudinal monitoring [6-8], while EVs themselves can serve as delivery vehicles, immune modulators, or therapeutic targets [9-11]. At the same time, conventional bulk-level analyses often mask functionally distinct EV subpopulations, reducing biomarker specificity and limiting the precision of EV-based therapeutic strategies. Therefore, the ability to resolve EV heterogeneity is essential for transforming EV biology into reliable clinical tools. Recent methodological advances are beginning to address this challenge. Single-vesicle technologies, multi-omics profiling, and data-driven analytical frameworks enable more precise characterization of EV subpopulations and their disease-specific signatures [12-14]. In parallel, advances in EV engineering, cargo loading, surface modification, and bio-mimetic vesicle design are expanding the therapeutic potential of EV-based platforms [15-18]. Together, these developments are shifting the field from descriptive EV profiling toward functional dissection and rational translational design.

Despite rapid progress, current studies often address EV biology, biomarker development, and therapeutic applications in isolation, lacking a unified framework that links EV heterogeneity to translational outcomes. In this review, we systematically integrate these dimensions by summarizing the origins and functional implications of EV heterogeneity in CRC, and by discussing how this heterogeneity informs biomarker discovery and therapeutic design. We further highlight emerging technologies that enable high-resolution EV analysis and scalable engineering, thereby bridging the gap between fundamental EV biology and clinical application. Together, this review provides a conceptual and translational framework for leveraging EV heterogeneity in precision medicine for CRC.

2. EV Heterogeneity and Functional Consequences in CRC

To clarify how EV heterogeneity shapes CRC biology, this section dissects EV heterogeneity across four interconnected dimensions: biogenesis pathway, molecular cargo composition, cellular origin, and species source. These dimensions are not independent categories, but jointly determine EV identity, functional specificity, and context-dependent effects within the CRC ecosystem. By organizing EV heterogeneity in this way, we provide a framework for understanding how heterogenous EV populations contribute to tumor progression, immune modulation, metastasis, and therapeutic response.

2.1 Biogenesis-Dependent EV Heterogeneity and Nomenclature

EVs are a heterogeneous group of lipid bilayer-enclosed particles released from cells that are incapable of replication. Their heterogeneity arises from distinct biogenesis pathways and molecular packaging mechanisms. Historically, EVs have been classified according to proposed biogenesis pathways into endosome-derived exosomes and plasma membrane-derived ectosomes (also referred to as microvesicles) [19]. In this classical framework, exosomes are generated as intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) and released upon fusion of MVBs with the plasma membrane, whereas ectosomes bud directly from the plasma membrane [19]. However, vesicle size and commonly used markers such as CD9, CD63, and CD81 are not sufficient to establish a specific biogenesis pathway. Accordingly, distinguishing these populations remains challenging in most experimental settings.

Recent MISEV2023 guidelines recommend the use of operational terms based on measurable characteristics, such as size (small EVs <200 nm; medium/large EVs >200 nm), density, biochemical composition, or cellular origin [20]. This framework promotes standardized EV characterization. Consistent with MISEV2023, this review therefore uses the generic term "EV" or, where supported by the reported physical characteristics, operational terms such as "small EV" when subcellular biogenesis has not been experimentally demonstrated. The terms "exosome" and "exosomal" are reserved for studies in which an endosomal origin has been established or are retained when directly reproducing the terminology used in the cited study, a formal assay name, or a commercial product name. In such cases, use of the original terminology should not be interpreted as independent evidence of endosomal biogenesis.

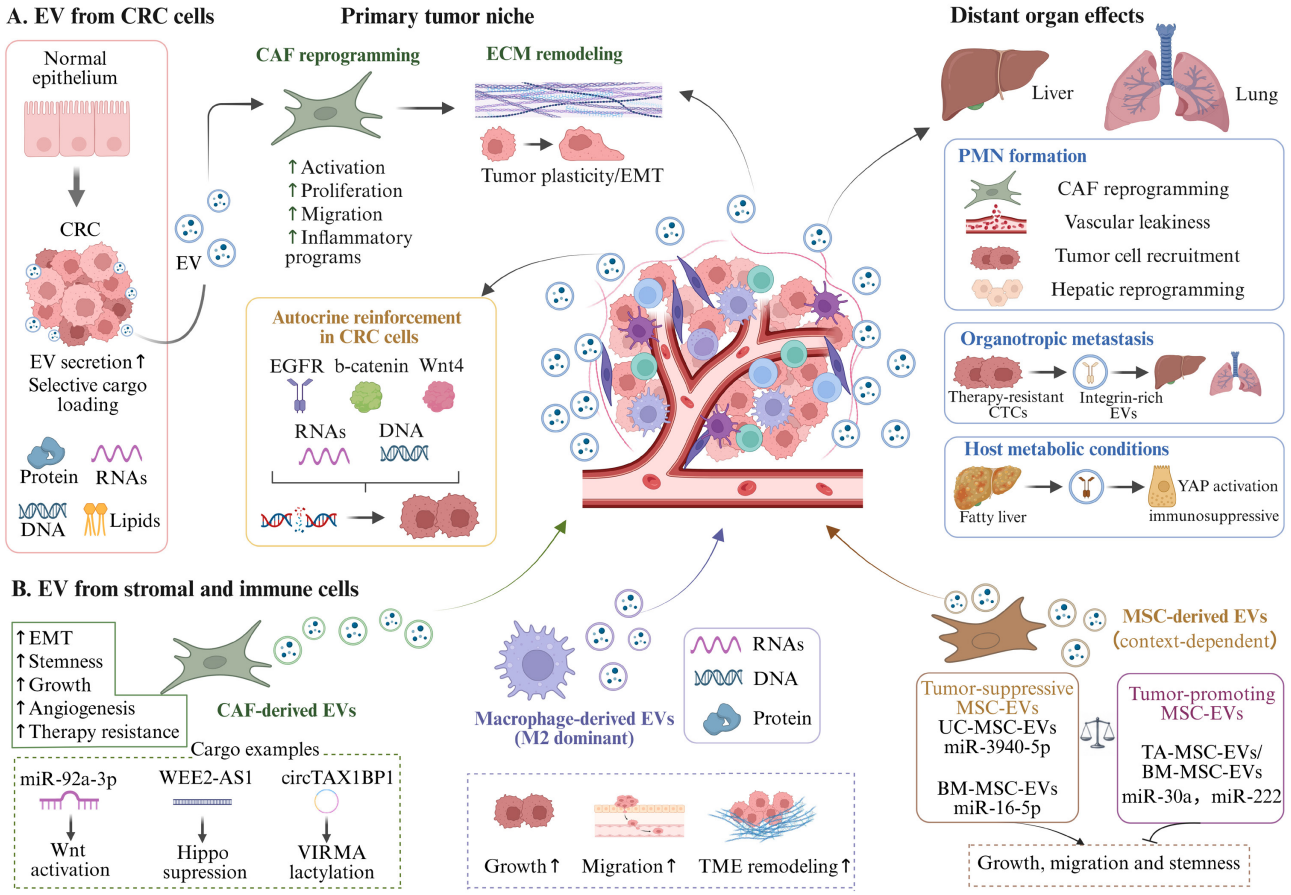


Figure 1. EV-mediated communication in colorectal cancer progression. Extracellular vesicles (EVs) derived from distinct cellular sources mediate diverse and context-dependent functions in colorectal cancer (CRC). **(A)** CRC cell-derived EVs increase during malignant transformation and selectively carry proteins, RNAs, DNA, and lipids. These EVs remodel the primary tumor niche by reprogramming cancer-associated fibroblasts (CAFs), promoting extracellular matrix (ECM) remodeling, enhancing tumor plasticity and epithelial-mesenchymal transition (EMT), and reinforcing malignant programs through autocrine signaling. They also contribute to premetastatic niche formation, organotropic metastasis, and host metabolism-associated metastatic progression. **(B)** Stromal and immune cell-derived EVs further shape CRC progression. CAF-derived EVs promote EMT, stemness, angiogenesis, and therapy resistance; macrophage-derived EVs, particularly those from M2-like macrophages, support tumor growth, migration, and tumor microenvironment remodeling; whereas mesenchymal stromal/stem cell-derived EVs (MSC-EVs), including tumor-associated mesenchymal stem cell-derived extracellular vesicles (TA-MSC-EVs), exert source- and context-dependent tumor-suppressive or tumor-promoting effects. Together, the figure highlights cellular origin as a major determinant of EV cargo composition, functional specificity, and biological outcome in CRC. Schematic illustrations were created with BioRender.com.

2.2 Cargo-Level EV Heterogeneity and Functional Output in CRC

Building upon the heterogeneity of biogenesis pathways, EV cargo composition is likewise highly variable and represents a key determinant of their functional diversity. EVs exhibit substantial heterogeneity in molecular cargo, which reflects their biogenesis, cellular origin, and functional state. Based on spatial distribution, EV cargo in CRC can be broadly classified into three categories: (i) internal cargo (e.g., soluble proteins, RNAs, DNAs, and lipids), (ii) membrane-associated components (e.g., transmembrane proteins), and (iii) exofacial molecules located on the outer surface of EVs. These distinct cargo types collectively determine EV-mediated intercellular communication.

2.2.1 Surface Proteins and Biomolecular Corona

Surface-associated proteins regulate EV-mediated communication by mediating signaling and enabling cargo sorting, targeting, uptake, and long-range intercellular communication. EV-associated proteins in CRC span several functional classes, including signaling receptors (e.g., FZD10 and EGFR) [21], immune-checkpoint proteins (e.g., PD-L1), death ligands (e.g., TRAIL) [22, 23], adhesion- and extracellular matrix-related proteins (e.g., ITGBL1) [24], growth-factor ligands (e.g., Gas6 and AREG), stress-response proteins (e.g., HSP70), and intracellular signaling or trafficking proteins associated with EV membranes or cargo (e.g., KRAS and Rab13) [25-29]. Collectively, these protein classes contribute to epithelial plasticity, stromal activation [24], immune regulation [23, 26], and angiogenesis [30] in CRC. In addition to intrinsic membrane components, EV surfaces are dynamically remodeled by a biomolecular corona formed through interactions with extracellular proteins, lipids, proteoglycans, and nucleic acids. This corona represents an acquired layer rather than a passive

coating, and can constitute a substantial fraction of EV-associated proteins, in some cases exceeding luminal cargo. Notably, molecules previously regarded as co-isolated contaminants are now recognized as integral components of this structure [31]. Functionally, the biomolecular corona redefines the physicochemical and biological identity of EVs. By altering surface composition, it modulates EV stability, biodistribution, cellular uptake, and immune recognition [32-34], thereby shaping their pharmacokinetics, targeting specificity, and immunoregulatory signaling [35, 36]. In addition, the presence of a corona may introduce biases in nanoparticle-based detection and characterization methods [37]. Despite its emerging importance, the role of the biomolecular corona in CRC remains largely unexplored, warranting further investigation into its regulation of EV behavior within the tumor microenvironment.

2.2.2 RNA Cargo

EVs carry diverse RNA species reflecting the genetic and functional state of their cells of origin. These include small RNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear and nucleolar RNAs, Y RNAs, and ribosomal RNAs (rRNAs), as well as long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), messenger RNAs (mRNAs), transfer RNAs (tRNAs), and mitochondrial RNAs. These RNAs collectively participate in gene regulation, RNA processing, and intercellular communication, thereby influencing CRC. For example, EV miRNAs promote CRC progression by driving tumor cell proliferation, liver metastasis [38, 39], metabolic reprogramming [40], remodeling of the tumor microenvironment [39], and angiogenesis [41], while siRNAs mediate RNA interference and hold therapeutic potential [42]. circRNAs [43], lncRNAs [44] and tRNA [45] act as epigenetic regulators and molecular sponges, contributing to CRC progression and therapy resistance. The RNA cargo of EVs differs markedly from that of their parental cells, not only in composition but also in size distribution. EVs are typically enriched in small RNA species, whereas longer RNAs (e.g., full-length mRNAs) are often truncated, reflecting the limited nucleic acid capacity of small EVs. Mechanistically, RNA incorporation into EVs is regulated by RNA-binding protein (RBP)-mediated loading [46] and sequence-dependent sorting (e.g., GGAG and GGCU motifs recognized by hnRNPA2B1 and SYNCRIP) [47], coupled with EV biogenesis pathways involving VAP-A and CERT at ER membrane contact sites [48]. Functionally, EV-associated RBP such as HuR can modulate gene expression in recipient cells by stabilizing target mRNAs (e.g., c-Myc) [49]. Beyond RNA composition and sorting, recent evidence has demonstrated that cancer-specific RNA modifications in EVs promote CRC progression by modulating immune cells, particularly macrophages [50]. Despite these advances, the precise molecular mechanisms governing RNA loading and release remain incompletely understood.

2.2.3 DNA Cargo

EVs also carry DNA molecules, including double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), mitochondrial DNA (mtDNA), and plasmid DNA. These DNAs can be localized both within EVs and on their outer surfaces, with their distribution and composition varying among EV subtypes. ssDNA and dsDNA may originate from micronuclei, autophagy-related amphisomes, or cytosolic DNA sensing pathways (e.g., via cGAS), particularly under genomic instability or DNA damage. Notably, EV-associated dsDNA includes large genomic DNA (gDNA) fragments (100 bp-20 kbp) that can span the entire genome and reflect the mutational landscape of parental cells, supporting their utility in PCR-based mutation detection, CRC diagnosis and disease monitoring [51-53]. Functionally, EV-mediated DNA transfer can also modulate immune responses. In Crohn's disease, EVs transport pathogenic mtDNA and gDNA from damaged intestinal epithelial cells to macrophages, activating the STING pathway and triggering inflammatory responses [54]. In CRC, therapy-induced release of epithelial-derived gDNA via EVs can activate the AIM2 inflammasome, thereby promoting inflammation [55]. Collectively, understanding the biological significance of EV-mediated DNA release and its extrinsic effects may provide novel approaches for biomarker development and therapeutic intervention in CRC.

2.2.4 Microenvironmental Regulation of EV Cargo

EV cargo composition is dynamically shaped by the microenvironmental state of the parental cell. Stress conditions, differentiation status, and physical cues can alter both EV release and cargo selection, thereby changing their biological functions [5, 56, 57]. For example, increased matrix stiffness promotes the secretion of Jagged1-enriched EVs, which activate Notch signaling and facilitate tumor progression [58]. In CRC, hypoxia represents a particularly important microenvironmental driver. As tumor growth often exceeds vascular supply, CRC cells frequently experience hypoxic and mildly acidic conditions that enhance EV release and reprogram EV cargo [59]. Specifically, EV-associated miR-135a-5p derived from

hypoxic CRC cells can promote metastatic adaptation [60]. Additionally, EVs released from aerobic CRC cells induce doxorubicin resistance in hypoxic CRC cells and macrophages, underscoring their role in chemoresistance [61]. Together, these findings indicate that EV cargo reflects not only the identity of the cell of origin but also the local ecological pressures imposed by the tumor microenvironment.

2.3 Cellular Origin-Dependent EV Heterogeneity in CRC

Given that EV cargo composition is largely determined by their cells of origin, heterogeneity at the cellular level represents another critical layer of EV diversity. EVs released by different cells demonstrate distinct functional specificities. Within the tumor microenvironment (TME), malignant cells, immune cells, and stromal cells establish a complex signaling network through EVs, collectively modulating disease progression. In this context, EVs can propagate both pro-tumor and anti-tumor signals, with their precise effects contingent on the characteristics of the originating and recipient cells.

2.3.1 CRC Cell-Derived EVs

CRC cell-derived EVs are critical mediators of intercellular communication between tumor cells and recipient cells in both local and distant microenvironments. Malignant transformation not only increases EV secretion compared with nonmalignant cells [62], but also enhances the selective packaging of bioactive cargo, including proteins and DNA [63], thereby reshaping both the quantity and molecular composition of EVs.

At the level of the primary tumor niche, CRC cell-derived EVs actively reprogram cancer-associated fibroblast (CAF), promoting their activation, proliferation, and migration, as well as the establishment of proinflammatory transcriptional programs [38, 64, 65]. In parallel, this CAF activation establishes a localized tumor-supportive microenvironment that enhance extracellular matrix (ECM) remodeling and tumor cell plasticity. For example, EV-derived miR-99a from highly metastatic CRC cells promotes tumor cell migration by inducing CAF secretion of CCL7, which in turn drives epithelial-to-mesenchymal transition (EMT) via activation of the CCR5-mTOR-p70S6K axis [64]. Beyond microenvironmental remodeling, EV-mediated signaling also reinforces malignant traits within tumor cells. Notably, EVs carrying non-coding RNAs and oncogenic proteins, including EGFR [66], β-catenin [67], and Wnt4 [68], can induce genetic instability and accelerate neoplastic transformation in CRC.

Beyond remodeling the primary tumor niche, CRC cell-derived EVs contribute to the establishment of premetastatic niche (PMN) in distant organs by reprogramming CAFs [24, 69], enhancing vascular leakiness and tumor cell recruitment [41, 70, 71], while simultaneously reprogramming hepatic cells toward a prometastatic phenotype [39, 72]. These changes collectively establish a permissive environment for metastatic colonization. Beyond niche formation, EVs further contribute to organ-specific metastatic behavior [73]. Notably, EVs released by therapy-resistant circulating tumor cells are enriched in integrin-family proteins that preferentially target organs such as the liver and lungs, increasing metastatic risk in CRC [74]. In addition, host metabolic conditions can further amplify EV-mediated metastatic progression. For example, fatty liver promotes the production of hepatocyte-derived EVs, which enhance CRC liver metastasis by activating Yes-associated protein signaling and reinforcing an immunosuppressive microenvironment [75].

2.3.2 Stromal and Immune Cell-Derived EVs

The CRC TME comprises diverse non-malignant cellular populations, including CAFs, mesenchymal stromal/stem cells (MSCs), immune cells, endothelial cells, pericytes, adipocytes, neurons, and other stromal elements. These cells are no longer viewed as passive bystanders, but as active regulators of tumor evolution. EVs represent a major route of this intercellular communication, enabling stromal and immune cells to transfer regulatory cargos that reshape malignant phenotypes.

CAFs represent the best-characterized stromal source of EVs in CRC. CAF-derived EVs promote multiple hallmarks of tumor progression, including EMT [43, 76], stemness maintenance [76, 77], tumor growth [78, 79], angiogenesis [80], and therapy resistance [76, 77, 80]. Mechanistically, these effects are mediated by diverse EV cargos, such as miR-92a-3p-induced activation of Wnt/β-catenin signaling [76], lncRNA WEE2-AS1-dependent suppression of the Hippo pathway [79], and circTAX1BP1-mediated regulation of VIRMA lactylation in CRC cells [43]. Together, these findings establish CAF-EVs as a prototypical pro-tumorigenic stromal EV population in CRC. MSCs represent another important stromal EV source, but their effects in CRC are more context-dependent. EVs from certain MSC sources suppress CRC progression. Umbilical cord-derived MSC-EVs transfer miR-3940-5p to CRC cells, leading to ITGA6 downregulation and inhibition of tumor growth and metastasis [81]. Similarly, bone marrow-derived MSC-EVs enriched in miR-16-5p or miR-22-3p suppress CRC progression via targeting ITGA2 or modulating the PI3K/AKT pathway [82, 83]. In contrast, MSC-EVs can also promote malignant

phenotypes under specific biological contexts. CRC-associated MSC-EVs deliver miR-30a and miR-222 to tumor cells, directly targeting the tumor suppressor MIA3 and enhancing proliferation and migration [84]. Moreover, bone marrow MSC-derived EVs carrying miR-142-3p expand the population of CRC stem-like cells [85]. These findings suggest that MSC-EVs are not intrinsically tumor-suppressive or tumor-promoting; rather, their function is determined by MSC origin, activation state, and cargo composition.

Immune cell-derived EVs further expand the complexity of TME-mediated communication. Among them, macrophage-derived EVs, particularly those released by M2-like macrophages, have been closely associated with CRC progression. By transferring oncogenic miRNAs [86-88], lncRNAs [89], and proteins [90, 91], macrophage-derived EVs reprogram cancer cell behavior, promote tumor growth and metastasis, and remodel the tumor-supportive microenvironment. In this context, immune cell-derived EVs provide a functional link between immune polarization, tumor cell plasticity, and metastatic progression.

2.4. Non-Mammalian EVs and Their Functional Roles in CRC

In addition to mammalian cell-derived EVs, EVs from other species, particularly gut microbiota and plants, have emerged as important modulators of CRC. These non-mammalian EVs exhibit distinct biogenesis pathways, cargo compositions, and functional properties, adding another layer of heterogeneity to the EV landscape in CRC.

2.4.1 Gut Microbiota-Derived EVs

The colon harbors a dense and diverse microbial ecosystem, and dysbiosis is a key driver of CRC [92]. Emerging evidence indicates that microbiota-derived EVs, rather than bacteria per se, function as critical mediators that translate microbial dysbiosis into host cellular reprogramming. These vesicles exhibit substantial heterogeneity in biogenesis, structure, and molecular cargo, reflecting differences in microbiota species, genetic background, and physiological state, thereby enabling broad functional capacity and dynamic adaptation to environmental cues. In the colorectal lumen, microbiota-derived EVs interact with host cells through ligand-receptor signaling or are internalized via endocytosis, phagocytosis, or membrane fusion, thereby activating intracellular pathways.

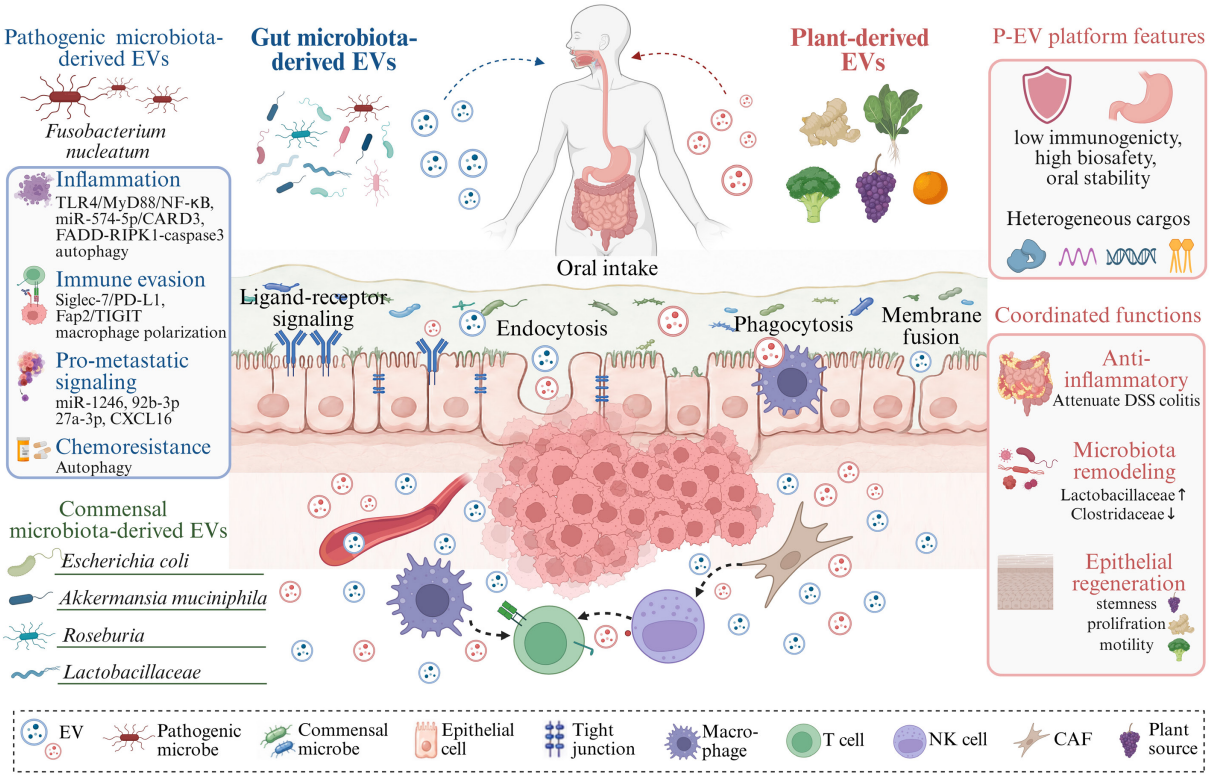


Figure 2. Non-mammalian EVs in colorectal cancer. Extracellular vesicles (EVs) derived from gut microbiota and plants add an additional layer of EV heterogeneity in colorectal cancer (CRC). Microbiota-derived EVs interact with host cells through ligand-receptor signaling, endocytosis, phagocytosis, or membrane fusion, thereby reshaping epithelial, immune, and stromal responses. Pathogenic microbiota-derived EVs promote CRC by inducing inflammation, barrier disruption, immune evasion, pro-metastatic signaling, and chemoresistance, whereas EVs from commensal or probiotic microbes can exert barrier-protective, anti-inflammatory, and antitumor effects. Plant-derived EVs (P-EVs), acquired through oral intake, carry diverse bioactive cargos and exhibit low immunogenicity, high biosafety, and oral stability. Functionally, they help maintain intestinal homeostasis by attenuating inflammation, remodeling gut microbiota, and promoting epithelial regeneration. Schematic illustrations were created with BioRender.com.

Microbiota-derived EVs initiate tumorigenesis by establishing a chronic inflammatory and barrier-disruptive niche. *Fusobacterium nucleatum*-derived EVs (FnEVs) activate TLR4/MyD88/NF-κB signaling and induce pro-inflammatory cytokines, while compromising epithelial integrity through miR-574-5p/CARD3-dependent autophagy, tight-junction disruption, and FADD-RIPK1-caspase-3-mediated epithelial injury [93-95]. Beyond this initiating niche, pathogenic EVs further accelerate CRC progression by coordinating immune evasion, intercellular signaling, and stress-adaptive autophagy. FnEVs suppress antitumor immunity through Siglec-7-dependent PD-L1 upregulation, macrophage polarization, and Fap2-mediated TIGIT engagement on NK and T cells [96, 97]. They also disseminate pro-metastatic cues by transferring miR-1246, miR-92b-3p, miR-27a-3p, and CXCL16 [98], while activating autophagic flux to support tumor cell survival, recurrence, and chemoresistance [94, 99, 100]. Notably, EVs derived from both pathogenic and commensal bacteria can globally reshape gene expression profiles in colon cancer cells, highlighting their role as systemic regulators of tumor cell states [101, 102].

Importantly, microbiota-derived EVs exert context-dependent and bidirectional effects. While pathogenic EVs promote tumorigenesis, EVs from commensal and probiotic bacteria exhibit barrier-protective, anti-inflammatory, and antitumor activities. For example, *Escherichia coli*-derived EVs can induce apoptosis or ROS-mediated mitophagy in CRC cells [103]. EVs from *Akkermansia muciniphila* and *Roseburia intestinalis* enhance barrier integrity and suppress inflammation [104-106], while Amuc_2172 (an *Akkermansia muciniphila*-derived protein) enhances cytotoxic T-cell activation and synergizes with immune checkpoint blockade [106, 107]. Similarly, Lactobacillaceae-derived EVs inhibit CRC progression by modulating PDK1/AKT/BCL-2 signaling, suppressing TLR4/NF-κB/NLRP3 pathways, reversing chemoresistance, and enhancing anti-PD-1 efficacy [108-110]. Collectively, microbiota-derived EVs function as context-dependent molecular switches that integrate inflammatory signaling, immune evasion, autophagy, and intercellular communication networks in CRC. Their bidirectional activity underscores their potential as both biomarkers and therapeutic targets, offering new avenues for precision intervention in CRC.

2.4.2 Plant-Derived EVs

Plant-derived EVs (P-EVs) are membrane-bound nanostructures released by plant cells. They exhibit heterogeneity in size, density, cellular origin, and biochemical composition, reflecting differences in plant tissue and physiological state [111-113]. Despite the presence of a rigid cell wall, accumulating evidence demonstrates that plants actively produce EVs through multiple intracellular pathways, including multivesicular bodies, exocyst-positive organelles, Pen1-positive compartments, vacuoles, and autophagosomes [114-116]. Similar to mammalian EVs, P-EVs carry diverse bioactive cargos including proteins, lipids, RNAs, and metabolites, which underlie their functional versatility. Notably, P-EVs exhibit low immunogenicity, high biosafety, and robust stability after oral administration, underscoring their translational potential [117].

Emerging evidence indicates that P-EVs are bioactive regulators of intestinal homeostasis with substantial anti-inflammatory and regenerative potential. Vesicles derived from edible plants such as ginger [118, 119], grape [120], broccoli [121], red cabbage [122], Portulaca oleracea L [123], and grapefruit [124] have been shown to attenuate dextran sulfate sodium (DSS)-induced colitis, supporting their broad capacity to restrain intestinal inflammation. Beyond direct anti-inflammatory effects, P-EVs actively reshape the gut microbiota by enriching beneficial taxa such as *Lactobacillaceae* and *Bacteroidaceae* while reducing pathogenic *Clostridaceae* [125], thereby contributing to microbial rebalance. They also influence epithelial repair programs. For example, grape-derived EVs promote $Lgr5^+$ stem cell expansion via Wnt/β-catenin signaling [120], whereas ginger-derived EVs enhance epithelial survival and proliferation and suppress colitis-associated cancer [126]. In addition, P-EVs can modulate intestinal physiology, as illustrated by broccoli-derived EVs, which improve gut motility and alleviate loperamide-induced constipation [127]. Collectively, these findings position P-EVs as multifunctional modulators of the intestinal ecosystem, with potential relevance for preventing inflammation-driven CRC initiation and improving CRC-associated gut dysfunction.

3. EVs as a Source of Biomarkers

The clinical translation of EVs as liquid biopsy templates relies on their ability to preserve molecular cargo from systemic degradation. However, a substantial translational gap remains. This is largely driven by the low throughput of gold-standard isolation methods and the complexity of biological fluids such as blood, where low-abundance tumor-derived EVs are often masked by a vast background of non-vesicular proteins and commensal vesicles. Consequently, the transition from benchtop discovery to high-throughput clinical assays requires both a deep understanding of the CRC-specific molecular

landscape and the integration of advanced analytical platforms.

3.1 Current Landscape of EV-based Biomarkers in CRC

To address the need for early diagnosis and longitudinal monitoring, research has focused on the high molecular diversity of EV cargo. By identifying CRC-specific alterations across different molecular layers, EVs offer a multidimensional resource for biomarker discovery and disease stratification.

3.1.1 RNA Biomarkers

EVs encapsulate multiple RNA species, representing the most extensively studied class of EV-derived biomarkers. Protected by the lipid bilayer, EVs-associated RNAs exhibit enhanced stability compared with freely circulating counterparts, supporting their utility as minimally invasive biomarkers. Accumulating evidence indicates that these RNAs reflect tumor-associated alterations and hold promise for CRC diagnosis, prognosis, and monitoring. Representative studies from the past five years are summarized in Table 1 [12, 128-155], detailing RNA species, sample size, specimen type, and diagnostic performance.

Table 1. Extracellular vesicle-derived RNAs as diagnostic, prognostic, and predictive biomarkers in colorectal cancer

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
Cui X et al. 2020 [128]	Serum	miR-1539	Diagnosis	CRC [n=51] vs healthy [n=49]	Single	0.673 (0.568-0.779)	RT-qPCR	ExoQuick™ exosome precipitation solution
Yang CK et al. 2022 [129]	Plasma	miR-320c	Diagnosis	mCRC [n=18] vs healthy [n=30]	Single	0.896	RT-qPCR	ExoEasy Plasma Handbook
Qiao D et al. 2022 [130]	Plasma	miR-3937	Diagnosis	CRC [n=34] vs healthy [n=18]	Single	0.827 (0.712-0.942)	RT-qPCR	BestBio-Exosome kit
					Combined +CEA+CA 199	0.889 (0.801-0.977)		
Kim S et al. 2022 [131]	Plasma	miR-92a, miR-141	Diagnosis	CRC [n=21] vs healthy [n=5]	Single	0.781-0.895	DNA barcode-based nucleic acid lateral flow assay	qEV original column
					Combined	0.952		
Li T et al. 2023 [132]	Plasma	circTADA2A (hsa_circ_0043278), circGAPVD1(hsa_circ_0003270), circMYO9B (hsa_circ_0004071), and circTJAP1 (hsa_circ_0092310)	Diagnosis	CRC [n=35] vs healthy [n=35]	Single	0.614-0.750	Microarray and RT-qPCR	Total Plasma Exosome Isolation Kit
Miyazaki K et al. 2023 [133]	Serum	A panel (miR-181b, miR-193b, miR-195, and miR-411)	Diagnosis	T1 CRC with LNM [n=7] vs without LNM [n=51]	Single	0.69-0.80	RT-qPCR	Total Exosome Isolation Kit
					Combined	0.86 (0.70-1.00) (Train)		
Wu Y et al. 2024 [134]	Serum	miR-1470	Diagnosis	CRC [n=168] vs healthy [n=168]	Single	0.740 (0.688-0.792)	qRT-PCR	Ultracentrifugation
Peng J et al. 2024 [135]	Serum	3'tRF-ThrCGT, 3'tRF-mtlleGAT	Diagnosis	CRC [n=205] vs healthy [n=201]	Single	0.656-0.669	RT-qPCR	Centrifugation
					Combined	0.777 (0.732-0.822)		
					Combined +CEA+CA 724	0.938 (0.913-0.963)		
Mannucci A et al. 2026 [136]	Plasma	4-miRNA panel (hsa-miR-32-5p, -486-3p, -550a-3-5p, -625-3p)	Diagnosis	EOCRC [n=251] vs non-disease [n=211]	Combined	0.946 (0.917-0.975) (Train) 0.923 (0.879-0.966) (Test)	RT-qPCR	ExoRNeasy Midi kits
Chen M et al. 2025 [137]	Plasma	miR-23a, miR-21	Diagnosis	CRC [n=5] vs non-cancer [n=9]	Single	0.889-1.000	CRISPR/Cas13 a	ExoQuick ULTRA EV Isolation Kit
Dana F et al. 2025 [138]	Plasma	lncRNA CCAT1, lncRNA XIST	Diagnosis	CRC [n=62] vs healthy [n=62]	Single	0.650-0.860	RT-qPCR	Centrifugation
Xu C et al. 2025 [139]	Plasma	miR-452-5p, miR-487-5p, miR-500a-5p, miR-7976 miR-664a-3p, miR-184, miR-4454, miR-218-5p, miR-3200a-3p	Prognosis	Good responders [n=57] vs poor responders [n=37]	Combined	0.81	qRT-PCR	ExoRNeasy Midi Kit
Handa T et al. 2021 [140]	Serum	miR-4323, miR-4284, miR-1290, miR-1246	Early diagnosis	Pretreatment and posttreatment of CRA [n=26]	Single	0.639-0.705	RT-qPCR	Polymer precipitation
					Combined	0.691 (0.528-0.817)		
				Pretreatment and posttreatment of large CRA [n=13]	Combined	0.834 (0.628-0.938)		

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
Nakayama S et al. 2025 [141]	Plasma	miR-122-5p, miR-130a-3p, miR-146a-5p, miR-15b-5p, and miR-126	Early diagnosis	Stage I CRC [n=60] vs healthy [n=60]	Single	0.730-0.788	RT-qPCR	QIAzol Lysis Reagent
Han L et al. 2021 [142]	Serum	miR-15b, miR-16, miR-21, and miR-31	Diagnosis and early diagnosis	CRC [n=150] vs healthy [n=123]	Single	0.58-0.86	RT-qPCR	ExoQuick kit
				CRC [n=123] vs CRA [n=117]	Single	0.55-0.84		
Shi Y et al. 2021 [143]	Serum	miR-126, miR-1290, miR-23a, and miR940	Diagnosis and early diagnosis	CRC [n=100] vs healthy [n=35]	Single	0.88-0.94	RT-qPCR	RiboTM Exosome Isolation Reagent
				stage I CRC [n=30] vs healthy [n=35]	Single	0.83-0.90		
Yu M et al. 2021 [144]	Serum	FOXD2-AS1, NRIR, XLOC_009459	Diagnosis and early diagnosis	CRC [n=203] vs healthy [n=201]	Single	0.660-0.728	RT-qPCR	Ultracentrifugation
					Combined	0.736		
				Early-stage CRC [n=80] vs healthy [n=201]	Single	0.660-0.743		
					Combined	0.758		
Wang L et al. 2022 [145]	Serum	miR-377-3p, miR-381-3p	Diagnosis and early diagnosis	CRC [n=175] vs healthy [n=172]	Single	0.792-0.798	RT-qPCR	Ultracentrifugation
					Combined	0.807 (0.7612-0.8520)		
				Early-stage CRC [n=69] vs healthy [n=172]	Single	0.798-0.803		
					Combined	0.815 (0.7596-0.8704)		
Guo TA et al. 2022 [146]	Plasma	A panel (HIST2H2AA4, H2BFS, UQCRHL, XCL2, AC008269.1, DMC1, RAB6D, KLHDC8B, CA3, APOL4, HIST1H2AI, ANKAR, SGMS1, CYP20A1, HIST1H2BB, STK3, CBWD1)	Diagnosis and early diagnosis	CRC [n=48] vs healthy [n=82] (Train) CRC [n=24] vs healthy [n=40] (Test) CRC [n=14] vs healthy [n=32] (Validation)	Combined	0.938 (Train); 0.943 (Test); 0.947 (Validation)	RNA-seq	ExoRNeasy Serum/Plasma Kit
				CRA [n=42] vs healthy [n=80]	Combined	0.993 (Train); 0.978 (Test)		
Shi YJ et al. 2023 [147]	Plasma	miR-185-5p	Diagnosis and early diagnosis	III-IV stage CRC [n=35] vs healthy [n=22]	Single	0.803 (0.678-0.896)	RT-qPCR	EZBioscience kit
				I-II stage CRC [n=70] vs healthy [n=50]		0.887 (0.767-0.959)		
				CRA [n=65] vs healthy [n=44]		0.702 (0.607-0.786)		
Zhang C et al. 2024 [148]	Plasma	miR-99b-5p, miR-409-3p	Diagnosis and early diagnosis	CRC [n=68] vs healthy [n=27]	Single	0.604-0.660	RT-qPCR	ExoRNeasy Midi Kit
					Combined +CEA	0.863 (0.774-0.952)		
				Early CRC [n=28] vs healthy [n=27]	Single	0.611-0.735		
					Combined +CEA	0.812 (0.679-0.945)		
Min L et al. 2024 [149]	Plasma	miR-425–5 p, Let-7f-5p, C19orf43, TOP1, PPDPF, LNC-EV-9572, lnc-MKRN2-42:1, HIST2H2AA4, (MT-ND2)	Diagnosis and early diagnosis	CRC [n=47] vs control [n=53]	8-RNA signature	0.87	RT-qPCR	Ultracentrifugation
				CRA [n=24] vs control [n=53]	9-RNA signature	0.88		
Koo B et al. 2025 [12]	Plasma	miR-23a-3p, miR-92a-3p, miR-125a-3p and miR-150-5p	Diagnosis and early diagnosis	CRC [n=80] vs healthy [n=20]	Single	0.66-0.87	RT-qPCR	ZAHVIS platform
					Combined	0.986 (0.936-1.0)		
				Early-stage CRC [n=40] vs healthy [n=20]	Single	0.61-0.89		
					Combined	0.99		
					Combined	0.900 (Train); 0.900 (Test)		
Vychytilova-Faltejskova P et al. 2025 [150]	Serum	SOS1-IT1, RP11-110G2, CSRP1-AS1, DKPK1-AS, SMARCA4-AS, FAR1-IT1, ENSG00000261765, SLC7A9-AS, PAX5-AS, PHB-AS, RP11-190A12, FLOT2-AS, UNC113A-AS, EGR1, RGS2, CXCR4, ITM2B, PTPRCAP, LASP1-AS	Diagnosis and early diagnosis	CRC [n=66] vs healthy [n=66] (Train) CRC [n=105] vs healthy [n=66] (Test)	Combined	0.854 (Train); 0.852 (Test)	RT-qPCR	qEVsingle/35 nm or qEVoriginal/35 nm columns
				PL [n=20] vs healthy [n=66] (Train) PL [n=20] vs healthy [n=66] (Test)		0.977 (Train); 0.983 (Test)		
Zhang Z et al. 2025 [151]	Serum	tsRNA-Gly-5-0007	Diagnosis and early diagnosis	CRC [n=172] vs healthy [n=164]	Single	0.781 (0.731-0.832)	qRT-PCR	Ultracentrifugation
					Combined +CEA+CA 19-9	0.930 (0.877-0.984)		
				CRC [n=79] vs healthy [n=164]	Single	0.773 (0.714-0.832)		
					Combined +CEA+CA 199	0.901 (0.822-0.980)		
Rodríguez-Cobos J et al. 2021 [152]	Plasma	ΔNp73, Δ133p53	Diagnosis, early diagnosis and	CRC [n=42] vs healthy [n=29]	Single	0.650-0.679	RT-qPCR	Ultracentrifugation
				PL [n=49] vs healthy [n=29]	Single	0.723 (ΔNp73)		

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
			prognosis	CRC [n=42]	Single	HR = 0.21 (0.06-0.68) (Δ133p53)		
Zheng R et al. 2022 [153]	Plasma	circLPAR1	Diagnosis and prognosis	CRC [n=112] vs healthy [n=60]	Single	0.858 (0.786-0.930)	RT-qPCR	ExoQuick Plasma Prep with Thrombin kit
					Combined +CEA+CA 19-9	0.875 (0.809-0.941)		
				CRC [n=112]	Single	HR = 0.46 (0.24-0.87)		
Hao YJ et al. 2022 [154]	Plasma	miR-21	Prognosis	mCRC [n=11]	Single	HR = 0.909 (1.429-68.72)	RT-qPCR	miRCURY Exosome Serum/Plasma kit
Yang Y et al. 2023 [155]	Plasma	GOLPH3L, RPL21P1, ANKRD20A17P, CCL2, EPS8L1, CUBN, PODXL, ALG9, TLR5, CLK4, PRICKLE2, HMGB1P24, ME3, RPL13P12, MIB2, SLC5A6, NUDT19, ELMOD3, PGAM5, ERICH6-AS1, BACE1, MYL12BP1	Prediction of treatment response	mCRC [n = 190]: training cohort [n = 80], testing cohort [n = 62], and external validation cohort [n = 48]	Combined	0.986 (0.968-1.000) (Train); 0.821 (0.711-0.931) (Test); 0.820 (0.702-0.937) (External validation)	NR	NR

CRC, colorectal cancer; mCRC, metastatic colorectal cancer; EOCRC, early-onset colorectal cancer; CRA, colorectal adenoma; PL, premalignant lesion; LNM, lymph node metastasis; AUC, area under the curve; HR, hazard ratio; CI, confidence interval; NR, not reported; EV, extracellular vesicle; RNA, ribonucleic acid; miRNA, microRNA; lncRNA, long non-coding RNA; circRNA, circular RNA; tsRNA, transfer RNA-derived small RNA; mRNA, messenger RNA; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; PCR, polymerase chain reaction; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724; ROC, receiver operating characteristic; EV nomenclature follows MISEV2023. Unless endosomal biogenesis was experimentally established, vesicles are referred to as EVs or by appropriate operational terms. The terms "exosome" and "exosomal" are retained only in formal commercial product names and published article titles or when directly reproducing the terminology of the original study.

3.1.1.1 Non-Coding RNAs

miRNAs represent the most extensively investigated EV-derived RNA biomarker in CRC [8, 156]. Numerous studies have demonstrated that specific EV-miRNAs are differentially expressed in CRC patients compared with healthy individuals, often exhibiting superior diagnostic performance to conventional serum markers such as carcinoembryonic antigen (CEA) and CA19-9 [147, 157]. Importantly, several EV-miRNAs retain discriminatory power in colorectal adenomas, early-stage CRC and early-onset CRC, underscoring their potential for early detection. While single-miRNA biomarkers show limited robustness, multi-miRNA panels substantially improve diagnostic accuracy [140]. Beyond diagnosis, EV-miRNAs are closely associated with treatment response, disease progression, and patient survival. Meta-analyses further support their prognostic relevance, with dysregulated circulating EV-miRNAs associating with poorer overall survival (HR = 2.928) and disease-free survival (HR = 2.105) in CRC patients [8].

Beyond miRNAs, other EV-associated non-coding RNAs, including lncRNAs, circRNAs, and tRNA-derived small RNAs (tsRNAs), provide complementary biomarker layers that enhance diagnostic and monitoring capacity. EV-lncRNAs (AUC 0.65-0.96) and circRNAs (AUC 0.61-0.85) have demonstrated moderate-to-high diagnostic performance [132, 158, 159], with increasing evidence supporting multi-marker panels as more robust classifiers than individual transcripts [144]. Notably, EV-derived circLPAR1 exhibit dynamic expression patterns during CRC progression and after surgical resection, supporting their utility in longitudinal disease monitoring.

Emerging evidence also highlights tsRNAs as stable and informative EV cargoes. Specifically, EV-associated tsRNA-Gly-5-0007 demonstrated robust diagnostic efficacy for early-stage CRC (AUC = 0.773), outperforming traditional tumor markers [151]. Moreover, combining tsRNA signatures with established serum markers markedly improves diagnostic accuracy (AUC=0.930-0.938) [135, 151]. Collectively, integrated EV-derived ncRNA signatures provide a multidimensional framework for CRC detection and prognostic stratification.

3.1.1.2 mRNA

Compared with non-coding RNAs, EV-derived mRNAs are generally less abundant and exhibit variable enrichment across transcripts. Nevertheless, EV transcriptomes partially reflect cellular gene expression, supporting their potential as cancer-specific biomarkers [160-162]. In CRC, Cha et al. employed a two-phase approach to identify EV mRNAs associated with diagnostic relevance. Eight candidate mRNAs (MYC, VEGF, CDX2, CD133, CEA, CK19, EpCAM, and CD24) enriched in cancer-derived EVs demonstrated moderate diagnostic performance (AUC 0.54-0.86) in plasma samples, while combinatorial analysis of VEGF and CD133 achieved an AUC of 0.96 with 100% sensitivity and 80% specificity [163]. The clinical utility of EV-mRNAs can be further improved through integrative analysis and statistical modeling. For instance, a six-EV-mRNA (H3F3A, MYL6, FBXO7, TUBA1C, MEF2C, and BANK1) signature derived from combined exoRBase and GEO datasets, constructed using a logistic

regression model, demonstrated robust diagnostic performance across training and validation cohorts [164]. In addition, EV-associated transcriptional isoforms expand the biomarker landscape beyond conventional gene-level analysis. Isoforms such as ΔNp73 and Δ133p53 are detectable at premalignant lesion stage (AUC = 0.723 and 0.641, respectively), and EV-derived Δ133p53 show inverse associations with disease-free survival (HR = 0.21) and overall survival (HR = 0.23) [152], highlighting their potential for early detection and prognostic stratification. Collectively, EV-derived mRNAs, particularly when integrated into multi-marker and isoform-resolved frameworks, represent a complementary biomarker layer in CRC.

3.1.2 DNA Biomarkers

EV-derived DNA carries tumor-specific mutations, offering potential as a biomarker for CRC, although research in this area remains limited relative to RNA [165]. In CRC, Fricke et al. identified frameshift mutation patterns in microsatellite stretches of TGFBR2 and other microsatellite instability target genes within CRC cell-derived EVs at the DNA level [166]. Similarly, KRAS and BRAF mutations can be sensitively detected in serum EVs, and EV-KRAS DNA levels increased with tumor progression and decreased after treatment [7, 167]. Moreover, mutant KRAS DNA copy number correlated with tumor size and liver metastasis. Notably, 91% of KRAS-mutated mCRC patients converted to wild type after first-line chemotherapy, with reversion to mutant upon progression in 80% of cases, supporting EV DNA as a tool for monitoring disease dynamics and drug resistance [7]. Current evidence remains limited and systematic validation in large, well-controlled cohorts are still required to establish clinical utility.

3.1.3 Protein Biomarkers

EV-associated proteins have emerged as a promising class of CRC biomarkers, reflecting the phenotypic state of tumor cells more directly than nucleic acids. Recent developments in this area over the past five years are summarized in Table 2 [6, 168-187]. Numerous studies have demonstrated that specific EV proteins are differentially expressed in CRC compared with healthy individuals, often showing diagnostic performance superior to conventional serum markers such as CEA and CA19-9 [169, 175]. Importantly, several EV proteins retain discriminatory power in early-stage CRC, CEA-negative CRC, and colorectal adenomas, highlighting their value for early detection [176, 178, 182].

Table 2. Extracellular vesicle-derived proteins as diagnostic, prognostic, and predictive biomarkers in colorectal cancer

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
Nazarova I et al. 2021 [168]	Plasma	CD63 and CD9	Diagnosis	CRC [n=48] vs healthy [n=50]	Single	0.79-0.81	Flow Cytometry	Plasma Two-Phase Polymer System
Ganig N et al. 2021 [169]	Plasma	QSOX1	Diagnosis	CRC [n=48] vs control [n=18]	Single	0.904 (0.831-0.977)	ELISA	Total Exosome Isolation kit
Park J et al. 2021 [170]	Plasma	EGFR, EpCAM, CD24, GPA33, CD133	Diagnosis	CRC [n=102] vs non-CRC [n=40]	Single	0.65-0.91	HiMEX	Immunomagnetic capture
					Combined	0.99		
Sun Z et al. 202 [171]	Plasma	FGB and β2-GP1	Diagnosis	CRC [n=30] vs healthy [n=20]	Single	0.834-0.871	ELISA	Exo-Quick™ solution
					Combined	0.915 (0.845-0.987)		
Rong W et al. 2023 [172]	Plasma	MARCKSL1	Diagnosis	mCRC [n=38] vs non-mCRC [n=40]	Single	0.6125	ELISA	Hieff® Quick exosome isolation kit
					Combined+CA125+lymphocyte	0.748		
Zhou GY et al. 2023 [173]	Tissue	NHP2, OLFM4, TOP1, SAMP, TAGL, TRIM28	Diagnosis	CRC tissue [n=10] vs adjacent normal [n=10]	Single	0.75-0.97	LC-MS/MS	Size-exclusion chromatography
Feng Y et al. 2025 [174]	Serum	PD-L1, c-Met, PTK7, CD71	Diagnosis	CRC [n=44] vs healthy [n=18]	Single	0.802-0.922	ICP-MS	Ultracentrifugation
					Combined	0.975 (0.946-1.000)		
Dash S et al. 2022 [175]	Plasma	CD59, TSPAN9, ADAM10	Diagnosis and early diagnosis	CRC [n=73] vs healthy [n=80]	Single	0.83-0.95	Targeted MS	Ultracentrifugation
					Combined	0.98		
				Stage I/II CRC [n=32] vs healthy [n=80]	Single	0.86-0.96		
					Combined	0.99		
Li L et al. 2023 [176]	Plasma	SERPINA1, PLG	Diagnosis and early diagnosis	CRC [n=155] vs healthy [n=153]	Single	0.799-0.934	ELISA	Differential centrifugation
					Combined	0.938 (0.903-0.973)		
					Combined+CEA+CA19-9	0.967 (0.943-0.991)		
				Early-stage CRC [n=80] vs healthy [n=153]	Single	0.762-0.919		
					Combined	0.919 (0.866-0.973)		

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
					Combined+CEA+CA19-9	0.950 (0.910-0.990)		
Guo W et al. 2023 [177]	Plasma	ITGAM, ITGB3, CD151, ADAM10	Diagnosis and early diagnosis	CRC [n=30] vs healthy [n=58]	Single	0.800-0.856	Proximity barcoding assay	Differential centrifugation
				PreCan [n=14] vs healthy [n=28]		0.783-0.835		
				Stage I CRC [n=11] vs healthy [n=28]		0.784-0.850		
Li P et al. 2023 [178]	Serum	SORL1	Diagnosis and early diagnosis	CRC [n=79] vs healthy [n=75]	Single	0.98	Quantum dot labeling	Microfluidic chip
				CEA-negative CRC [n=56] vs healthy [n=100]		0.99		
				Early CRC [n=81] vs healthy [n=100]		0.99		
Huang Z et al. 2024 [179]	Serum	FIBG, PDGF-β, TGF-β	Diagnosis and early diagnosis	CRC [n=135] vs healthy [n=79](Train) CRC [n=84] vs healthy [n=56](Test)	Single	0.743-0.796	4D-DIA quantitative proteomic	Ultracentrifugation
					Combined+CEA	0.882 (Train); 0.937 (Test)		
				AA [n=25] vs healthy [n=79](Train) AA [n=25] vs healthy [n=56](Test)	Combined+CEA	0.822 (Train); 0.767 (Test)		
Zhang J et al. 2024 [180]	Plasma	APOA4, SERPINA3, GPX3, SNCA, YKT6, RAN, ENPP2, ANTXR1, THBS4, SPP2	Diagnosis and early diagnosis	CRC [n=10] vs healthy [n=10]	Combined	0.97	DIA-MS	DSPE-functionalized beads
				CRC [n=10] vs polyp [n=8]		0.94		
				Polyp [n=8] vs healthy [n=10]		1		
Yin H et al. 2024 [181]	Serum	PF4, AACT	Diagnosis and early diagnosis	CRC [n=25] vs healthy [n=12]	Single	0.892-0.984	4D-DIA proteomics + ELISA	Size-exclusion chromatography
					Combined	0.999		
				CRC [n=195] vs healthy [n=96] (Train) CRC [n=161] vs healthy [n=112] (Test) CRC [n=98] vs healthy [n=60](External validation)	Combined+CEA+CA19-9	0.960 (Train); 0.963 (Test); 0.895 (External validation)		
				Early-stage CRC [n=50] vs healthy [n=112]	Combined+CEA+CA19-9	0.905		
Wei Y et al. 2025 [182]	Plasma	EpCAM, CD24, EGFR	Diagnosis and early diagnosis	CRC [n=35] vs healthy [n=10]	Single	0.88-0.97	Bubble Multimer-Enhanced Fluorescence	Ultracentrifugation
					Combined	0.94		
				Early-stage CRC [n=8] vs healthy [n=10]	Single Combined	0.89-0.96 0.93		
Wang J et al. 2025 [183]	Plasma	CTTN, HNRNPK, PSMC6	Diagnosis and early diagnosis	CRC [n=20] vs healthy [n=20]	Single	0.550-0.750	Staged MS + ELISA	ExoEasy Maxi Kit
				CRC [n=123] vs healthy [n=54](Train) CRC [n=53] vs healthy [n=23](Test) CRC [n=75] vs healthy [n=32](Internal validation) CRC [n=142] vs healthy [n=66](External validation)	Combined	0.992 (Train); 0.990 (Test); 0.986 (Internal validation) 0.974 (External validation)		
				Early-stage (TNM stage I) CRC [n=7] vs CRA [n=27] (Internal validation) Early-stage (TNM stage I) CRC [n=29] vs CRA [n=46] (External validation)	Combined	1.000 (Internal validation) 0.969 (External validation)		
Gu C et al. 2023 [184]	Serum	CD147	Diagnosis and prognosis	CRC [n=120] vs healthy [n=48]	Single	0.827 (0.764-0.891)	Western blot	Ultracentrifugation
					Combined+CEA+CA19-9	0.852 (0.792-0.912)		
				Metastasis [n=60] vs non-metastasis [n=60]	Single	0.644 (0.545-0.743)		
				CRC [n=120]	Single	HR = 4.306 (1.417-13.083)		
Lin K et al. 2022 [6]	Serum	CD14, SerpinA4, CFP, LBP	Prognosis	CRLM [n=320]	Single	CD14: HR = 1.226 (1.082-1.389) LBP: HR = 394.615 (4.059-38360) CFP: HR = 0.992 (0.985-0.998) SerpinA4: HR = 0.924 (0.878-0.973)	ELISA	NR
					Combined	HR = 3.78 (2.339-6.110)		

Study	Sample Type	Biomarker	Clinical Application	Comparison (Sample Size)	Strategy	AUC / HR (95% CI)	Detection Method	EV Isolation Method
Raciti G et al. 2025 [185]	Plasma	IL-6, CD81, Cyclin D1	Prognosis	Recurrence [n=27] vs non-recurrence [n=6]	Single	0.55-0.63	dSTORM imaging	Ultracentrifugation
					Combined	0.83		
Brocco D et al. 2022 [186]	Plasma	CD133	Prognosis and prediction of treatment response	mCRC [n=33]	Single	HR = 1.72 (1.24-2.39)	Flow cytometry	Flow cytometry
				Responders [n=14] vs non-responders [n=16]	Single	0.728 (0.542-0.913)		
Strybel U et al. 2022 [187]	Serum	C8G, SERPINF, CFHR3, THBS4, HGFAC, PGLYRP2, SH3BGRL3, SERPINA3	Prediction of treatment response	Good responders [n=17] vs poor responders [n=23]	Single	0.88-0.91	LC-MS/MS	Size-exclusion chromatography

CRC, colorectal cancer; mCRC, metastatic colorectal cancer; CRLM, colorectal cancer liver metastasis; AA, advanced adenoma; CRA, colorectal adenoma; PL, premalignant lesion; HR, hazard ratio; AUC, area under the curve; CI, confidence interval; NR, not reported; EV, extracellular vesicle; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; DIA-MS, data-independent acquisition mass spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; MS, mass spectrometry; HiMEX, high-throughput integrated magneto-electrochemical device; dSTORM, direct stochastic optical reconstruction microscopy. EV nomenclature follows MISEV2023. Unless endosomal biogenesis was experimentally established, vesicles are referred to as EVs or by appropriate operational terms. The terms "exosome" and "exosomal" are retained only in formal commercial product names and published article titles or when directly reproducing the terminology of the original study.

Given the heterogeneous nature of EV protein expression, multi-protein panels more accurately capture tumor-specific profiles and enhance diagnostic precision. For example, EV-protein panels incorporating tumor-associated surface markers have achieved excellent diagnostic accuracy, with AUC values approaching or exceeding 0.9 across independent cohorts [170, 179, 183]. Beyond diagnosis, EV-associated proteins are closely linked to treatment response, recurrence risk, and survival outcomes. For instance, multi-protein signatures (CD14, SerpinA4, CFP, LBP) have demonstrated the ability to stratify recurrence and mortality in metastatic CRC across discovery, internal, and external validation cohorts [6], while specific EV protein subpopulations ($CD133^+$ EVs) correlate with both overall survival and treatment response (AUC = 0.728) [186]. Moreover, combinatorial EV protein clusters (IL-6, CD81, Cyclin D1) further enhance discrimination of CRC recurrence [185], supporting their application in longitudinal monitoring. Collectively, these findings support EV proteins as a complementary biomarker modality for CRC, with applications spanning early detection, prognostic stratification, and disease monitoring.

3.1.4 Lipid Biomarkers

EV-associated metabolites encompass a broad spectrum of molecular classes, among which lipids represent the most extensively characterized components. Beyond their structural roles in EV membranes, lipids contribute to EV biogenesis, cargo sorting, and vesicle release, underscoring their functional relevance. Although comprehensive metabolomic profiling of EVs remains limited, accumulating evidence indicates that EV lipid composition is dynamically altered in CRC and reflects tumor-associated metabolic reprogramming.

Multiple studies have identified disease-specific EV lipid signatures in CRC across both experimental models and patient samples. CRC-derived EVs are enriched in lipid classes such as glycerophospholipids and sphingolipids, with alterations consistently observed in both cell line-derived and clinical EVs [14, 188, 189]. Importantly, specific lipid species have been linked to disease progression; for instance, distinct ceramide profiles are elevated in metastatic CRC, suggesting their potential as indicators of tumor aggressiveness [188]. In parallel, systematic shifts in fatty acid composition and phospholipid species enable discrimination among healthy individuals, patients with adenomas, and CRC cases, supporting their utility in early detection and disease stratification [190]. Similar lipid alterations have also been observed in fecal EVs, reinforcing the concept of disease-specific lipid fingerprints [191]. Collectively, EV lipid profiling holds potential as a noninvasive biomarker for CRC detection and disease monitoring. However, routine clinical application remains distant, and large-scale validation studies with standardized detection methods are required.

3.1.5 Bacterial EV Biomarkers

Bacterial extracellular vesicles (BEVs) have emerged as promising non-invasive biomarkers for CRC. As nanoscale vesicles enriched in proteins, nucleic acids, lipids, and metabolites, BEVs reflect microbial composition and activity, thereby capturing disease-associated dysbiosis beyond conventional microbiome profiling. Increasing evidence indicates that the composition of fecal BEVs differs significantly between CRC patients and healthy individuals, with reduced microbial diversity and distinct shifts in BEV-producing taxa [192]. Notably, Ruminococcus 2-derived BEVs were significantly decreased in distal CRC compared with proximal CRC. In addition, Alistipes-derived EVs have been proposed as biomarkers for

CRC diagnosis and stage prediction [192]. Integration of BEV-derived microbial genera with metabolite data further improves diagnostic accuracy, highlighting the advantage of multi-omic approaches. For example, combining leucine and oxalic acid with BEV-producing genera (Collinsella and Solanum melongena) in fecal samples achieved an AUC of 1.0 for CRC detection [191]. However, discrepancies across sample types, such as the limited discriminatory power observed in urinary BEVs, underscore the need for robust methodological standardization [193].

BEV protein markers have also shown high diagnostic and prognostic potential. BEV-derived protein markers (CD147 and A33) have demonstrated high accuracy in distinguishing CRC from healthy donors, with combinatorial panels outperforming conventional biomarkers such as CEA. Moreover, longitudinal analysis of fecal samples demonstrated that CD147/A33-enriched BEVs can track CRC prognosis over time [194]. In addition, BEV-derived microRNAs have shown remarkable sensitivity for CRC detection, with multi-miRNA panels (miR-16-2-3p, miR-375-3p, miR-378a-3p, and miR-7-5p) substantially outperforming traditional serum markers [195]. Collectively, these findings highlight the considerable potential of BEVs as biomarkers for CRC diagnosis, early detection, and prognosis monitoring. However, further studies are required to evaluate their clinical utility, cost-effectiveness, and performance relative to existing diagnostic models.

3.1.6 Positioning EV Biomarkers Relative to Established CRC Screening Tests

Although several EV-based biomarker studies have reported AUC values approaching or exceeding 0.90, these findings should not be interpreted as evidence of superiority over clinically validated CRC screening assays. Established noninvasive approaches have generally undergone larger prospective validation, whereas many EV studies remain retrospective or case-control in design and rely on study-specific analytical thresholds. Representative benchmarks include plasma methylated-SEPT9 testing, next-generation multitarget stool DNA testing, and the cfDNA-based Shield assay, while the recent ENCODER study provides an example of an EV/cell-free miRNA-based approach for early-onset CRC detection (Table 3) [136, 196-198]. Importantly, the reported performance estimates are not directly comparable across studies because the study populations, endpoint definitions, and validation designs differ substantially. EV-based assays may offer additional molecular information by integrating protected nucleic acids, proteins, lipids, and defined EV subpopulations, but they currently lack standardized pre-analytical workflows, universally accepted normalization strategies, locked diagnostic thresholds, and prospective validation in population-based screening cohorts. Accordingly, EV biomarkers should currently be regarded as investigational complements rather than replacements for established CRC screening tests.

Table 3. Clinical positioning of extracellular vesicle-based biomarkers relative to representative established noninvasive colorectal cancer screening assays

Study	Sample Type	Biomarker / Principle	Validation Context	CRC Sensitivity	Specificity	Major Advantages	Major Limitations / Clinical Positioning
ENCODER EV/cell-free miRNA assay [136]	Plasma	Six circulating cell-free and EV-associated miRNA biomarkers plus machine learning	International multicenter, multiphase EOCRC study with independent external testing	0.916 (0.842–0.957); stages I–III: 0.973 (0.906–0.993)	0.875 (0.794–0.927)	High EOCRC discrimination; external testing; combines cell-free and EV-associated miRNA signals	Not an average-risk population screening trial; assay/model-specific; requires prospective screening validation and standardized preanalytics
mSEPT9 assays (Epi proColon as a clinical example) [196]	Plasma	Methylated SEPT9 cfDNA; PCR-based detection	Established blood-based screening modality; pooled evidence across mSEPT9 studies	0.81 (0.77–0.84)	0.88 (0.79–0.93)	Blood-based sampling; regulatory precedent for blood-based CRC screening; simple PCR workflow	Lower sensitivity for precursor lesions than stool DNA approaches; performance varies by assay and algorithm
Next-generation mt-sDNA (BLUE-C) [197]	Stool	Multitarget stool DNA plus hemoglobin	Prospective average-risk screening study (n = 20,176)	0.939 (0.871–0.977)	0.906 (0.901–0.910)	High CRC sensitivity; improved precursor detection; large prospective validation	Stool collection; lower specificity than FIT; positive results require colonoscopy
Shield cfDNA blood test (ECLIPSE) [198]	Plasma	cfDNA genomic, epigenomic, and fragmentomic features	Large prospective average-risk screening cohort	0.831 (0.722–0.903)	0.896 (0.888–0.903)	Blood-based; prospective multicenter validation; clinically deployed assay	Limited sensitivity for advanced precancerous lesions (~13%); positive results require colonoscopy

CRC, colorectal cancer; cfDNA, cell-free DNA; mSEPT9, methylated SEPT9; mt-sDNA, multitarget stool DNA; FIT, fecal immunochemical test; EV, extracellular vesicle; EOCRC, early-onset colorectal cancer; CI, confidence interval.

3.2 Emerging Analytical Strategies for EV-based Biomarkers in CRC

Despite the molecular promise of EV-associated cargo, the practical utility of these biomarkers is frequently compromised by the limitations of traditional analytical strategies. These limitations primarily arise from three key challenges, including vesicle heterogeneity, low abundance of tumor-derived signals, and the high dimensionality of EV-associated data.

Specifically, single-vesicle analysis resolves inter-vesicular heterogeneity by characterizing individual vesicles. Multi-omics integration captures complementary biological insights across molecular layers, improving biomarker robustness. AI-powered analytics interprets high-dimensional data and enables predictive modeling. Collectively, these advancements have the potential to redefine analytical paradigms in CRC precision oncology, shifting from bulk measurements toward integrated and high-resolution profiling strategies.

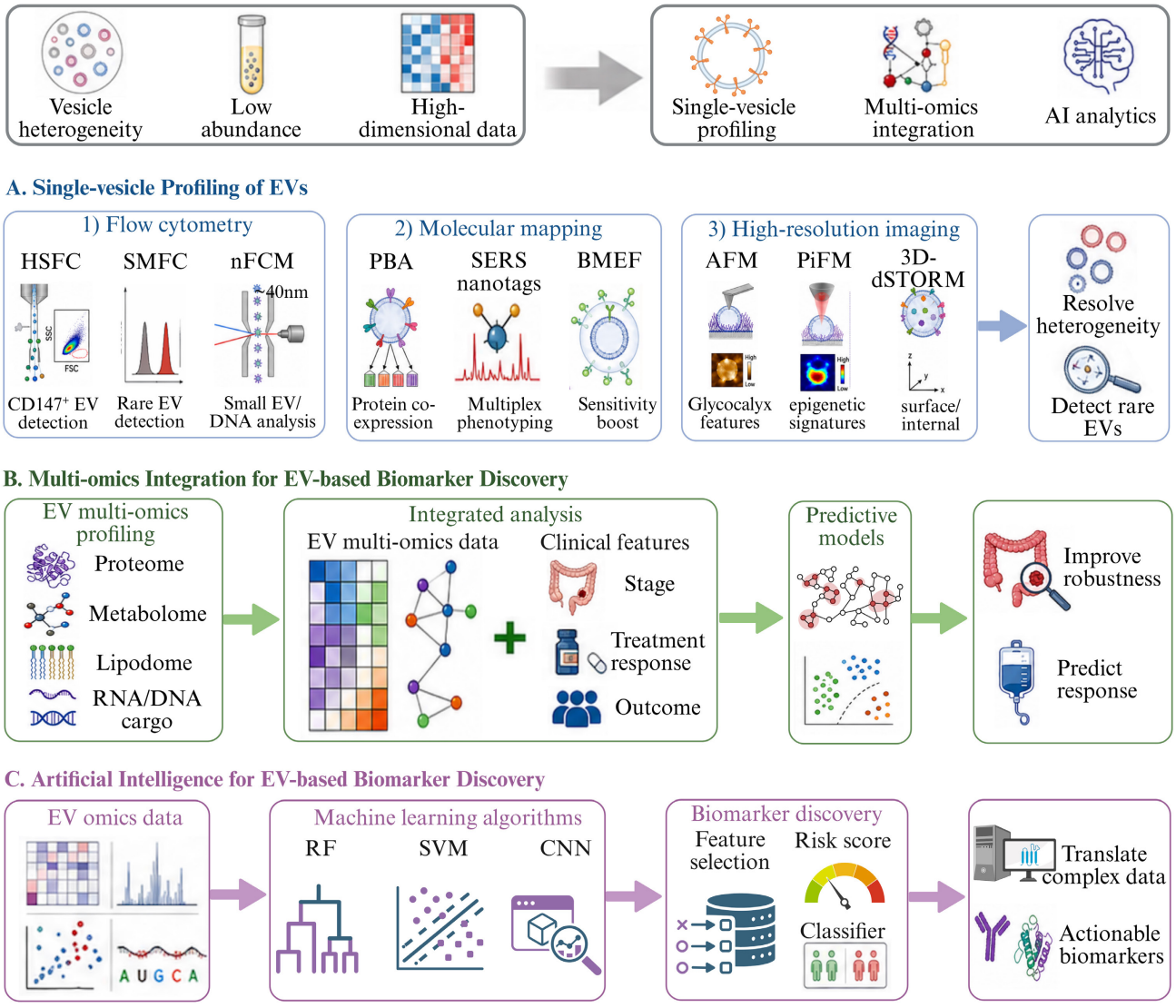


Figure 3. Emerging analytical strategies for EV-based biomarker discovery in colorectal cancer. Extracellular vesicle (EV)-based biomarker development in colorectal cancer (CRC) is challenged by vesicle heterogeneity, the low abundance of tumor-derived signals, and the high dimensionality of EV-associated data. **(A)** Single-vesicle profiling resolves inter-vesicular heterogeneity by characterizing individual EVs through flow cytometry-based platforms, molecular mapping approaches, and high-resolution imaging techniques, enabling the detection of rare and clinically informative EV subpopulations. **(B)** Multi-omics integration combines EV proteomic, metabolomic, lipidomic, and nucleic acid profiles with clinical features, thereby improving biomarker robustness and supporting predictive models for diagnosis, treatment response, and outcome stratification. **(C)** Artificial intelligence and machine learning algorithms further enhance EV-based biomarker discovery by extracting informative features from complex omics datasets, constructing classifiers or risk scores, and translating high-dimensional EV signals into clinically actionable biomarkers. These strategies shift EV biomarker research from bulk-level measurements toward high-resolution, integrated, and data-driven analytical frameworks for CRC precision oncology. Schematic illustrations were created with BioRender.com.

3.2.1 Single-vesicle Profiling of EVs

Single-vesicle analysis enables characterization of individual EVs rather than population-averaged signals. This is particularly important in CRC, where tumor-derived EVs may represent only a minute fraction of total circulating vesicles, especially in early-stage disease. By resolving EV heterogeneity at the single-particle level, these approaches provide the sensitivity needed to identify rare but biologically and clinically informative subpopulations that are obscured by bulk analysis [13].

Flow cytometry-based platforms represent a cornerstone of this field, with high-sensitivity flow cytometry (HSFC), single-molecule flow cytometry (SMFC), and nano-flow cytometry (nFCM) facilitating the precise quantification of surface protein copy numbers and particle distribution. In CRC, HSFC identified a significant enrichment of $CD147^+$ EV in plasma, achieving superior diagnostic performance compared with total EV concentration metrics (AUC: 0.93 vs 0.63) [199]. Comparative studies further suggest that SMFC offers improved sensitivity for detecting small and low-abundance EV subpopulations [200], while nFCM extends analysis to EVs as small as 40 nm and has revealed heterogeneous distributions of EV-associated DNA, including distinct dsDNA-positive subpopulations responsive to genotoxic treatment [201]. Beyond quantitative profiling, emerging molecular mapping and spectroscopic tools provide deeper phenotypic resolution. Proximity barcoding assay (PBA) enables high-resolution mapping of surface protein co-expression and has identified CRC-associated EV subsets with distinct biological relevance and diagnostic potential [177]. Similarly, surface-enhanced raman spectroscopy (SERS) nanotags allow multiplexed phenotyping of CRC-related EV markers [202], whereas bubble multimer-enhanced fluorescence (BMEF) substantially improves analytical sensitivity for stage-discriminating markers [182]. High-resolution imaging techniques further add spatial and structural dimensions to single-vesicle analysis. Atomic force microscopy (AFM) has revealed altered surface glycocalyx features in CRC-derived EVs [203], while photo-induced force microscopy (PiFM) identified CRC-specific epigenetic signatures, including methylation-related features linked to molecular subtypes [204]. Additionally, three-dimensional direct stochastic optical reconstruction microscopy (3D-dSTORM) enabled simultaneous analysis of surface and internal protein distributions, with demonstrated value in distinguishing recurrent from non-recurrent CRC and correlating EV phenotypes with clinicopathological features [185]. Collectively, these advances move single-vesicle analysis beyond bulk quantification toward integrated molecular and structural profiling, thereby providing a powerful framework for the discovery of sensitive and specific EV-based biomarkers in CRC.

3.2.2 Multi-omics Integration for EV-based Biomarker Discovery

Integrative multi-omics profiling of EVs has emerged as a high-dimensional strategy to enhance biomarker discovery in CRC by capturing the coordinated alterations of multiple molecular layers. Compared with single-omics approaches, multi-omics integration enables comprehensive characterization of EV-associated molecular alterations, thereby enhancing the specificity and clinical relevance of identified signatures.

In CRC, integrated analyses of EV proteome, metabolome, and lipidome have effectively discriminated malignant from non-malignant states, with pathway-level insights highlighting metabolic reprogramming, particularly dysregulated fatty acid and amino acid metabolism, as a hallmark of CRC-associated EVs [14]. Similarly, integrated proteomic and lipidomic profiling of serum-derived EVs from rectal cancer patients has demonstrated that EV protein signatures outperform metabolite-based panels in predicting response to neoadjuvant radiotherapy [187]. Notably, this combined analysis further reveals shared pathways related to immune response, complement activation, and cancer-associated signaling that underpin therapeutic sensitivity [187, 205]. Beyond molecular profiling, integration of EV multi-omics datasets with clinical features facilitates the construction of robust disease classification and prediction models. Although still emerging in CRC, evidence from other malignancies, such as pancreatic cancer, demonstrates that machine-learning-driven models incorporating thousands of multi-omics and clinical features significantly outperform single-omics approaches in predicting patient outcomes [205]. Such frameworks underscore the translational potential of multi-dimensional data integration for CRC liquid biopsy.

3.2.3 Artificial Intelligence for EV-based Biomarker Discovery

The complexity and high dimensionality of EV-derived omics data necessitate analytical frameworks beyond conventional statistical approaches. Artificial intelligence (AI), particularly machine learning (ML), provides a robust framework to decipher the non-linear correlations within heterogeneous datasets, thereby unlocking the full diagnostic and prognostic potential of EV-based biomarkers.

Currently, various ML algorithms, including random forest (RF), support vector machine (SVM), and convolutional neural networks (CNN), have been applied for CRC detection. For example, combining 4D-DIA proteomics with an RF model enabled the identification of PF4 and AACT as serum EV biomarkers, which demonstrated strong early-stage CRC diagnostic performance and effective discrimination from benign colorectal conditions [181]. Similarly, an SVM-based model incorporating a five-metabolite EV signature achieved the superior discriminatory power for CRC screening (AUC = 0.985), significantly outperforming alternative statistical approaches [206]. Beyond biomarker selection, AI also strengthens the translational potential of EV-based assays by enabling integrated and scalable diagnostic platforms. For instance, the ZAHV-AI system integrates efficient EV isolation with AI-based analysis to support rapid and cost-effective nucleic acids profiling. Using plasma EV-derived miRNAs (miR-23a-3p, miR-92a-3p, miR-125a-3p, and miR-150-5p) integrated with carcinoembryonic antigen (CEA) yielded an AUC of 0.9861 for overall CRC detection and near-perfect discrimination of stage 0-I disease, surpassing individual markers [12]. Beyond primary CRC detection, ML applications demonstrate remarkable utility in classifying cancers of unknown primary origin [207]. A plasma EV-derived 29-protein signature, optimized via RF algorithms, successfully distinguished melanoma, pancreatic, lung, and colorectal cancers, with its feasibility further confirmed in patient-derived xenograft models [208]. Collectively, these advances position AI as a critical enabler for translating complex EV-derived signals into robust and clinically actionable biomarkers in CRC.

4. Therapeutic Applications of EVs in CRC

Sections 4 and 5 address two complementary levels of EV-based therapeutics. Section 4 focuses on what EV-based interventions are designed to accomplish in CRC, including therapeutic cargo delivery, vaccination, immune microenvironment reprogramming, checkpoint modulation, and disruption of tumor-promoting EV communication. Section 5 focuses on how these therapeutic functions can be technically realized and translated, including cargo-loading strategies, source selection, surface engineering, administration and biodistribution, comparison with established delivery platforms, and scalable manufacturing. Thus, Section 4 is primarily application-oriented, whereas Section 5 addresses the engineering and translational infrastructure required to convert these applications into clinically viable therapeutic platforms.

4.1 EV-Mediated Delivery of Therapeutic Cargos

EVs have emerged as a versatile and biologically compatible nanoplatform for targeted therapy in CRC. Their endogenous membrane architecture confers intrinsic stability, low immunogenicity, and efficient cellular uptake, while enabling immune evasion and prolonged circulation, collectively supporting their use as effective carriers for therapeutic delivery [10].

As natural nucleic acid delivery vehicles, EVs have been engineered to transport miRNA inhibitors [209, 210], miRNA mimics [211], and siRNAs [212, 213] for precise intervention in CRC-associated oncogenic pathways. These engineered EVs have demonstrated therapeutic efficacy by suppressing tumor growth [153], reversing EMT [211], and reducing chemoresistance and metastatic potential [71, 212] in CRC. A representative example is the EV-mediated delivery of siRNA targeting BRAFV600E, which selectively inhibited mutant CRC cells in vitro and in vivo while sparing BRAF wild-type cells [213], highlighting the potential of mutation-specific gene therapy. Beyond nucleic acids, EVs also support the delivery of other therapeutic modalities. They can be engineered for intracellular delivery of bioactive proteins, protecting them from enzymatic degradation and enhancing intracellular bioavailability to improve therapeutic efficacy. For example, EV-mediated delivery of the ribosome-inactivating protein gelonin significantly suppressed tumor growth, resulting in a 14-fold increase in therapeutic efficacy [214]. Similarly, encapsulation of drugs such as oxaliplatin or doxorubicin into EVs improves solubility, tumor accumulation, pharmacokinetics, and systemic tolerability in CRC models [215-217]. In addition, combinatorial cargo strategies further expand the therapeutic potential of EV-based platforms by enabling co-delivery of chemotherapeutic agents with regulatory RNAs [209, 212, 217] or integration of gene-silencing RNAs with metabolic inhibitors [218]. Collectively, these studies establish EVs as programmable therapeutic carriers capable of delivering diverse cargos, including nucleic acids, proteins, small-molecule drugs, and combinatorial therapeutic payloads. Strategies that further improve tumor specificity and delivery precision are discussed in Section 5.3.

4.2 Engineered EV-Based Vaccine Platforms

EV-based vaccines have emerged as a promising cell-free immunotherapeutic strategy in CRC by enabling efficient antigen delivery and immune activation while overcoming the limitations of

conventional cell-based vaccines. Unlike traditional approaches, EVs provide a stable and scalable platform for antigen presentation, offering improved safety, manufacturability, and therapeutic versatility.

4.2.1 DC-Derived EV Vaccines

DC-derived EVs (DEVs) represent the most extensively studied EV-based cancer vaccine platform [219]. Enriched in major histocompatibility complexes and costimulatory molecules, DEVs retain intrinsic antigen-presenting capacity, enabling the activation of tumor-specific immune responses without the limitations associated with live DC-based vaccines [220]. Therapeutically, DEV-based vaccines have demonstrated robust antitumor efficacy in preclinical models. Neoantigen-loaded DEV can elicit strong adaptive immune responses, resulting in tumor suppression and durable immune memory [221]. Their efficacy can be further enhanced through engineering strategies. For example, bispecific DEV designed to simultaneously target tumor antigens and modulate immune checkpoints have shown improved therapeutic outcomes in CRC models [222], highlighting the benefit of combining antigen presentation with immune regulation. Importantly, the immunostimulatory activity of DEV has been validated in early-phase clinical studies in melanoma and non-small cell lung cancer, supporting their translational potential [223, 224]. Although clinical evidence in colorectal cancer remains limited, these findings establish DEV as a promising platform for EV-based cancer vaccination.

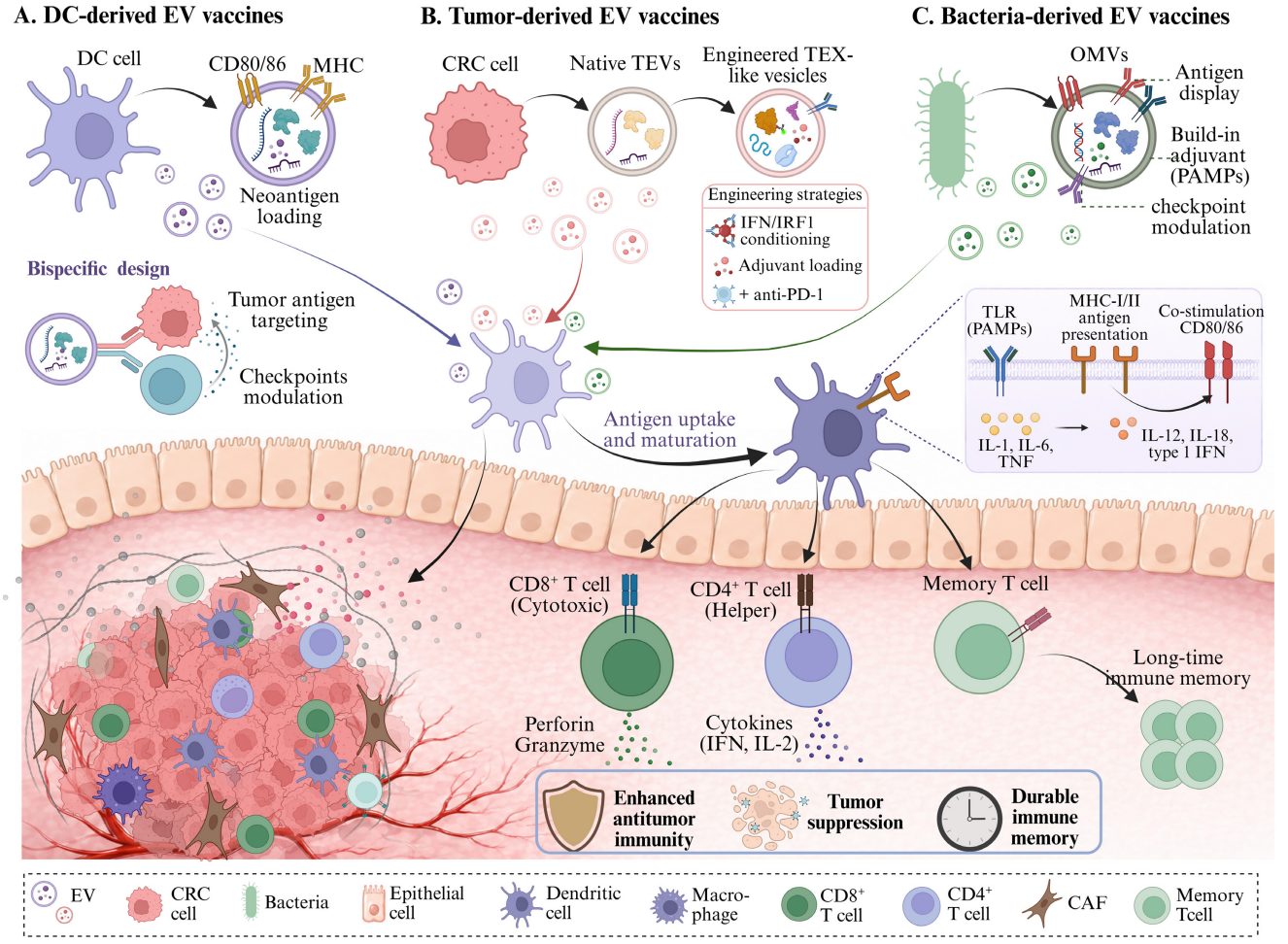


Figure 4. Engineered EV-based vaccines for colorectal cancer therapy. Engineered extracellular vesicle (EV)-based vaccines provide cell-free platforms for antigen delivery and immune activation in colorectal cancer (CRC). **(A)** Dendritic cell-derived EVs retain antigen-presenting and costimulatory capacity, which can be enhanced by neoantigen loading or bispecific designs. **(B)** Tumor-derived EVs carry endogenous tumor antigens but require engineering, such as IFN/IRF1 conditioning, adjuvant loading, anti-PD-1 combination, or TEV-like vesicle design, to improve immunogenicity and reduce immunosuppressive effects. **(C)** Bacteria-derived EVs, particularly outer membrane vesicles, provide built-in adjuvant activity through pathogen-associated molecular patterns and can be engineered for antigen display or checkpoint modulation. Together, these platforms promote dendritic cell maturation, activate cytotoxic and helper T-cell responses, induce memory T cells, and enhance durable antitumor immunity. Schematic illustrations were created with BioRender.com.

4.2.2 Tumor-Derived EV Vaccines

Tumor-derived EVs (TEVs) provide an alternative vaccine strategy by leveraging their endogenous repertoire of tumor-associated antigens. Preclinical studies have shown that TEV-based vaccination can induce antitumor immune responses and inhibit tumor progression in CRC models [225]. To improve therapeutic efficacy, current strategies focus on enhancing TEV immunogenicity and functionality, including modulation of parental tumor cells, incorporation of immune adjuvants, and combination with immune checkpoint blockade. For instance, interferon- or IRF-1-conditioned TEVs exhibit enhanced antigen-presenting capacity and T cell activation [226], while TEV-based vaccines combined with GM-CSF or anti-PD-1 therapy further augments therapeutic efficacy [227]. However, a major limitation of TEV-based vaccines is their potential to induce immunosuppressive signaling [228]. To address this, engineered TEV-like nanovesicles have been developed to enhance antigen specificity while minimizing immunosuppressive effects [229, 230]. For instance, FAP-targeting TEV-like vesicles can simultaneously induce cytotoxic T cell responses against tumor cells and stromal components, thereby improving therapeutic efficacy [230]. Overall, TEV-based vaccines exploit the intrinsic antigenic landscape of tumor cells while requiring careful engineering to balance immunogenicity and immunosuppression.

4.2.3 Bacteria-Derived EV Vaccines

Bacteria-derived EVs (BEVs), particularly outer membrane vesicles (OMVs), have recently emerged as a highly immunogenic vaccine platform. Unlike mammalian EVs, BEVs intrinsically contain pathogen-associated molecular patterns (PAMPs), which provide a built-in adjuvant effect and support their use as immunogenic vaccine carriers [231]. Preclinical studies showed that systemically administered OMVs preferentially accumulated in tumors, inhibited tumor growth in an IFN-γ-dependent manner, and induced durable immune memory, establishing their feasibility as vaccine-like immunostimulatory nanoplatforms [232]. To improve specificity, bioengineering approaches have been developed to display tumor antigens on OMV surfaces, enabling multi-antigen presentation and enhanced immune activation [233]. This strategy has been further extended to neoantigen delivery, as recombinant OMVs expressing CT26-derived polytope neoantigens showed strong immunogenicity and antitumor activity in vitro and in vivo [234-236]. Moreover, BEV-based vaccines can be combined with immune-modulating strategies to further improve therapeutic efficacy. For example, OMV-based platforms have been engineered to deliver tumor antigens alongside immune checkpoint modulators, resulting in synergistic antitumor effects [237]. These systems also support personalized vaccine design through modular antigen display technologies. Importantly, the clinical use of licensed OMV-containing vaccines such as Bexsero® demonstrates that selected bacterial OMV formulations can be manufactured and administered in humans [238]. However, this experience should not be extrapolated to establish the safety or efficacy of OMV-based cancer vaccines. In CRC, evidence for engineered OMV vaccine platforms remains predominantly preclinical, and key translational issues, including tumor-antigen selection, systemic reactogenicity, biodistribution, dose optimization, and manufacturing consistency, require dedicated clinical evaluation. Thus, BEV/OMV-based cancer vaccines represent a promising but still early-stage therapeutic platform for CRC.

4.3 Engineered EVs for Remodeling the Immunosuppressive TME

Engineered EVs have emerged as programmable platforms to remodel the immunosuppressive TME through coordinated reprogramming of multiple immune compartments. Rather than acting on single pathways, EV-based strategies simultaneously target TAMs, DCs, and T cell subsets, thereby converting an immune-suppressive niche into an immune-reactive state.

Reprogramming TAMs represents a central therapeutic entry point. EV-mediated delivery of STAT6 antisense oligonucleotides effectively induces M2-to-M1 polarization, restores $CD8^+$ T cell cytotoxicity, and leads to robust tumor regression in preclinical models [9]. This effect can be further enhanced by engineering EVs with targeting and functional modules. Dual-targeted EVs enable selective delivery to M2 TAMs and improve reprogramming efficiency, while M1 macrophage-derived EVs integrated with therapeutic components such as photosensitizers or nitric oxide-generating systems further amplify antitumor efficacy through combinatorial mechanisms [239, 240]. Beyond macrophage targeting, engineered EVs also reprogram adaptive immunity. Delivery of immunomodulatory cargos such as miR-155 restores DC function and enhances downstream T cell-mediated antitumor responses, thereby alleviating immunosuppression [241]. In parallel, engineered EVs directly correct T cell imbalance within the TME. Delivery of cargos exemplified by miR-206 enhances cytotoxic T cell activity while limiting Treg expansion,

reinforcing antitumor immunity [242]. Collectively, engineered EVs remodel the immunosuppressive TME through coordinated, multi-layered immune reprogramming, establishing a therapeutically actionable framework for CRC immunotherapy.

4.4 Targeting EV-Associated Checkpoint Signaling to Enhance Immunotherapy

In addition to cellular reprogramming, EVs critically regulate immune checkpoint signaling and influence the efficacy of immune checkpoint blockade (ICB) therapies. A major mechanism involves modulation of PD-L1 expression and its EV-associated forms, which play key roles in systemic immunosuppression. Pharmacological inhibition of upstream pathways, such as JAK2/STAT3, reduces PD-L1 levels in both tumor cells and EVs, thereby restoring T cell activity [243]. In parallel, EV cargo remodeling can improve responsiveness to ICB [244]; for example, EVs enriched in chemokines promote recruitment of $CD8^+$ T cells and NK cells, enhancing sensitivity to PD-1 blockade [245]. Building on these mechanisms, engineered EVs have been developed to directly inhibit checkpoint signaling. EVs loaded with PD-L1 and CTLA-4 siRNA effectively suppress checkpoint signaling, leading to enhanced $CD8^+$ T cell activity and reduced Treg infiltration in vivo [246]. Similarly, milk-derived EV-based nanoplatforms such as Super PD-L1exo downregulate PD-L1 expression and promote cytotoxic T cell infiltration [247]. More advanced platforms integrate checkpoint inhibition with cytotoxic modules; for example, KDEL-modified EVs co-delivering PD-L1 siRNA induce immunogenic cell death and amplify antitumor immune responses, achieving synergistic therapeutic effects [248]. Importantly, EV-associated PD-L1 is also a functional handle for improving adoptive T cell therapies. Tumor-derived $PD\text{-}L1^+$ EVs can facilitate T cell migration to tumor sites and enhance infiltration of effector memory $CD8^+$ T cells, thereby improving the tumor-targeting capacity of CAR-T cells [249].

Collectively, EVs occupy a dual position in checkpoint immunotherapy: they act as mediators of immune evasion and therapeutic resistance, yet also provide exploitable platforms for checkpoint-targeted intervention and combinatorial immunotherapy. This duality makes EV-associated checkpoint signaling, particularly PD-L1-centered pathways, a promising axis for optimizing ICB efficacy and overcoming resistance.

4.5 Targeting the Endogenous EV Life Cycle to Disrupt Tumor Communication

Given the central role of EVs in CRC progression and the increased EV output of cancer cells relative to normal cells, inhibition of EV biogenesis, secretion, or uptake has emerged as a rational therapeutic strategy.

One major strategy is to inhibit EV biogenesis and secretion. EV formation occurs within multivesicular bodies (MVBs) and is regulated by both ESCRT-dependent and ESCRT-independent pathways [250]. In the ESCRT-dependent pathway, components such as TSG101 and CHMP5 regulate EV production, and their depletion reduces EV secretion in CRC [251, 252], highlighting the ESCRT machinery as a potential therapeutic target. In contrast, the ESCRT-independent pathway is primarily linked to ceramide metabolism, where pharmacological inhibition of ceramide generation (e.g., GW4869) reduces EV release and associated cargo, with demonstrated antitumor activity in CRC [11]. In addition to MVB formation, EV secretion is regulated by vesicle trafficking machinery, including SNARE proteins, Rab GTPases, and Ca^{2+}-dependent processes [62]. In CRC, dysregulation of these pathways (e.g., SEC22B upregulation or KRAS-RAB13 signaling) enhances EV release, whereas their inhibition suppresses EV-mediated effects [27, 28, 253].

A second therapeutic approach is to inhibit EV uptake by recipient cells, thereby disrupting EV-mediated communication. EV internalization occurs via membrane interaction, fusion, and endocytic pathways, which are amenable to pharmacological inhibition [254, 255]. In this context, EV surface molecules represent key determinants of uptake specificity and provide actionable therapeutic targets [175, 256, 257]. In RAS-transformed cells, oncogenic RAS has been shown to promote CRAF-dependent macropinocytosis and enhanced EV uptake [256], while CD166 (ALCAM) has been implicated in the binding and uptake of cancer-derived EVs [257]. Beyond targeting individual molecules, modulation of EV surface properties offers an additional strategy to regulate cellular interactions. Surface glycosylation, for instance, influences EV uptake [258], and artificial glycosylation can be exploited to improve therapeutic delivery [254]. Similarly, engineering the EV biomolecular corona provides a means to modulate EV behavior, as exemplified by albumin enrichment, which prolongs circulation and reduces macrophage uptake, while removal of endogenous components can enhance functional activity [255]. Notably, EV uptake exhibits heterogeneity across CRC cell populations, as illustrated by IFITM1-dependent differences in uptake capacity [259], suggesting that cell-intrinsic variability should be considered in the design of EV-targeted therapies.

5. Engineering and Translational Optimization of EV Therapeutics in CRC

While EV-based therapeutic strategies have demonstrated substantial preclinical efficacy, their successful clinical translation critically depends on rational engineering design and scalable manufacturing approaches.

5.1 Engineering Strategies for Cargo Loading

Whereas Section 4.1 focuses on the therapeutic cargos delivered by EVs and their biological effects in CRC, this section focuses specifically on the engineering methods used to incorporate these cargos into EVs. Advanced bioengineering techniques have refined the methods for loading therapeutic cargo into EVs, broadly categorized into pre-loading (endogenous loading), post-loading (exogenous loading), each tailored to different applications [16]. In brief, pre-loading exploits EV biogenesis to achieve endogenous cargo encapsulation and is simple and suitable for nucleic acids and proteins, but is limited by transfection efficiency and may perturb cellular homeostasis. In contrast, post-loading introduces cargos after EV isolation, offering greater control; methods such as electroporation, ultrasound, and freeze-thaw enhance loading efficiency (with ultrasound often showing superior performance), but may compromise membrane integrity and cargo stability [260]. To overcome the limitations of conventional loading techniques, next-generation technologies have been developed. Microfluidic systems have been shown to significantly enhance loading efficiency under controlled flow conditions, for example increasing doxorubicin encapsulation from 7.86% to 31.98%. In parallel, acoustofluidic platforms enable one-step, reproducible drug loading with improved scalability [261, 262]. These technologies may be particularly attractive for CRC therapy, where controlled dosing, scalability, and regulatory compliance are essential for clinical translation [16, 261, 262].

5.2 Optimizing EV Sources for Therapeutic Application

The choice of donor source fundamentally shapes EV composition, therapeutic function, and translational feasibility. In CRC therapy, selection of an appropriate donor source must account for scalability, immunogenicity, intrinsic bioactivity, and compatibility with intended therapeutic payloads.

5.2.1 Mammalian Cell-Derived EVs

EVs derived from stem cells, tumor cells, and immune cells exhibit distinct biological properties, informing their application-specific suitability. MSC-derived EVs are widely used due to scalable culture conditions, high proliferation, and strong immunomodulatory activity. For instance, MSC-EVs have been engineered to deliver functional miRNAs or chemotherapeutic agents, demonstrating effective cargo delivery and enhanced intratumoral accumulation in CRC models [81, 210, 263]. Tumor-derived EVs, such as from CT26 cells, exhibit preferential uptake by parental tumor cells and superior tumor penetration, although safety concerns necessitate cautious selection [264]. HEK293T-derived EVs are frequently used due to their high production efficiency and ease of genetic modification, enabling delivery of therapeutic RNAs and chemotherapeutic agents [217, 228]. Immune cell-derived EVs exhibit intrinsic immunostimulatory properties, making them suitable for delivering immune modulators and enhancing antitumor immunity [260].

5.2.2 Non-Mammalian Cell-Derived EVs

Alternative EV sources, including bacteria, milk, and plants, have emerged as complementary platforms to enhance therapeutic performance. Bacterial OMVs exhibit strong immunogenicity and enable efficient antigen delivery, supporting their use in immunotherapy-oriented strategies [233]. Milk-derived EVs demonstrate high stability in the gastrointestinal tract and are well suited for oral delivery, offering a practical route for non-invasive administration [265]. Plant-derived EVs offer advantages such as low toxicity, high yield, and cost-effective production. A recent study developed a hybrid therapeutic platform combining plant-derived EVs with a dendrimer-based micelle nanoparticle loaded with an miR-146 mimic. This system achieved a fivefold increase in miRNA uptake compared with either component alone and demonstrated effective cargo delivery and antitumor activity in mouse models [266].

5.2.3 Bio-mimetic EV Platforms

Bio-mimetic EVs, including artificially synthesized EV-like nanoparticles, hybrid EVs, EV membrane-camouflaged nanoparticles and physical-origin nanovesicles, have recently been developed to overcome the key limitations of natural EVs [17]. These platforms may address some limitations of natural EVs by increasing production yield and enabling design flexibility, although compositional control and manufacturing standardization remain important translational challenges [267, 268]. Different bio-mimetic strategies provide complementary advantages. Artificial EV-like nanoparticles enable precise compositional control and reproducible fabrication;

hybrid EVs combine the biological functionality of natural EVs with the engineering flexibility of synthetic nanocarriers; EV membrane-camouflaged nanoparticles integrate immune evasion and targeting capability with high loading efficiency; and EV-mimetic nanovesicles achieve substantially increased production yield while retaining essential biomimetic properties. In a CT26 colon adenocarcinoma mouse model, doxorubicin-loaded nanovesicles inhibited tumor growth as effectively as natural EVs, and achieved 20-fold greater efficacy than free doxorubicin, without obvious systemic side effects [268], suggesting that nanovesicles are a promising alternative to natural EVs for chemotherapeutic delivery.

5.3 Strategies to Enhance EV Targeting Specificity

Distinct from Section 4.5, which focuses on therapeutic disruption of endogenous tumor-associated EV biogenesis, secretion, and uptake, this section addresses the engineering of administered therapeutic EVs to enhance tissue- and cell-specific delivery. The therapeutic efficacy of EV-based platforms depends not only on cargo potency but also on their ability to reach tumor cells or disease-relevant microenvironments with sufficient specificity. However, like many nanocarriers, systemically administered EVs are frequently sequestered by the liver, which limits delivery efficiency to extrahepatic tumors such as CRC.

To overcome this limitation, surface engineering strategies have been developed to enhance targeting specificity. A key approach involves functionalizing EV membranes with targeting ligands such as peptides [18], antibodies, or aptamers [263, 269] to enable receptor-specific delivery to tumor cells. Peptide-mediated targeting has been widely explored due to its small size and ease of modification. For example, DOX-loaded EVs modified with the EGFR-targeting peptide GE11 demonstrated enhanced uptake in EGFR-positive tumor cells [18]. Aptamer-based targeting strategies further expand the versatility of EV engineering. For example, AS1411 (anti-nucleolin) or MUC1 aptamer-functionalized EVs enable prolonged circulation and preferential tumor accumulation, thereby enhancing the delivery and intra-tumoral retention of chemotherapeutic agents such as doxorubicin in CRC models [269]. In CRC, an anti-PD-L1-functionalized exosome platform carrying apatinib has been investigated to combine tumor-targeted delivery with immune reprogramming [15]. Dual-targeted EV platforms combining tumor-targeting ligands with immune-modulatory components have demonstrated the capacity to simultaneously enhance tumor specificity and remodel the tumor microenvironment, thereby improving overall therapeutic outcomes [15]. These strategies indicate that EV surface engineering should be viewed not merely as a delivery enhancement technique, but as a key determinant of therapeutic selectivity, biodistribution, and clinical translatability.

5.4 Administration Routes and Biodistribution in CRC

The route of administration is a major determinant of EV biodistribution and should be matched to the anatomical and metastatic characteristics of CRC. Intravenous administration provides systemic exposure and is therefore attractive for disseminated or metastatic disease; however, intravenously administered EVs are rapidly exposed to phagocytic clearance and frequently accumulate in the liver and spleen. In CRC, this hepatic distribution may reduce delivery to extrahepatic primary lesions, whereas in colorectal liver metastasis it could potentially be exploited for liver-directed therapy if tumor-selective uptake can be distinguished from nonspecific uptake by resident phagocytic cells.

Intraperitoneal administration provides an alternative strategy for peritoneal dissemination by increasing local exposure while limiting systemic distribution. In a preclinical colorectal carcinomatosis model, intraperitoneally administered MSC-derived EVs loaded with the photosensitizer mTHPC showed markedly greater tumor selectivity than either the free drug or a liposomal formulation [270]. Nevertheless, penetration into larger lesions, dose reproducibility, and clinical scalability remain unresolved. Oral administration has been explored particularly with milk- and plant-derived EVs because some preparations can retain vesicular integrity or protect therapeutic cargo under gastrointestinal conditions and access the intestinal mucosa [271]. However, gastrointestinal stability is source- and formulation-dependent, and mucus penetration, epithelial uptake, gastrointestinal variability, and quantitative dose delivery remain important barriers. Rectal or intracolonic administration offers a potential route for localized colonic exposure without upper gastrointestinal transit. Recent proof-of-concept studies in inflammatory colonic disease have demonstrated that locally administered EV formulations can enhance mucosal retention and sustained delivery, although CRC-specific evidence remains limited [272]. Thus, local retention, mucosal coverage, and formulation design require further investigation.

Thus, administration route should be integrated with EV source, cargo properties, targeting strategy,

primary tumor location, and metastatic pattern rather than treated as a purely procedural variable.

5.5 Positioning EVs Relative to Established Therapeutic Delivery Platforms

EVs should be considered alongside established therapeutic delivery systems rather than in isolation (Table 4) [273-277]. Lipid nanoparticles (LNPs) provide efficient nucleic-acid encapsulation and comparatively mature, scalable manufacturing, although systemic formulations remain strongly influenced by hepatic biodistribution and extrahepatic targeting remains challenging [273]. Adeno-associated virus (AAV) vectors can support efficient and durable transgene expression but are constrained by limited cargo capacity, pre-existing or treatment-induced immunity, and difficulties with repeated administration [275]. Liposomes and polymeric nanocarriers offer chemically defined and highly tunable formulations, but tumor penetration, targeting efficiency, formulation reproducibility, and scale-up remain context-dependent challenges [276, 277]. EVs differ from these platforms in their biomimetic membrane composition and capacity to transport diverse molecular cargos, but they face substantial limitations in product heterogeneity, cargo loading, dose definition, batch consistency, systemic clearance, and large-scale manufacturing [273, 274]. Thus, EVs should not be viewed as intrinsically superior to established delivery systems. In CRC, platform selection should instead be guided by the therapeutic payload, required duration of activity, target site, need for repeated administration, and manufacturing feasibility.

5.6 Manufacturing and Purification Strategies for Clinical Translation

Despite advances in EV engineering, their therapeutic potential in CRC is ultimately constrained by challenges in scalable production, standardization, and purity. Intrinsic heterogeneity in EV size, composition, and morphology further complicates clinical-grade manufacturing.

Table 4. Translational comparison of extracellular vesicles with established therapeutic delivery platforms

Platform	Typical Cargo / Functional Features	Targeting and Biodistribution	Major Advantages	Major Limitations	Clinical / Translational Status
Extracellular vesicles (EVs) [273, 274]	Nucleic acids, proteins, small molecules; native membrane proteins and lipids can contribute to cell interactions	Source-dependent biodistribution and tropism; substantial mononuclear phagocyte system uptake; targeting can be enhanced by surface engineering	High biocompatibility; natural intercellular cargo transfer; accommodates diverse cargo classes; membrane can support biological targeting	Source and product heterogeneity; variable yield and cargo loading; rapid systemic clearance; batch consistency, potency assays, and GMP manufacturing remain challenging	Preclinical and early clinical development; active bioengineering, manufacturing, and regulatory standardization efforts
Lipid nanoparticles (LNPs) [273]	Primarily mRNA, siRNA, and other nucleic-acid therapeutics; chemically defined lipid formulations	Systemic formulations often show liver-biased distribution; ionizable lipid composition and ligand engineering can modify tissue targeting	Efficient nucleic-acid encapsulation and cytosolic delivery; scalable and reproducible manufacturing; multiple approved products	Extrahepatic targeting remains difficult; endosomal escape is incomplete; formulation-dependent inflammatory or tolerability issues may occur	Clinically mature nucleic-acid delivery platform with approved siRNA and mRNA products; extensive manufacturing and regulatory experience
Adeno-associated virus (AAV) [275]	DNA transgenes with durable expression; packaging capacity is limited (~4.7 kb)	Serotype- and capsid-dependent tissue tropism; capsid engineering can alter organ and cell targeting	High transduction efficiency in selected tissues; sustained transgene expression; several approved gene therapies	Limited cargo capacity; pre-existing and treatment-induced immunity; redosing is difficult; dose-related toxicity and off-target transduction remain concerns	Clinically validated gene-transfer platform with multiple approved products; generally optimized for durable, often single-administration gene delivery
Liposomes [276]	Hydrophilic or lipophilic small molecules; selected nucleic acids and combination formulations	Passive tumor accumulation is variable; PEGylation, ligands, and stimuli-responsive designs can modify circulation and targeting	Clinically established; flexible drug encapsulation; can improve pharmacokinetics and reduce toxicity of conventional drugs	Variable tumor penetration and EPR dependence; stability and premature drug release can limit performance; reticuloendothelial clearance and repeated-dose reactions may occur	Mature nanomedicine platform with multiple approved formulations, including established oncology products
Polymeric nanoparticles / micelles [277]	Small molecules, proteins, and nucleic acids; tunable polymers enable controlled release and surface functionalization	Size, charge, polymer composition, and ligands can be engineered for passive or active targeting	Highly tunable physicochemical properties; controlled drug release; modular surface chemistry	Complex formulation optimization; scale-up and batch reproducibility; purification, storage, and regulatory standardization remain barriers	Broad preclinical and clinical investigation, but overall clinical translation is more limited than for lipid-based nanocarriers

EV, extracellular vesicle; LNP, lipid nanoparticle; AAV, adeno-associated virus; siRNA, small interfering RNA; mRNA, messenger RNA; EPR, enhanced permeability and retention; GMP, good manufacturing practice.

To address these limitations, emerging bioengineering strategies have focused on improving both yield and quality. Microfluidic systems enable precise control of culture conditions, enhancing EV yield and quality. For example, shear stress-regulated microfluidic culture (0.5 – 5 dyn/cm²) markedly increases EV production from MSCs while preserving vesicle integrity. Beyond production, microfluidic platforms have also emerged as efficient tools for EV isolation, integrating size-based separation and affinity capture to overcome the low recovery and poor scalability of conventional methods such as ultracentrifugation. For instance, a porous microfluidic chip coupled with anti-CD9 capture significantly improves EV enrichment efficiency compared with traditional approaches [178]. In parallel, affinity-based bead-assisted strategies provide a simple and rapid alternative for EV enrichment. DSPE-functionalized beads enable direct capture of EVs from biological fluids within minutes without the need for specialized equipment, achieving efficient and broadly applicable EV enrichment [129]. Collectively, these strategies establish a more efficient and scalable framework for EV production and purification, thereby strengthening the translational potential of EV-based therapeutics in CRC.

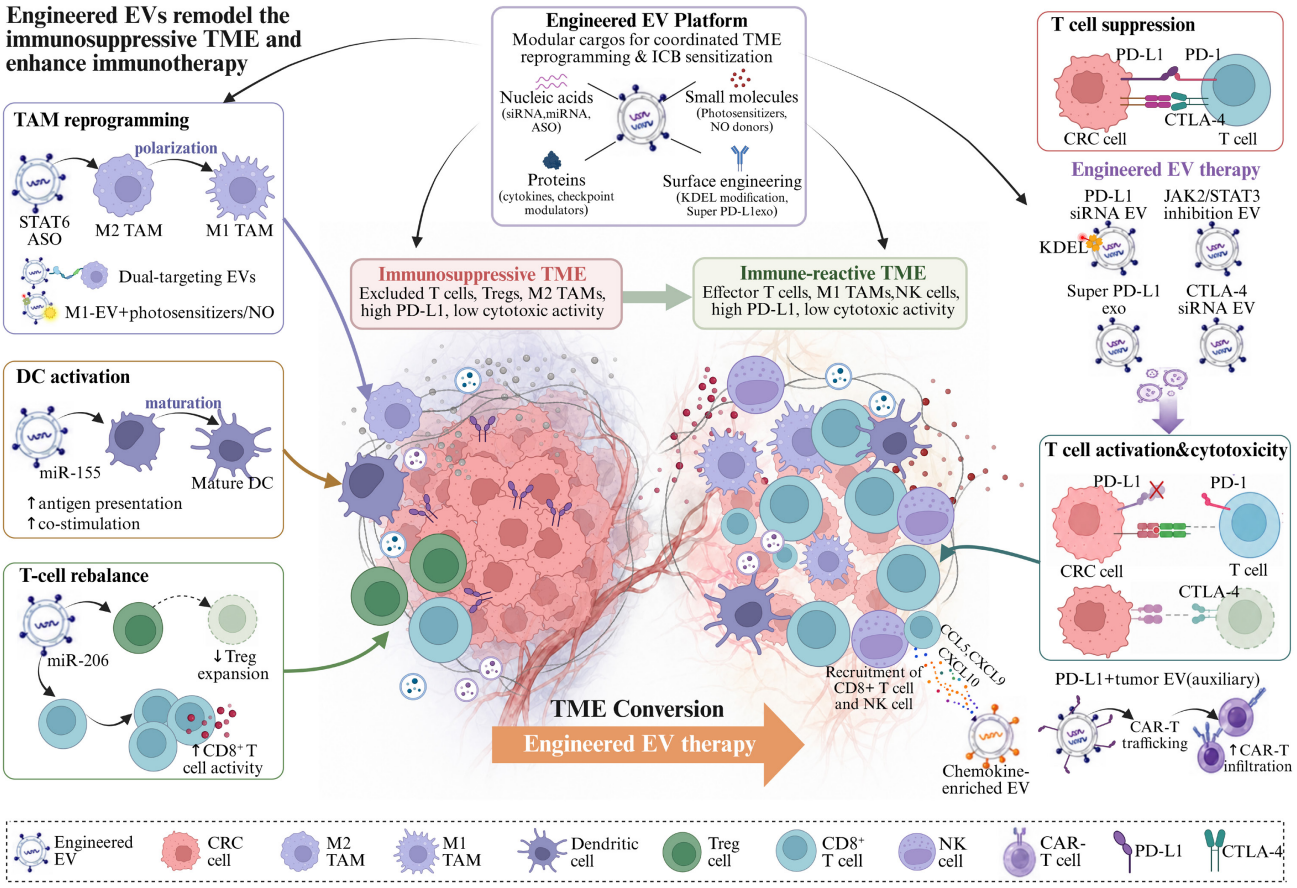


Figure 5. Engineered EVs remodel the immunosuppressive tumor microenvironment in colorectal cancer. Engineered extracellular vesicles (EVs) provide programmable platforms for converting an immunosuppressive tumor microenvironment (TME) into an immune-reactive state in colorectal cancer (CRC). By delivering modular cargos, including nucleic acids, proteins, small molecules, and surface-engineered functional components, EV-based strategies can coordinately reprogram multiple immune compartments. These approaches promote M2-to-M1 tumor-associated macrophage (TAM) polarization, enhance dendritic cell (DC) maturation and antigen presentation, restore cytotoxic $CD8^+$ T-cell activity, and limit regulatory T-cell expansion. In parallel, EV-based platforms can target checkpoint-associated signaling by suppressing PD-L1/PD-1 or CTLA-4 pathways, while chemokine-enriched EVs facilitate recruitment of $CD8^+$ T cells and NK cells. Tumor-derived PD-$L1^+$ EVs may also be exploited to improve CAR-T cell trafficking and tumor infiltration. Schematic illustrations were created with BioRender.com.

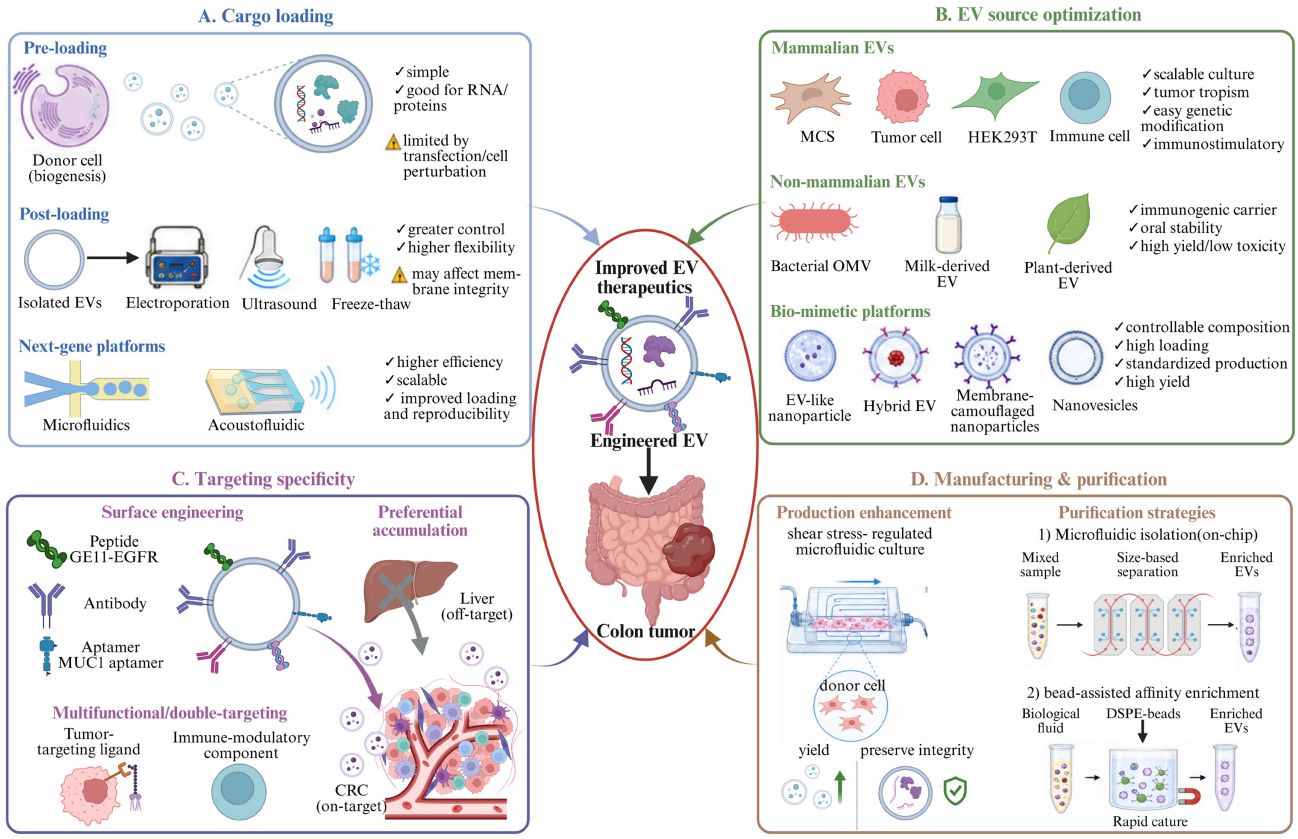


Figure 6. Strategies to improve the therapeutic potential of EVs in colorectal cancer. The clinical translation of extracellular vesicle (EV)-based therapeutics in colorectal cancer (CRC) depends on coordinated optimization of engineering design and manufacturing workflows. **(A)** Cargo loading strategies include pre-loading through donor-cell engineering, post-loading into isolated EVs, and next-generation platforms such as microfluidic and acoustofluidic systems, which improve loading efficiency and reproducibility. **(B)** EV source optimization involves selecting appropriate mammalian, non-mammalian, or bio-mimetic platforms according to scalability, bioactivity, immunogenicity, and manufacturability. **(C)** Targeting specificity can be enhanced through surface engineering with peptides, antibodies, or aptamers, as well as multifunctional or dual-targeting designs that improve tumor accumulation and reduce off-target delivery, particularly liver sequestration. **(D)** Manufacturing and purification strategies, including shear stress-regulated microfluidic culture, on-chip microfluidic isolation, and bead-assisted affinity enrichment, further improve EV yield, purity, and scalability. Schematic illustrations were created with BioRender.com.

6. Challenges and Future Perspectives

Despite substantial progress, the clinical translation of EV-based strategies in CRC remains constrained by several interrelated challenges. Intrinsic EV heterogeneity arising from biogenesis, cargo composition, cellular origin, and species source creates context-dependent functions that complicate mechanistic studies, biomarker discovery, and therapeutic design. Different EV subpopulations may exert opposing pro- or anti-tumor effects, making functional interpretation challenging. Population-level analyses yield averaged metrics essential for scalable biomarker discovery and production yet obscure rare and functionally distinct EV subpopulations. High-resolution analytical technologies, including single-vesicle profiling combined with multi-omics integration, computational modeling, and AI-assisted analytics, enable linkage of EV heterogeneity with clinical phenotypes. However, low abundance of tumor-derived EVs, molecular variability, cargo instability, and insufficient cohort validation remain critical barriers.

Beyond heterogeneity, the design and optimization of EV-based therapeutics face multiple interrelated challenges, including EV source selection, engineering strategies, and in vivo barriers. EV source critically influences immunogenicity, yield, safety, and functional cargo compatibility. Autologous EVs offer low immunogenicity but limited scalability, whereas allogeneic or engineered sources provide higher yield yet introduce donor variability or tumorigenicity risks. Engineering strategies, including cargo loading, surface modification, targeted delivery, and immune modulation, aim to enhance therapeutic efficacy while preserving native biological activity. However, engineered EVs face batch-to-batch variability, unstable modifications, suboptimal in vivo stability, poor targeting specificity, and unassessed long-term safety. Additional limitations include rapid clearance by the mononuclear phagocyte system, inefficient intracellular trafficking, and incomplete cargo release.

Manufacturing and standardization remain

major hurdles, as efficient, scalable, and reproducible EV isolation, characterization, and production are lacking. Current isolation methods (e.g., ultracentrifugation, precipitation) face trade-offs between yield, purity, and scalability, while emerging microfluidic and affinity-based approaches require further validation for clinical-grade production. Microfluidic technologies offer several advantages over conventional EV isolation methods. They enable precise fluid control, reduced reagent consumption, and automated workflows, leading to improved yield, purity, and consistency while minimizing sample processing time. By integrating size-based filtration, immunoaffinity capture, or inertial focusing, microfluidic platforms can achieve high recovery efficiency and enhanced selectivity for rare functional EV subpopulations. Additionally, closed-loop designs with in-line quality control sensors facilitate real-time monitoring of EV size, concentration, and purity, supporting scalable and reproducible production. However, current microfluidic systems face challenges in throughput, cost-effective fabrication, and sterility assurance at clinical scales, and their widespread adoption for GMP-compliant EV manufacturing remains limited, requiring further validation and standardization.

Clinical translation remains constrained by inadequate preclinical models that incompletely recapitulate the human tumor microenvironment, limited clinical trial data, and insufficient characterization of optimal dosage, administration routes, predictive biomarkers, long-term efficacy, and adverse effects across CRC subtypes, including metastatic, chemotherapy-resistant, and MSI/MSS tumors. The absence of large-scale, randomized controlled trials further hinders clinical adoption.

Importantly, these translational challenges are unlikely to be uniform across CRC clinical contexts. In dMMR/MSI-H CRC, where immune-checkpoint blockade can produce substantial and durable responses, EV profiling may provide a dynamic complement to tissue-based biomarkers for monitoring immune escape and acquired therapeutic resistance. By contrast, pMMR/MSS CRC remains substantially less responsive to checkpoint blockade and is biologically heterogeneous; here, EV-defined immune, stromal, or metabolic states may help identify biologically selected subgroups that are more likely to benefit from rational combination immunotherapy. In metastatic CRC, particularly in patients with liver metastases, EV-based approaches face a distinct biodistribution problem: the prominent hepatic sequestration of systemically administered EVs may represent both an off-target clearance mechanism and a potential opportunity for liver-directed delivery, provided that reticuloendothelial uptake can be distinguished from metastasis-selective targeting. In chemotherapy-resistant disease, longitudinal EV RNA, protein, and DNA signatures may enable dynamic tracking of treatment-induced adaptation and emerging resistance. However, prospective comparison with established approaches, including ctDNA, conventional serum markers, and imaging, will be essential before these applications can enter routine clinical practice.

From a translational perspective, future priorities should also be distinguished by clinical readiness. In the near term, the most feasible goals include standardization of pre-analytical workflows, reproducible EV isolation and characterization, prospective validation of focused EV biomarker panels, integration of EV readouts with established clinical assays, optimization of administration routes, and development of relatively simple targeting modifications compatible with GMP manufacturing. These approaches build directly on technologies that are already experimentally mature and can therefore be evaluated in prospective clinical cohorts. In the longer term, population-scale single-EV multi-omics, AI-guided patient-specific EV signatures, personalized EV vaccine platforms, multifunctional EVs carrying multiple therapeutic cargos, and closed-loop manufacturing with real-time quality control could enable more individualized intervention. However, these strategies will require substantially greater advances in analytical standardization, manufacturing reproducibility, safety assessment, and prospective clinical validation.

7. Conclusions

In conclusion, EVs serve as key mediators of intercellular communication and have witnessed substantial research and technological advances. These include precise identification through single-vesicle analysis and surface-enhanced Raman spectroscopy, functional characterization via multi-omics integration and single-vesicle sequencing, and efficient isolation using microfluidic platforms. These innovations have deepened our understanding of EV biology, revealing their roles in tumor progression, metastasis, and immune modulation. Moreover, they have catalyzed the development of EV-based applications in liquid biopsy diagnostics and therapeutic platforms. Despite these advances, challenges remain, including standardization of isolation and characterization methods, scalable production, optimization of in vivo stability and targeting specificity, and rigorous validation in clinical settings. Addressing these challenges will be essential to translate EV research into precision diagnostic and

therapeutic strategies, thereby realizing the full theranostic potential of EVs in CRC and other malignancies.

Abbreviations

CRC: colorectal cancer; EVs: extracellular vesicles; ILVs: intraluminal vesicles; MVBs: multivesicular bodies; MISEV: Minimal Information for Studies of Extracellular Vesicles; PD-L1: programmed death-ligand 1; TRAIL: TNF-related apoptosis-inducing ligand; miRNAs: microRNAs; siRNAs: small interfering RNAs; rRNAs: ribosomal RNAs; lncRNAs: long noncoding RNAs; circRNAs: circular RNAs; mRNAs: messenger RNAs; RBP: RNA-binding protein; dsDNA: double-stranded DNA; ssDNA: single-stranded DNA; mtDNA: mitochondrial DNA; gDNA: genomic DNA; cGAS: cyclic GMP-AMP synthase; STING: stimulator of interferon genes; AIM2: absent in melanoma 2; TME: tumor microenvironment; CAFs: cancer-associated fibroblasts; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; PMN: premetastatic niche; MSCs: mesenchymal stromal/stem cells; MSC-EVs: mesenchymal stromal/stem cell-derived extracellular vesicles; FnEVs: Fusobacterium nucleatum-derived extracellular vesicles; NK cells: natural killer cells; P-EVs: plant-derived extracellular vesicles; HR: hazard ratio; AUC: area under the receiver operating characteristic curve; tsRNAs: tRNA-derived small RNAs; ncRNAs: non-coding RNAs; GEO: Gene Expression Omnibus; mCRC: metastatic colorectal cancer; BEVs: bacterial extracellular vesicles; DCs: dendritic cells; DEVs: dendritic cell-derived extracellular vesicles; TEVs: tumor-derived extracellular vesicles; IFN: interferon; IRF-1: interferon regulatory factor 1; GM-CSF: granulocyte-macrophage colony-stimulating factor; OMVs: outer membrane vesicles; PAMPs: pathogen-associated molecular patterns; FDA: Food and Drug Administration; TAMs: tumor-associated macrophages; STAT6: signal transducer and activator of transcription 6; ASO: antisense oligonucleotide; Tregs: regulatory T cells; ICB: immune checkpoint blockade; PD-1: programmed cell death protein 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CAR-T cells: chimeric antigen receptor T cells; ESCRT: endosomal sorting complex required for transport; BM-MSCs: bone marrow-derived mesenchymal stromal/stem cells; UC-MSCs: umbilical cord-derived mesenchymal stromal/stem cells; 4D-DIA: four-dimensional data-independent acquisition; AAV: adeno-associated virus; AFM: atomic force microscopy; AI: artificial intelligence; BMEF: bubble multimer-enhanced fluorescence; cfDNA: cell-free DNA; CNN: convolutional neural network; dMMR: deficient mismatch repair; DSS: dextran sulfate sodium; DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; ER: endoplasmic reticulum; FAP: fibroblast activation protein; GMP: good manufacturing practice; HSFC: high-sensitivity flow cytometry; IFN-γ: interferon gamma; LNPs: lipid nanoparticles; ML: machine learning; MSI: microsatellite instability; MSI-H: microsatellite instability-high; MSS: microsatellite-stable; nFCM: nano-flow cytometry; PBA: proximity barcoding assay; pMMR: proficient mismatch repair; PiFM: photo-induced force microscopy; RF: random forest; ROS: reactive oxygen species; SERS: surface-enhanced Raman spectroscopy; SMFC: single-molecule flow cytometry; SVM: support vector machine; 3D-dSTORM: three-dimensional direct stochastic optical reconstruction microscopy; TA-MSC-EVs: tumor-associated mesenchymal stem cell-derived extracellular vesicles; ctDNA: circulating tumor DNA; VAP-A: vesicle-associated membrane protein-associated protein A; CERT: ceramide transfer protein.

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Author contributions

Yining Sun: Writing, original draft preparation, drawing, and figure-making. Xian Zhang: Writing, original draft preparation, drawing, and figure-making. Feifan Chen: Critical revision. Binyang Luo: Critical revision. Zhu Wang: Conception, critical revision. Jin-Lin Yang: Conception, critical revision.

Competing Interests

The authors have declared that no competing interest exists.

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