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Review article

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Clinical relevance of extracellular vesicles in cancer — therapeutic and diagnostic potential

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Abstract

Extracellular vesicles (EVs) encompass a multitude of lipid bilayer-delimited particles, of which exosomes are the most widely studied. Bidirectional cell–cell communications via EVs have a pivotal role in the physiology of multicellular organisms. EVs carry biological cargoes (including proteins, RNA, DNA, lipids and metabolites) capable of mediating a range of pleiotropic cellular functions. Over the past decade, EVs released by cancer cells (onco-EVs) have been shown to promote cancer progression including tumour outgrowth and metastatic dissemination. Furthermore, the innate ability of EVs to protect vulnerable molecular cargoes (such as RNA, DNA or proteins) from enzymatic degradation, their presence in most biofluids and the ability to transverse biological barriers to reach distant organs make them ideal targeted drug delivery systems, including in patients with cancer. Many of these properties also support investigations of EVs as biomarkers with potential roles in both diagnosis and treatment monitoring. In this Review, we describe advances in the development of EVs as cancer therapeutics or biomarkers, including cancer vaccines, targeted drug delivery systems and immunotherapies, as well as potential roles in early cancer detection, diagnosis and clinical management. We also describe the potential of emerging technologies to support further discoveries as well as the clinical translation of EVs into diagnostic and therapeutic clinical tools. We highlight the potential of single-EV and onco-EV detection and discuss how advances in multi-omic and artificial intelligence-enabled integration are providing new biological insights and driving clinical translation.

Sections

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Key points

- Extracellular vesicles (EVs) are directly released from cancer cells and thus provide a direct but accessible source of information on tumour biology with potential for implementation as biomarkers to guide the management of patients with cancer.
- EVs released from cancer cells are able to interact with the tumour microenvironment and with non-malignant cells at distant anatomical locations, thus enabling the progression and metastatic dissemination of cancer.
- EV-mediated cell–cell communication and cargo transfer between tumour and non-tumour cells are involved in all stages of cancer — from development to metastatic dissemination and modulation of the host immune response.
- EVs can be engineered and have potential clinical utility as next-generation drug delivery platforms for cancer therapeutics as well as a potential role as cancer vaccines or for the delivery of immunotherapies.
- Thus far, the clinical development and implementation of EV-based biomarkers or therapeutics have been limited owing to various technical challenges.
- Technological advances and improved integration of omics and other technologies are expected to continue to advance the clinical implementation of therapeutics or diagnostics involving EVs.

Introduction

Extracellular vesicles (EVs) comprise a diverse range of membrane-encapsulated organelles containing various molecular cargoes. Research from the past 20 years has demonstrated an important role of EVs as mediators of bidirectional communications between cells and their microenvironment, including in both non-pathological and pathological physiology[1–4]. Although much progress has been made in EV biology, including our knowledge of the roles of EVs in various diseases, knowledge in other areas, such as the extent of EV subtype diversity (including morphology, size, biophysical properties, biogenesis and molecular cargoes) as well as functional traits remains limited[5,6]. Since initial reports from the late 1990s and early 2000s describing EVs as potential cancer vaccines[7–10], we are now beginning to piece together the various roles of EVs including as drivers of tumour development and as mediators of metastatic dissemination (Fig. 1), described in detail elsewhere[3,11,12]. This knowledge has led to considerable research interest in the development of biologically informed tools for the delivery of personalized cancer vaccines and anticancer drugs as well as various liquid biopsy biomarkers for the detection of early-stage cancers, as well as informing about risk stratification, and the management of patients with cancer treatment at various stages of their disease trajectory[13–16] (Fig. 1).

Two broad categories of extracellularly released particles are now recognized: membranous EVs (EVs encapsulated in lipid bilayers, such as exosomes and microparticles) and non-vesicular extracellular particles (NVEPs) (such as exomeres and supermeres). Here, we focus on membranous EVs, which are often abundant in biofluids and include several subtypes that differ markedly in their biogenesis, biophysical and biochemical properties (Table 1) (described in detail elsewhere[17]).

Membranous EVs include exosomes, microparticles, microvesicles, endosomes, ectosomes and apoptotic bodies. Exosomes (with diameters of 50–150 nm) originate from multivesicular bodies of late endosomal origin, which traffic to and fuse with the cell membrane followed by release into the extracellular environment either via endosomal sorting complex required for transport (ESCRT)-dependent or ESCRT-independent mechanisms[3,18–23]. Conversely, microparticles whose size range extends to up to 1,500 nm in diameter form by direct outward budding and fission of the plasma membrane[24,25]. Although microparticle biogenesis is still poorly understood, accruing evidence indicates that the required membrane blebbing involves protrusion of the plasma membrane, entailing changes in membrane lipid composition and cytoskeletal remodelling[26,27]. Membrane-encapsulated midbody remnants (diameter of 200–600 nm) are a type of EV that can be generated during cytokinetic abscission followed by release into the extracellular space[28–31]. Midbody remnants are molecularly distinct from exosomes and microparticles[28,32], although, similar to other EVs, they can be taken up by non-sister cells[33] and can elicit a phenotypic response. Over the past decade, different cell types have

Fig. 1 | Onco-extracellular vesicles as diagnostic and therapeutic targets. Primary tumour cells secrete extracellular vesicles (EVs) capable of reprogramming resident cells in their microenvironment, including cancer-associated fibroblasts, endothelial cells and immune cells to drive the emergence of and regulate various hallmark effects of cancers (1). Activated fibroblasts then secrete extracellular matrix (ECM) and signalling factors capable of supporting the expansion of neoplastic cells, endothelial cells and forming new blood vessels that provide nutrients, while guiding the polarization of immune cells towards a pro-inflammatory phenotype. As cancer-related alterations accumulate and promote tumorigenesis, such alterations are also reflected in the molecular composition of EVs, enabling them to acquire de novo functions, such as transformation of the extracellular and cellular microenvironment, enabling further development in a malignant phenotype. During this invasive outgrowth, EVs can profoundly reprogramme the ECM and related cell–cell adhesion networks as well as enter the circulation or lymphatic systems. EVs from various cellular and organ sources have surface protein signatures (such as integrins or cytokines) that mediate their systemic dissemination to specific target organs. For example, EVs might accumulate in the vascular beds of the lungs or disseminate to the liver (as often observed with EVs derived from primary pancreatic cancer). This dissemination results in the profound reprogramming of target tissues (towards increased vascular permeability and altered expression of ECM proteins for increased adhesion and chemotaxis), which enhances the seeding of circulating tumour cells. Cancer cell-derived EVs are also able to recruit and polarize immune cells to activate signalling pathways favouring tumour cell tissue invasion and metastatic dissemination. This priming precedes the arrival of disseminated tumour cells and is therefore referred to as pre-metastatic niche formation (2). These pre-metastatic niches promote not only seeding but also eventual outgrowth to establish metastatic foci in distant organs. Circulating EVs can also migrate to bone marrow, in which they mobilize bone marrow-derived cells (BMDCs) home to pre-metastatic niches and reprogramme stromal recipient cells at these sites and thus promote the development of metastases (3). Indeed, such reprogrammed BMDCs then mediate their ability to generate a pre-metastatic niche and influence other cells within the niche to create a permissive environment for cancer metastasis. NK, natural killer.

been shown to generate various other membranous protrusions; these include trailing retraction fibres of migrating cells (referred to as migrasomes[34–37]), pearling and/or vesiculation of filopodia and retraction fibres[27,38], tips of filopodia or microvilli and tunnelling nanotubes[39] (for example, nanotubes between neuronal and microglial cells[27,40,41]). Apoptotic bodies (~50–5,000 nm in diameter) are the products of cells undergoing apoptosis and are another distinct EV class arising from plasma membrane blebbing or actomyosin contraction (also referred to as apoptopodia)[42–44]. Other EV subtypes include autophagy-linked secreted EVs[45,46], mitovesicles (and mitophers, which are enriched in mitochondria)[47], blebbisomes[48], oncosomes[25,49], cardiac or muscle exophers[50,51], mitochondrial enriched EVs[52,53], arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs)[54,55], and EVs derived from various non-apoptotic regulated cell death processes[56].

For many studies investigating EVs, uncertainty exists within the nomenclature regarding the nature of the samples being investigated. Different opinions exist on the use of the term 'exosome' versus 'EV'. Nonetheless, the consensus recommendation of the International Society for Extracellular Vesicles on nomenclature is to apply EV as the 'generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate'[57] and to further define EV on the basis of a set of clear, measurable characteristics, including cell and/or tissue of origin, molecular markers,

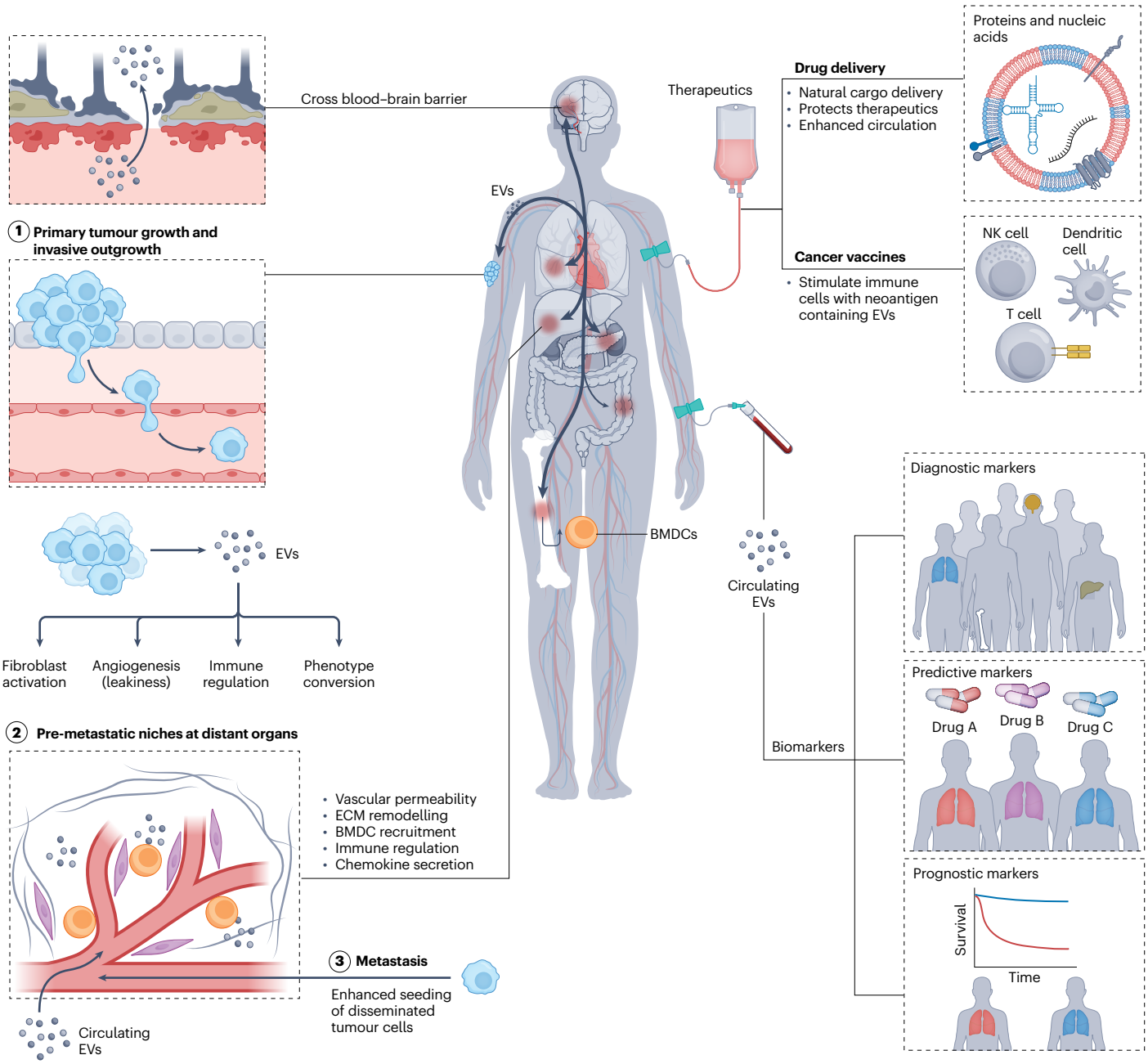

Table 1 | Diversity of extracellular vesicles and extracellular particles

Subtype	Mechanisms of biogenesis	Markers	Particle diameter	Buoyant density
Membranous EVs[a]				
Exosomes[22,89,102,121,403–405]	Endosomal pathway	CD63, CD81, SDCBP, ESCRT complex proteins: ALIX/PDCD6IP and TSG101	50–150 nm	1.08–1.14 g/ml
Microparticles (also known as ectosomes and microvesicles)[24,89,119,406]	Plasma membrane budding	ANXA1, RPS7, ARF6	100–1,500 nm	1.08–1.14 g/ml
Midbody remnants (MBRs)[28]	Cytokinesis (late-stage symmetrical cytokinetic abscission)	KIF23, RACGAP1[b]	200–600 nm	1.22–1.30 g/ml
Exophers[407]	Plasma membrane budding	Phosphatidylserine, LC3, Tom20	3.5–4 μm	–
Blebbisomes[48]	Plasma membrane budding (cell motility)	Cellular organelle markers; VDAC2 (mitochondria), GM130 (Golgi), RPS8/10 (ribosome), EEF2 (nuclear), myosin IIA, actin (cytoskeleton)	5–20 μm	–
Large oncosomes[408]	Plasma membrane budding	CK18, GOT1	1–10 μm	–
Migrasomes[34,35]	Plasma membrane budding (cell motility)	TSPAN4, ITGA5	0.5–3 μm	–
Apoptotic bodies[409]	Plasma membrane budding	Phosphatidylserine	50 nm–5 μm	–
NVEPs[c]				
Exomeres[111]	Unknown	HSP90-b, ENO1, GANAB	<50 nm	–
Supermeres[410]	Unknown	TGFBI, HSPA13, ENO1, ENO2	<50 nm	–

ALIX, ALG-2-interacting protein X; ANXA1, annexin A1; ARF6, ADP-ribosylation factor 6; CK18, cytokeratin 18; EEF2, elongation factor 2; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; GM130, Golgin subfamily A member 2; GOT1, glutamic-oxaloacetic transaminase 1; ITGA5, integrin α-5; KIF23, kinesin family member 23 — also known as mitotic kinesin-like protein 1 (MKLP1); LC3, microtubule-associated protein 1 light chain 3; NVEP, non-vesicular extracellular particle; PDCD6IP, programmed cell death 6-interacting protein; RACGAP1, rac GTPase-activating protein 1; RPS7, RNA-binding protein ribosomal protein S7; RPS8/10, 40S ribosomal protein S8/S10; SDCBP, syndecan binding protein; Tom20, translocase of the outer membrane (TOM) complex in the outer mitochondrial membrane; TSG101, tumour susceptibility gene 101; TSPAN4, tetraspanin-4; VDAC2, voltage-dependent anion channel 2. [a]Extracellular vesicles that are encapsulated in a membranous lipid bilayer. [b]KIF23 and RACGAP1 are uniquely expressed in midbodies and secreted midbody remnants. [c]Non-vesicular extracellular particles that do not have a membranous lipid bilayer (also known as nanoparticles).

size distribution, density and biological function[58]. Accordingly, we use the all-encompassing term EVs to denote different vesicle types[58,59].

EVs are thought to be evolutionary conserved as they are released from cells across several different kingdoms, including Archaea, Bacteria (both Gram-positive and Gram-negative) as well as Eucharia (including animals, plants, fungi and protists)[60–64]. All cells are known to release EVs both as part of their physiological functions and when affected by specific pathologies; their attendant molecular cargoes can elicit phenotypic changes in target recipient cells, leading to pleiotropic functional capabilities[3,65] (for example, pre-metastatic niche formation[66–74] or non-malignant effects such as promotion of neurodegeneration or cardiac cell repair[65], as well as amelioration of immunosuppression in the tumour microenvironment (TME)[15]). EVs are also found in almost all biofluids and this opportunity for largely non-invasive sampling makes them prime candidates for cancer detection, staging, monitoring and identification of potential therapeutic targets[75–78]. EVs have now emerged as a focus for the development of nanotherapeutics[65], particularly by modulating mechanisms of biogenesis to curb TME formation[79] and the distribution of oncogenic programmes[80–83].

In this Review, we discuss foundational applications of EVs as tools for therapeutic interventions in patients with cancer, including therapeutic EV-based cancer vaccines and the development of EVs as drug delivery systems. We summarize the utility of EVs for clinical translation as liquid biopsy analytes and for the potential early detection and prevention of cancer. We also describe how EVs can be utilized as technology platforms in cancer therapeutics and diagnostics and how this can be accomplished using various bioengineering strategies that target, regulate and deliver various molecular and bioactive cargo molecules (described in detail elsewhere[13,65,84–86]). We also illustrate how EV-based cancer therapeutic strategies are now informing late-phase clinical trials, including those testing cancer vaccines, immunotherapies and patient-tailored therapies.

EV heterogeneity and complexity

Despite the clinical potential of EVs, their detection and analysis are non-trivial. EVs comprise a heterogeneous group of vesicles that vary in size and molecular composition, potentially down to the single-cell secretome level[87,88]. This subtype diversity is reflected in the range of molecular cargoes and distinct functionalities[28,48,89,90] (Table 1 and Fig. 2). Small EVs, owing to their small size, protective lipid bilayer and presence of surface receptors, can mediate paracrine signalling and interorgan systemic crosstalk[5,28,79,91–95], whereas larger-sized EVs (such as microparticles) function predominantly in local intercellular communication[26,96,97]. The relative abundance of EV subtypes (and variations in cargoes) is selectively determined during biogenesis[24,26,98] and varies according to subtype and state or type (for example, differentiated cell type[99] or cell phenotype[100]) of the producing cell[5,97,101–103] as well as the source (cells or tissues)[4,93] (Table 1). Vesicular secretion of EV marker proteins in small EVs has been postulated to be dependent on an endocytosis-independent pathway, suggesting that EVs accumulate and are released along the plasma and endosome membranes[104]. Small EVs contain a core protein signature comprising highly expressed vesicular proteins commonly shared between EVs of diverse parent cell origin (such as tetraspanins CD63, CD9 or CD81 (refs. 28,102,104–106)).

The molecular heterogeneity of EVs is the source of much controversy concerning the properties of different EVs[89], such as whether specific types of EV are capable of cell-specific uptake and whether their associated nucleic acid complexes (such as microRNA (miRNA) biogenesis machinery and RNA-induced silencing complex[107]) are functional upon delivery[4,108]. Studies are continuing with the aim to better understand the biomolecular content of EVs (and their subpopulations), including diversity of cargoes, the reasons for limited expression of extracellular RNA and double-stranded DNA[89] as well as the fate of other EV-delivered biomolecules[109] following transfer[108,110].

Further to EV molecular and functional heterogeneity, EV isolation and analytical methods are also highly heterogeneous, with vastly different isolation workflows and sample preparation methods used for EV isolation and characterization in various studies. Substantial biological heterogeneity stems from the variable content of EVs secreted by a single cell type as well as differential packaging during biogenesis[89,104,111–113]. The cell-to-cell heterogeneity of tumour cells, which is evident at phenotypic and genetic levels, provides a further layer in the complexity affecting the development of clinical applications[114]. Tumour heterogeneity is probably a source of diversity in EV content, as demonstrated for various EV types[115–118]. At a single-EV level, heterogeneity might be attributed to differences in the chemical or physical traits ascribed to different types of EVs or within the same population, suggesting that all individual vesicles might not have all functional properties ascribed to a specific population. This subpopulation-level heterogeneity (for example, in EV secretion[119], the EV transcriptome or proteome[28,102,120,121], cell-surface expression profile[106] and biodistribution[79]) might have implications for the feasibility of both diagnostic and therapeutic clinical applications[122]. This heterogeneity in EV subpopulations might include differences in concentration and composition as well as variations in innate EV characteristics,

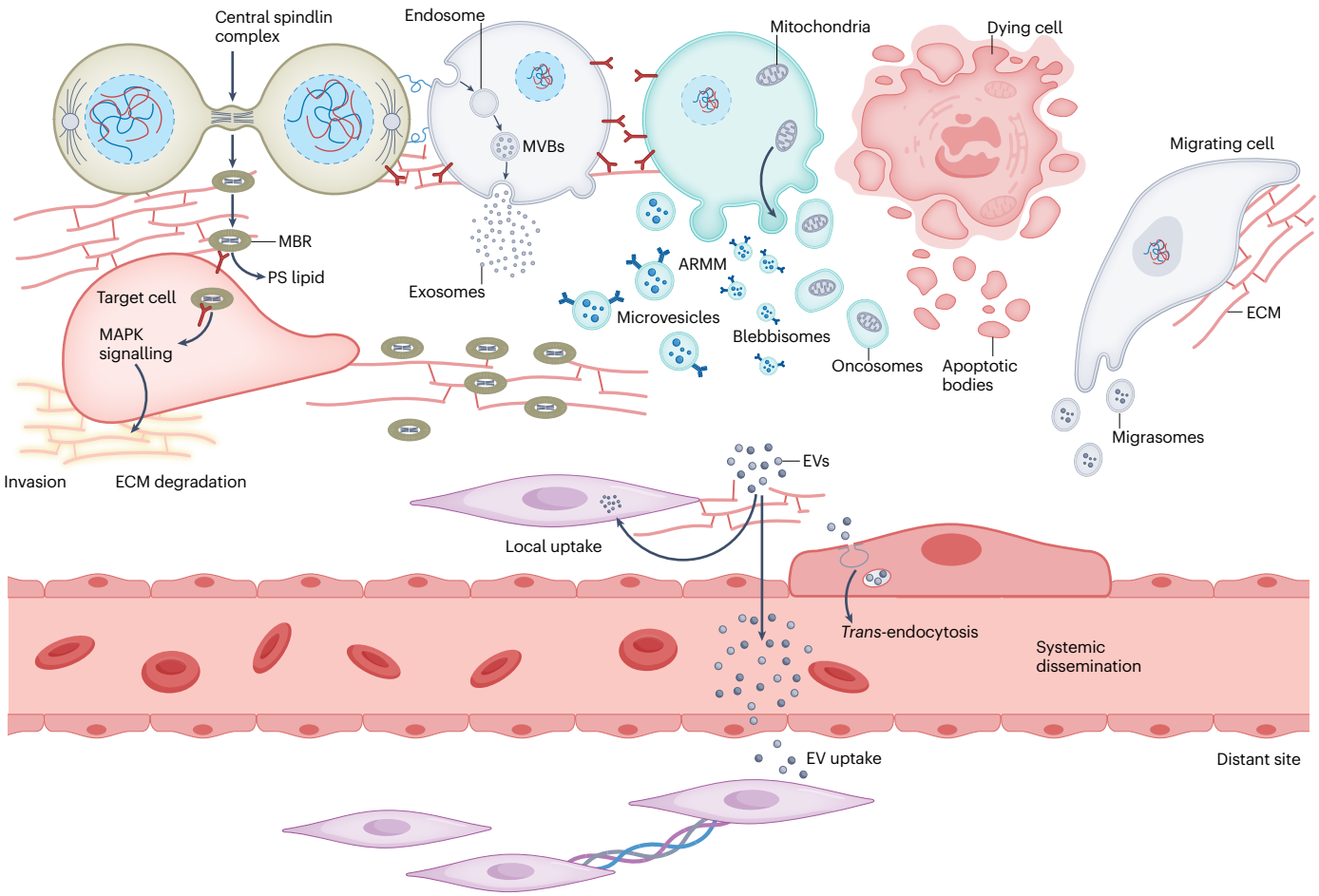

Fig. 2 | Extracellular vesicle biogenesis and heterogeneity. Extracellular vesicles (EVs) comprise a heterogeneous population of membrane-enclosed structures that are released into the extracellular environment. EV diversity arises from variations in their molecular composition, cells and/or tissues of origin as well as mechanisms of biogenesis. EV subtypes include exosomes, membrane-derived microvesicles (ectosomes), larger autophagosomes, secreted midbody remnants (MBRs) from dividing cells, released vesicles from migrating cells (migrasomes) and apoptotic bodies from dead or dying cells during apoptosis (Table 1). Endosomally derived exosomes are released through the maturation and exocytosis of multivesicular bodies (MVBs). Microvesicles, including ectosomes, are formed via plasma membrane blebbing at specific actin-rich domains. EVs can induce localized signalling via receptor–ligand interaction or can be internalized by endocytosis and/or phagocytosis or even fuse with the membranes of target cells to deliver their content into its cytosol, thereby altering the cellular machinery and modifying the physiological state of the recipient cell. EVs can mediate intercellular signalling through localized uptake to target cells or through *trans*-endocytosis to mediate systemic dissemination and clearance. ARMM, arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; PS, phosphatidylserine.

such as membrane structure, size and cargo and co-isolates, all of which might influence how EVs are isolated and processed for clinical applications[90,122,123]. Further implications in this heterogeneity include effects on the pharmacokinetic properties, distribution, cell-surface interactions and, importantly, therapeutic utility of EVs as well as affecting the approach to coverage analysis in biomarker discovery or diagnostic signatures (for example, the selection of affinity-based, global EV analysis versus analysis of predetermined mutational landscapes[115,124] and/or cancer-associated molecular cargoes[78]). Finally, research in this field requires higher standards of rigour and reproducibility to better understand, monitor and implement strategies to manage the challenges to clinical translation created by EV heterogeneity.

Onco-EVs and their oncogenic cargoes drive cancer progression

The role of EVs in cancer is highly dynamic as well as being specific to cancer type, genetic landscape, cancer stage and EV source (Fig. 3). The cargoes of cancer cell-derived EVs (subsequently referred to as onco-EVs) comprise diverse oncogenic molecules, including neoantigens and oncoproteins (such as MET, MIF, epidermal growth factor receptor (EGFR)vIII, CD147, PCA3, GPC1, PIGR or PD-L1)[10,125–127], peptides, fusion proteins[128] and various nucleic acids including tumour-specific genetic sequence alterations[129–131] as well as retrotransposon elements and amplified oncogene sequences[11,12,132] (Fig. 3). These EVs not only promote the progression of primary tumours but also support the enrichment of pre-metastatic niches that enhance metastasis[66,82,133]. Such cargoes can also influence various other cancer-associated functions including organotropism[80,95,134], cell invasiveness[127], metabolic reprogramming[135,136], regulation of apoptosis and gene expression[137], activation of receptor-induced intracellular signalling to regulate angiogenesis[138], macrophage[131,139,140] and fibroblast transformation[141–143], cell survival[144], cell migration and invasion[145,146], cell polarity and transformation[147] (including epithelial-to-mesenchymal transformation[148,149]), as well as immune cell modulation and immune

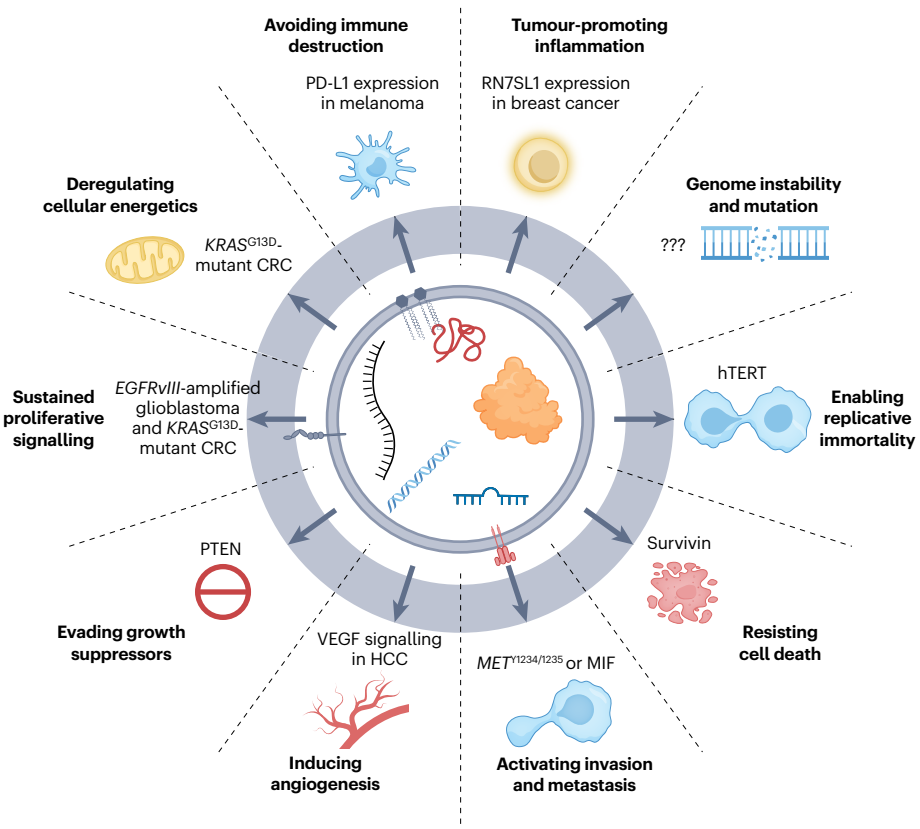

Fig. 3 | Cancer hallmarks of onco-extracellular vesicles. Cancer-derived extracellular vesicles (onco-EVs) are critical mediators of intercellular communication between tumour cells and stromal cells present in both local and distant microenvironments. Onco-EVs have a fundamental role in primary tumour growth and metastatic evolution, and orchestrate multiple systemic pathophysiological processes, such as coagulation, vascular leakiness and the reprogramming of stromal recipient cells to support pre-metastatic niche formation and/or metastatic dissemination. Onco-EVs carry various cancer-related cargoes including proteins, genes, peptides or lipids, which have important functional roles in intercellular crosstalk during tumour progression. Onco-EVs can orchestrate the hallmarks of cancer, such as avoidance of immune destruction, tumour-promoting inflammation, genome instability and mutation, enabling replicative immortality, resisting cell death, activating invasion and metastasis, inducing angiogenesis, drug resistance, evading growth suppressors, sustained proliferative signalling and deregulating cellular energetics. Moreover, an understanding of the synergy between onco-EVs and synthetic nanoparticles is emerging, whereby natural EVs can act as decoys for antibody-based therapeutics including binding with cancer therapeutics and shuttling them out of the tumour to the liver for destruction[228]. The biological molecules, mechanisms and effects listed are intended as illustrative examples of onco-EVs in the development and maintenance of the hallmarks of cancer. CRC, colorectal cancer; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; PTEN, phosphatase and tensin homologue; VEGF, vascular endothelial growth factor.

suppression[13,15,86,150–152]. Onco-EVs mediate these changes in cell transformation through various signalling pathways and networks including Wnt signalling[153,154], SMAD-dependent signalling or activation[155], activation of HER signalling pathways including active EGFR complexes and oncogenic EGFRvIII[156–160], VEGF[161], MET[66] and various other cell activation networks[141]. Other oncogenic cargo components such as mitochondrial DNA have been linked with cellular stress responses and activation of inflammatory responses as well as the innate immune system[142–146].

The potential of onco-EVs as a source of clinically relevant biomarkers enabling the early detection of cancer as well as those providing an accurate indication of prognosis is an area of considerable research interest[78,124,147–152] (Fig. 3). EVs are stabilized in the circulatory system by their protective lipid bilayer; this, in turn, allows quantification of circulating EVs and their cargoes, providing an indicator of overall tumour burden[124]. The heterogeneity of EV cargoes reflects the diversity of the cells of origin[89,121,153]. Carcinomas in situ release a diverse range of soluble factors (including oncoproteins) and EVs into the circulatory system before the release of circulating tumour cells (CTCs)[154]. These EVs, along with those derived from stromal and immune cells, can use both the lymphatic system and vasculature to colonize distant organs and awaken dormant disseminated tumour cells or facilitate microvascular hyperpermeability to facilitate organ-specific metastatic dissemination[67,69,81] (Fig. 1; described in detail elsewhere[155]). For example, miR-105 delivered by EVs from breast cancer cells dissociates vascular endothelial barriers in endothelial cell monolayers, facilitating the ability of CTCs to breach vascular barriers to entering the lung parenchyma by remodelling cellular adhesion networks[69]. Furthermore, select integrins on onco-EVs are preferentially internalized by lung-resident cells owing to the laminin-rich lung microenvironment, thus regulating the expression of metastasis-promoting factors, such as S100A4 (ref. 80). In 2024, the presence of onco-EVs containing S100A4 in peritoneal fluid was identified as a prognostic marker in patients with ovarian cancer, supporting a role of S100A4 in metastatic dissemination[148]. PD-L1^{+} onco-EVs have also been shown to absorb therapeutic anti-PD-L1 antibodies for subsequent transport to the liver for degradation, thereby acting as a decoy for systemic anti-PD-L1 antibodies in mouse models of colon and prostate cancers[156].

Encouragingly, DNA fragments and RNA species identified in preclinical models of metastatic prostate cancer reflect the genomic and transcriptomic features of the primary cancers and strongly correlate with matched patient biopsy samples and circulating tumour DNA (ctDNA) as well as being associated with disease progression[157]. Evidence is also emerging that these circulating EV cargoes are not mere bystanders, but rather active drivers of cancer evolution[17]. For example, oncogenic RNAs (and RNA fusions, such as repeat RNAs or DNA–RNA fusions) in circulating EVs can mediate induction of the G2/M checkpoint, mitotic spindle and DNA damage programmes, in addition to innate immune signalling and inflammation at distant sites[158]. Furthermore, surface-bound cytokines, RNAs and lipids on EV membranes have been shown to direct receptor–ligand interactions between tumour cells and distant organ microenvironments[159–165] and have a crucial role in preparing pre-metastatic niches in an organ-specific manner (described in detail elsewhere[166]). An even more complex interaction has emerged, whereby EVs are surrounded by a biomolecular corona consisting of components originating from the extracellular space or matrix[167], including other biological nanoparticles, such as lipoproteins[168]. The protein corona (a concept from therapeutic nanomedicine[169,170]) evolves on the surface of nanoscale materials when they are exposed to biological environments. This effect alters the physiochemical properties of the nanoparticle or EV and affects the behaviour of synthesized particulates in vivo, as well as subsequent interactions with biological systems including pharmacokinetic properties, cellular targeting, toxicities and immunoregulatory signalling as well as inducing errors in nano-based assays (described in detail elsewhere[167]). Researchers in the EV field are beginning to assess the specific effects of the biomolecular corona absorbed to the surfaces of a vesicle or particle, which is associated with the biological identity of EVs[171]. This corona is involved in complement activation, opsonization and regulation of phagocytosis, including how the corona influences transport and biodistribution[168,172], EV stability (for example, tissue protease resistance[173]) and uptake by target cells[172]. The EV surfaceome (the plasma membrane and surface-associated molecules of EVs) is recognized as a highly interactive and dynamic element that facilitates the interactions of EVs with the extracellular environment, target cell membranes and cell matrix[106]. Further understanding of the mechanical, electrostatic and molecular properties of the EV surface that underlies interactions between EVs and their targets[174] is warranted. Considering such interactions is important, given that the EV corona is a highly dynamic interactome with substantial potential as a source of biomarkers[175].

EV isolation and purification for clinical applications

Potential EV therapeutics

Despite the achievements of EV-based therapeutics in ongoing clinical trials[13], numerous technological challenges remain including scalability and yield, regulation and standardization. EVs have been isolated from various sources, including mammalian and prokaryotic cell cultures, blood plasma, blood cells, bovine milk and from plants. Various methods have been developed in an attempt to address the issues of low yield and thus improve the scalability of EV production, such as optimizing cell culture conditions, refining and modifying different cell types, use of bioreactors and enclosed cell systems and applying mechanical or chemical stimuli[176–180]. The use of plant and microalgae-derived systems for hybrid EV generation has several important advantages including limited immunogenicity, scalable generation and fewer ethical and regulatory concerns, compared with systems involving mammalian cells, implying that EVs generated using these methods are more likely to be safe in clinical settings[181].

Various diverse EV isolation strategies exist with variations in the optimal approach according to the type of biofluid, efficiency of isolation, differences in costs, sample volume and desired levels of purity[90,182,183]. Several workflows have been used for isolating EVs from a diverse range of cell types including differential centrifugation or ultracentrifugation, density-based ultracentrifugation, microfluidics, charge-based isolation, size-based isolation, peptide, protein or oligonucleotide-based immunoaffinity capture, tangential-flow filtration and array/capture platforms[184–188]. Given that EV subpopulations arise from distinct biogenesis pathways, and because their precise origins are difficult to ascertain in most scenarios, comprehensive characterization of the derived EVs is crucial. The heterogeneity of EVs encompasses a wide range of differences, including size, shape, composition and function[122]. The topology of EV surfaces includes various molecular features, such as proteins, polysaccharides and lipids, the composition of which varies depending on cell type and state, which provides a further layer of heterogeneity[106]. This heterogeneity poses challenges for EV identification and capture methods that

Box 1 | Extracellular vesicle-based therapeutics in clinical trials

Over the past decade, substantial developments in the field of extracellular vesicle (EV)-associated cancer therapeutics have occurred, progressing from promising preclinical findings to clinical trials involving patients with cancer. Several approaches are now gaining commercial interests as they enter phase I/II and randomized phase III trials as immunoregulation or tissue repair and regeneration strategies for various non-malignant indications (NCT05413148, NCT05354141, NCT04761562 and NCT05774509 (ref. 422)) described in detail elsewhere[65]. The initial groundbreaking application of EVs in antitumour immunotherapy[7] led to clinical trials in which autologous dendritic cell-derived EVs and modified variants (featuring modified tumour antigens) conferred immune responses to tumour antigens[219,220]. Since then, refinements in EV production and modification strategies have led to immunotherapies capable of inducing reductions in tumour size in various preclinical models[221–224] as well as additional early-phase trials attempting to leverage the immunomodulatory capabilities of such EVs in patients with cancer (NCT01550523, NCT01159288 and NCT02507583)[423–425]. The EV-based liquid biopsy assay ExosomeDx IntelliScore (EPI) test has been designated as an FDA Breakthrough Device for early risk stratification and biopsy-related decision-making in the management of patients with prostate cancer[216,347]. The use of EVs as antigen delivery vehicles is an emerging approach[221,426] and thus far the most successfully translated application[234,288,427–429]. Such studies have established the clinical feasibility and tolerability of EV vaccines; however, although clinical trials are mostly phase I/II, an unmet need exists to leverage the available knowledge as a foundation for investigating the feasibility and safety of EV-based vaccines. Synergy with other fields and advances in bioengineering and biotechnology have led to the development of bivalent and potent EV vaccines that have contributed to robust T_H1-type immune responses against COVID-19, as well as supporting the development of platforms such as StealthX that combine the advantages of mRNA and recombinant protein vaccines for potent and broader immunity. Such strategies hold immense potential to revolutionize vaccine development.

Despite such preclinical data and the conceptual appeal of cell-free production, the lack of clinical momentum supporting the development of EV-based therapeutics broadly reflects several ongoing scientific, translational and commercial challenges, including:

(i) The heterogeneity of cargoes among EVs derived from single-cell or multiple cellular sources as well as the reproducibility-related challenges associated with donor variation and manufacturing conditions;
(ii) An incomplete understanding of EV pharmacokinetics, including uptake kinetics, in vivo half-life and how cargo variability (RNA, protein and lipid) influences efficacy across different patients and disease settings;
(iii) Complexities associated with manufacturing scale-up and the challenges related to consistently generating clinical-grade EVs while maintaining potency and purity and refining the associated and universally accepted assays for regulatory approval;
(iv) The lack of defined regulatory frameworks for EV-based therapeutics, which creates ambiguity and might confer uncertainty;
(v) The limited high-quality clinical data showing robust efficacy and a lack of long-term safety data specifically in the field of oncology;
(vi) A lack of commercial interests and funding, with competing modalities (engineered proteins, mRNAs, chimeric antigen receptor T cells and novel small molecules) progressing rapidly through late-phase trials, thus focusing on industry attention and funding in other areas.

Translating the potential of EV-based therapeutics into clinically effective interventions will require standardized manufacturing, clear regulatory pathways, rational clinical trial design, universal assays, storage and stability metrics, protocols fulfilling good manufacturing practice criteria and implementation of cross-disciplinary expertise for such technologies and strategies for investment and translation.

involve surface engineering. Care needs to be taken that any EV surface modification strategies do not also have unintended effects such as altering the biophysical or biochemical characteristics of the EVs, their function and/or activity and other aspects of the co-isolates. Certain additives are often also required to stabilize EV content during isolation procedures (including RNAase-specific and/or DNAase-specific protease inhibitors) as well as broad recommendations on additives in formulations for short-term and extended storage[189–191].

Standardization, reporting and key metrics affecting EV research have been identified in an attempt to improve our understanding of clinical sample requirements. Biological reference materials now exist[192–194] for the purpose of comparing and/or standardizing EV purity — which is essential for interlaboratory comparisons. However, a lack of appropriate reference materials for assessing EV separation methods for biofluids continues to exist. With this issue in mind, snorkel-tag (a CD81 fusion protein designed to display a series of tags on the EV surface) was developed, providing potential benefit for use as a reference material for assessments of the functionality and cargoes of specific subsets of affinity-purified EVs. From a therapeutic perspective, EVs are regulated as drugs and biological products and, hence, will require transparent reporting on manufacturing and characterization, suitable quality control and to provide evidence of both safety and efficacy in one or more rationally designed clinical trials, ideally with long-term follow-up monitoring[178,195,196] (Box 1). EVs intended for clinical use can be derived from either autologous (self) cells[127] or standardized, generic sources[197,198]. The latter approach overcomes many of the challenges associated with autologous EVs in terms of the need to manufacture a custom product within a therapeutically relevant turnaround time, costs and scalability, as well as the potential to be derived from diverse cell sources while having a standardized, broad utility[199]. Although industrial-scale manufacturing pipelines have already been established in areas such as cell therapy, manufacturing clinical-grade EVs entails a unique set of considerations and challenges[65]. Currently, protocols fulfilling GMP criteria are sparse and regulators such as the FDA have yet to set guidelines for developing EV-based therapeutics. Furthermore, the development of therapeutic strategies for cancer-specific and EV-specific functions requires an important focus on how such products are isolated including selection

of the most appropriate source cells, ideally while also taking into account scalability, batch reproducibility, EV integrity and stability as well as safety and tolerability[65,178,200,201].

EV-based diagnostics

Key challenges in the field of circulating biomarker discovery include a lack of clinical relevance and translation of findings, as most current EV isolation methods are low throughput and not yet suitable for translation into high-throughput clinical assays[58,202]. Consistently detecting biomarkers in EVs presents difficulties given the often-low EV concentrations in samples from many patients, the need for highly sensitive detection technologies and the complexities of separating EVs from other proteins and non-vesicular components in biological fluids.

In a study published in 2020, investigators used ultracentrifugation workflows to define EVs and particles as diagnostic tools to analyse plasma samples and other bodily fluids from both patients and mouse models[78]. These findings provide potential markers for cancer detection, and determining cancer type (such as VCAN, TNC and THBS2) that distinguishes tumours from non-malignant tissues with 90% sensitivity and 94% specificity, and through predictive modelling, enables the classification of tumours of unknown primary origin[78]. However, such studies are limited in both the throughput and resolution of the isolation workflows, which enable only limited separation of vesicular and non-vesicular components, as well as a lack of cancer-specific isolation approaches. Novel tumour-specific EV isolation methods are urgently needed[203,204]. Various studies have explored approaches designed to extract cancer-specific EVs from plasma using a combination of isolation methods including size exclusion chromatography and immunoaffinity (including for proteoglycan CSPG4 (ref. 185) and TNC[205]) through to surfaceome capture and/or profiling of specific EV proteins from both malignant and non-malignant cells (cancer and non-neoplastic cell lines) and plasma samples from patients[204]. For example, *KRAS* mutations in total EVs were detected in 44.1% of patients with pancreatic cancer of various stages receiving active therapy, with this percentage increasing to 73.0% following EV surface capture. Such EV-based enrichment pipelines also allow for additional molecular profiling of EV cargoes, for example, to identify known driver mutations (such as those in *BRCA2*) and to further our understanding of mechanisms of therapeutic resistance[204]. By applying EV-based capture strategies to circulating EVs (for example, the use of different antibody cocktails, such as monoclonal antibody 763.74, which is specific for the CSPG4 epitope), such assays have consistently shown higher yields when applied to circulating marker detection compared with single-antibody isolation platforms[185,205].

However, isolation systems involving several antibodies are not scalable owing to the need for multiple isolation and incubation procedures and are thus limited in the extent of their capture efficiency and sensitivity. Developments in antibody target–capture optimization and use of microfluidic conjugate systems can facilitate highly sensitive isolation from a limited volume of sample[184,206,207]. For example, a highly sensitive EV-capture chip (^{EV}HB-Chip) has been shown to isolate tumour-specific EV-RNAs from plasma within 3 h at 94% tumour-EV specificity and a 10-fold increase in tumour RNA enrichment in comparison to other methods (using either direct immunoaffinity isolation with cetuximab or ultracentrifugation to capture tumour EVs)[184,208]. Dual isolation of CTCs and EVs from the same source using immunoaffinity-based microfluidic interfaces has also been demonstrated using the cancer-specific antibodies MCAM and MCSP. Molecular profiling reveals that both antibodies can isolate EVs expressing melanoma-associated cell-surface proteins[206]. Importantly, this dual isolation, high-throughput approach was able to differentiate patients with melanoma, with up to eightfold higher levels of CTCs and a fourfold increase in EV protein concentrations compared with individuals without cancer as well as having the potential to provide clinically relevant information on disease progression and/or the efficacy of ongoing treatment[209]. The use of alternating current electrokinetics has been highlighted as an EV-based method for rapid and accurate early detection of pancreatic, ovarian or bladder cancers[210]. This lab-on-a-chip, scalable platform has the potential to be integrated into high-throughput, automated systems.

Various EV isolation strategies exist, including advanced sorting strategies[211] and affinity enrichment approaches[212], although these come with certain challenges including the need for extensive processing, co-isolation of similar-sized protein aggregates, a lack of biomolecular confirmation of EV identity, limited resolution for certain types of EVs and the co-isolation of non-vesicle, free proteins. Advances in EV multiplexing tools have led to new fundamental insights into EV biology and heterogeneity as well as the potential for increasing diagnostic specificity[124,150,213]. For example, researchers developed a multichannel, fluorescence-based technique capable of profiling ~15 different EV markers, including core EV markers, and oncogene and tumour suppressor proteins to a near single-EV level[213]. Such technologies highlight the clinical feasibility of rapid, multiplexed and highly sensitive EV-based analysis of cancer biomarkers that could potentially be extended to additional EV subtypes (including single EVs and mutated protein analyses[124,212]). Developments in nanotechnology platforms might also support the clinical implementation of EV-biomarker assays. For example, templated plasmonics for exosomes (TPEX) technology[214] enables rapid (1 µl of sample in 15 min) and multiplexed analysis of EV protein targets (such as CD63, CD24, EpCAM and MUC1) with high levels of performance. Importantly, this technology could differentiate additional clinical characteristics (such as prognosis) over conventional singleplex enzyme-linked immunosorbent assay based on a TPEX signature with an improved sensitivity of >10^3-fold and an area under the curve (AUC) of 0.97 for colorectal and gastric cancers, relative to 0.76 for analyses of total target proteins[214]. These developments highlight the emergence of multiparametric EV diagnostic platforms capable of establishing distinct panel signatures based on the ability to simultaneously detect and quantify several EV biomarkers and validated in large-scale clinical studies[207,210,215,216].

Engineering EVs for cancer therapy

Considerable research interest exists in the development of EVs as cancer therapies. This interest has involved the direct targeting of cancer cells using EVs with antitumour payloads[217], as well as various less-direct approaches including: modulating innate immunity[7,218–224]; targeting immune checkpoints[225,226]; targeting specific cellular phenotypes (such as differentiation or cell quiescence[217,227]); interactions with other particles, including therapeutic nanoparticles[228]; and to enhance the effectiveness of immunotherapies, especially as antigen-carrying vehicles[226,229–234] in preclinical models (Fig. 4a–c). Selective depletion of circulating onco-EVs (or inhibition of secretion of onco-EVs) is another example of a preclinically effective anticancer strategy[235,236]. Novel therapeutic approaches include the use of nanoparticles equipped with EGFR-targeting aptamers, which are able to redirect fluorescence-labelled EGFR-expressing exosomes derived from a non-small-cell lung cancer (NSCLC) cell line towards the gastrointestinal tract, followed by excretion with a concomitant reduction in

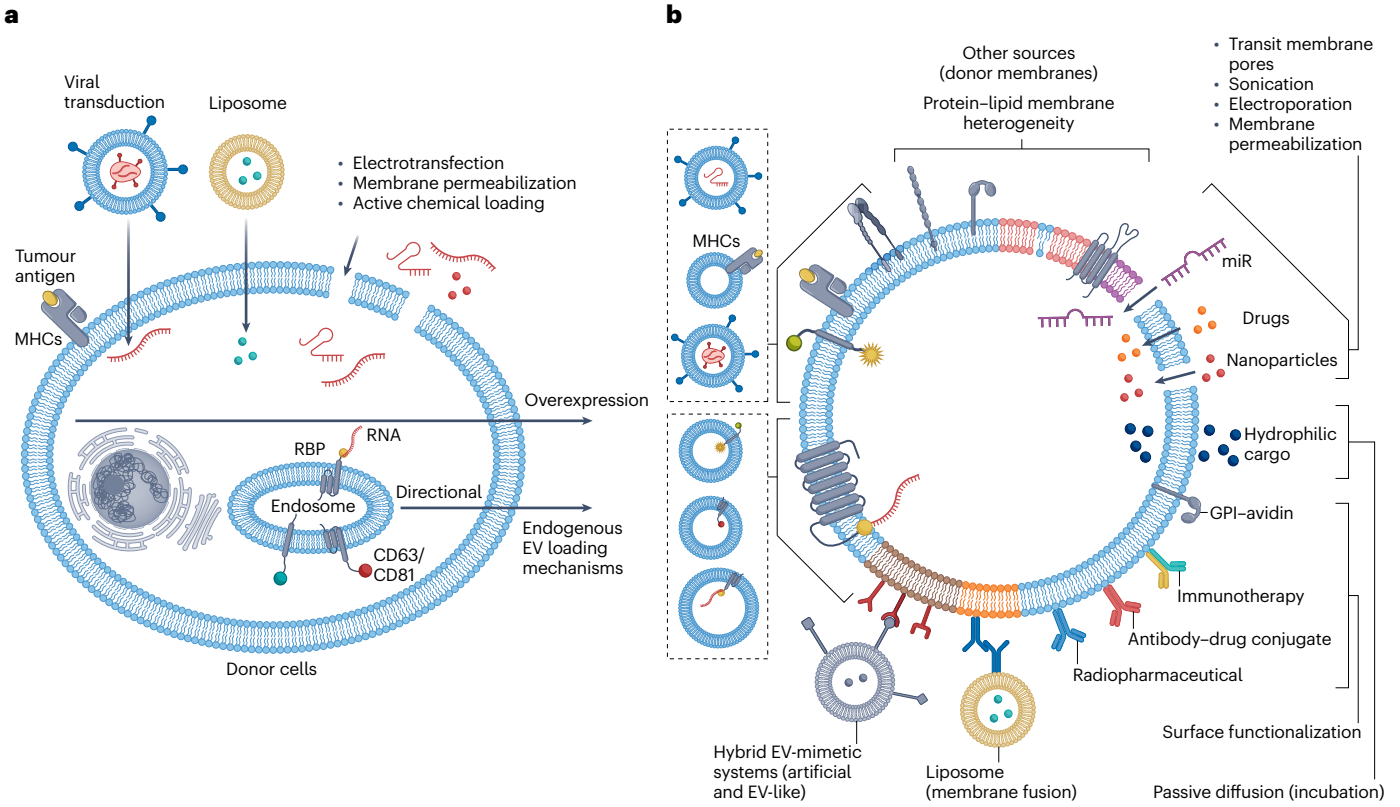

Fig. 4 | Engineering extracellular vesicles for cancer-targeted therapy. **a**, A diverse range of extracellular vesicle (EV) engineering strategies have been developed for the preparation of therapeutic EVs. Genetic engineering approaches have been applied to regulate the donor cell sources of therapeutic EVs. These approaches include using an expression vector to deliver genes encoding the desired cargo into the genetic material of the host cell and to enable the production of EVs with various delivery proteins expressed on the membranes and/or specific luminal cargoes. These vectors can include plasmids, retroviruses, lentiviruses, adenoviruses and adeno-associated viruses. **b**, Direct cargo loading strategies include modifications of isolated EVs using chemical, physical and/or mechanical approaches, including click chemistry, cloaking, biocoupling, ultrasonography, extrusion and electroporation. Other loading strategies include chemical transfection and co-incubation, which is reliant on passive diffusion. The surfaces of EVs can be decorated with targeting molecules using aptamers, click chemistry and covalent binding interactions, enzymatic conjugation, ligand–receptor binding or membrane fusion for more specific delivery to targeted cells. **c**, EVs can be modified using various innovative bioengineering strategies to enhance potency including via refined and more selective cargo-loading approaches, modulating donor cells to alter EV secretion, conferring responsiveness to stimuli (including those present within the tumour microenvironment), encapsulating anticancer drugs for delivery, delivery and/or display of tumour-specific antigens to directly engage the immune response (immunomodulation) and improved tumour targeting, and altered pharmacokinetic properties including extended circulation half-life, intracellular uptake, lysosomal escape and enhanced tumour penetrance. EVs have been further engineered to display tumour-specific antigens and engage therapeutic immunization, as anticancer vaccines. CPP, cell-penetrating peptide; DC, dendritic cell; GPI, glycosylphosphatidylinositol; RBP, RNA-binding protein; VSVG, vesicular stomatitis virus G glycoprotein.

the number of metastatic lung nodules in mouse xenograft models[235]. As drug delivery modalities, EVs have advantages over other delivery systems including their high levels of biocompatibility with limited immunogenicity, stability against degradation and the ability to cross tissue barriers[65,84,115,237,238]. EVs can also be modified using various bioengineering strategies to refine their luminal and surface cargo

(such as to improve their tumour targeting[239] and display tumour-specific antigens to directly engage the immune response[240]) and to modulate secretion and/or production (to enhance yield)[241]. These EV engineering approaches can confer altered pharmacokinetic properties such as extended circulation time, intracellular uptake, lysosomal escape and enhanced penetration retention effect[65,84,242,243] (Fig. 4c).

EV bioengineering strategies include cellular nanoporation[237], electroporation[115,244,245], electrotransfection[246], membrane permeabilization[247], genetic engineering (for example, modified donor cells[127,146], scaffold proteins for cargo loading[18] or cell-penetrating peptides[18]), endogenous EV loading mechanisms[248], extrusion[242], metabolic and chemical engineering (such as incorporation of ligands[115,127,249,250] or therapeutic molecules[251]) and physical engineering approaches (such as membrane fusion[252] and membrane coating[253,254]) by using cross-disciplinary innovations including aptamer technology and click chemistry (Fig. 4a,b). Other approaches include the development of hybrid EV-mimetic systems that combine EVs with synthetic nanoparticles. Such hybrid vesicles have an enhanced cargo-carrying capacity while also retaining the biocompatibility and targeting abilities of natural EVs[225,255–257]. These various approaches have the potential to pave the way towards more effective EV-based diagnostic and/or therapeutic applications[258] (Table 2 and Fig. 4).

Cargo loading strategies

Numerous strategies have been developed for loading of EVs with a desired specific cargo or 'payload'. Data from several preclinical studies demonstrate that EVs engineered to carry small-molecule chemotherapeutic drugs[259] are able to suppress tumour growth[256,260]. Furthermore, EV-based delivery vehicles incorporating antisense oligonucleotides[250,261], CRISPR–Cas9 (refs. 262–266), mRNAs or miRNAs[267–269] and small interfering RNAs (siRNAs) targeting oncogenes and various oncoproteins[81,115,270,271] have been used to elicit direct regulation of tumorigenesis in various preclinical models. Interestingly, EVs from a mouse pancreatic cancer cell line have been developed

Table 2 | Engineered extracellular vesicles as next-generation delivery platforms for cancer therapeutics

EV design and targeting	Outcomes	Ref.
Natural EVs		
EVs from human or mouse DCs exposed in vitro to tumour antigens for in vivo priming of T cell precursors	60% of tumour bearing xenograft mice were tumour-free at 60 days vs 0% of controls	7
EVs derived from various cancer cell lines were used to prime DCs for the presentation of cancer antigens for in vivo priming of T cell precursors	Immunization before injection of cancer cell lines significantly impaired tumour development in both syngeneic and allogeneic mouse models	10
Cell-based engineered EVs		
A DC-EV cancer vaccine expressing pMHC-I, anti-PD-1 antibody and B7 was engineered as a personalized cancer immunotherapy strategy to directly activate both native and exhausted T cells	Elicited 80% tumour regression in an LLC model, reflecting a robust $CD8^+$ T cell response owing to proficient neoantigen presentation	411
EVs derived from M1 macrophages were engineered to express intracellular catalases to relieve hypoxia as well as an anti-PD-L1 nanobody and loaded with the DDR inhibitor nedisertib	Synergized with radiotherapy to reduce tumour growth markedly in mouse xenograft models, resulting in 100% survival in mice receiving the fully functional vaccine	412
CAR T cell-derived EVs carrying peptide antigen and RNAs encoding RN7SL1 selectively accumulate in endogenous intratumoural immune cells	Expansion of effector-memory and tumour-specific T cells, leading to tumour rejection in mouse xenograft models	413
EVs secreted from fibroblast-like mesenchymal cells loaded with siRNAs or shRNAs targeting $KRAS^{G12D}$ with enhanced retention in systemic circulation relative to liposomes, probably owing to CD47-mediated protection	Reduced tumour size in orthotopic mouse xenograft models of $KRAS^{G12D}$-mutant PDAC compared with siRNA-loaded or shRNA-loaded liposome controls	115
EVs derived from Expi293F cells engineered with cell-surface antibodies against CD3, EGFR, PD-1 and OX40L	Induced antitumour immunity with robust reductions in tumour size in mouse xenograft models of breast cancer	226
Post-isolation EV engineering		
HEK293 T cell-derived EVs loaded with siRNAs targeting survivin and coated with RNA-aptamer PSMA, EGFR or the folate receptor ligand 3WJ-cholesterol	Demonstrated tumour regression in mouse orthotopic xenograft models of various solid tumours	414
Hybrids		
Grapefruit-derived nanovesicles decorated with activated leukocyte cell membranes and loaded with doxorubicin	Demonstrated the ability to home to inflammatory tumour tissues with reductions in tumour growth and improved survival in mouse xenograft models	415
Artificial nanovesicles		
EV-like nanovesicles derived from neutrophils and decorated with SPIONs via transferrin receptor interactions and loaded with 5-fluorouracil	Enables the efficient and safe drug delivery with tumour shrinkage in mouse xenograft models	416

CAR, chimeric antigen receptor; DC, dendritic cell; DDR, DNA damage repair; EGFR, epidermal growth factor receptor; EV, extracellular vesicle; HEK, human embryonic kidney; LLC, Lewis lung carcinoma; PDAC, pancreatic ductal adenocarcinoma; pMHC-I, peptide-bound MHC class 1 antigen; PSMA, prostate-specific membrane antigen; shRNA, short hairpin RNA; siRNA, small interfering RNA; SPION, superparamagnetic iron oxide nanoparticle.

for targeted delivery of a CRISPR–Cas9 system designed to suppress *KRAS*^{G12D} (ref. 272), with a similar system developed for the suppression of PARP1 expression in mouse models of ovarian cancer[263]. Several versatile platforms have been developed for EV-based delivery of CRISPR–Cas9 genome editing components alongside single-stranded DNA, single-guide RNA and siRNA to specific cells and tissues[264]. These strategies have certain advantages such as their greater stability in circulation, enabling long distance transmission and reduced immunogenicity compared with conventional synthetic lipid nanoparticles. These cargo loading platforms signal key therapeutic developments for EV-based gene editing applications.

Several strategies exist to load therapeutic drug cargoes into EVs. These include electroporation, EV–liposome hybrids, genetic modification and chemical modification approaches, such as introducing surface ligands[250], to functionalize EVs with topologically diverse macromolecules through luminal loading[261] (including cytokines, antibody fragments, RNA binding proteins, antigens, Cas9 and members of the tumour necrosis factor superfamily)[248]. Indeed, CD47-modified EVs loaded with short hairpin (sh) or siRNA-*KRAS*^{G12D} (including shRNA or siRNA targeting *KRAS*^{G12D}) have been shown to be more potent in inhibiting tumour progression compared with lipid nanoparticles loaded with the same cargo[115].

A popular strategy for cargo loading involves passive diffusion of hydrophilic cargoes into EVs through the lipid bilayer. To overcome challenges in passive diffusion of hydrophilic cargo in EVs (owing to the need to cross highly hydrophobic cell membranes), various alternative loading strategies have been developed. For protein-based loading approaches, these include ubiquitination[273], light or chemical-induced protein–protein interactions[274] or protein-cleavable systems[243] (Fig. 4a,b). Such strategies involve biological modifications of the source cells to promote the in situ encapsulation of RNA cargoes into EVs[54,146]. For example, exosome-total-isolation-chip (EXOtic) devices create functionally modified EV-producing cells in vivo, enabling constitutive in situ production and delivery of mRNA-containing EVs[146]. An alternative approach for targeted transcriptional manipulation and therapy involves the insertion of large mRNA molecules directly into cells via nanoporation to produce EVs containing high quantities of specific mRNAs (encoding PTEN)[237]. These modified EVs can also be engineered to display targeting peptides (such as CDX and CREKA) fused with CD47 via the N-terminus, which is typically localized on the external EV membrane surface[237]; this high-yield approach has been shown to increase the in vivo circulatory half-life of the modified EVs with no short-term effect on CD47 function (either in vivo toxicities or immunogenicity in mouse models) relative to EVs loaded via electroporation compared with non-targeted EVs and liposomes. Elsewhere, an 18-amino acid exosome-binding peptide sequence was identified through structure–function deletion analysis of Wnt proteins and was found to mediate localization to the EV surface[275]. Notably, linking of the exosome-binding peptide sequence to other proteins also resulted in their localization to the EV surface, creating opportunities for innovative therapeutic targeting strategies and systemic protein delivery[275]. Electroporation strategies designed to directly load EVs with antitumour miRNAs[276], enzymes and/or immunomodulators (such as immunogenic cell death inducers such as human neutrophil elastase (ELANE) and Toll-like receptor agonists (hiltonol)) have been shown to result in a higher vesicle loading capacity[277]. These latter EVs have also been demonstrated to form a long-term, stable in situ cancer vaccine capable of activating dendritic cells and resulting in select T cell responses in an in vivo model of triple-negative breast cancer[277].

The bioavailability of the cargoes in target cells is an important consideration for EV cargo selection and loading. In this regard, the engineered EV system, exosomes for protein loading via optically reversible protein–protein interactions (EXPLORs), has been developed. This strategy is based on a reversible protein–protein interaction module[274,278,279] that enables the in situ production of regulatory EV delivery systems in live cells (acting as a live cell-based EV cargo loading factory)[274]. By leveraging the innate machinery of the cell to load therapeutic agents into EVs, this approach overcomes the limitations associated with exogenous loading methods, such as cytoplasmic loading and delivery of soluble proteins, as well as the often limited encapsulation efficiency of such methods, in addition to providing an inherently biocompatible autologous source of EVs that is less likely to trigger an immune response compared with allogeneic EVs or synthetic nanoparticles[280]. Similarly, another system using the conjugation of rapamycin-sensitive fusion domains to specific target proteins has been shown to promote the incorporation of such proteins into EVs in the presence of rapamycin[281]. This fusion cargo loading approach packaged Cas9 proteins plus single-guide RNAs into EVs to induce therapeutic exon skipping in reporter mice and cells[282]. Further systems designed to incorporate target proteins into EVs include a ubiquitin-based technique, which involves Nedd4-mediated ubiquitination of the target protein, which confers subsequent preferential loading into EVs[273].

However, vesicle loading strategies involving electroporation can induce concomitant siRNA aggregation and thus impair EV pharmacokinetics, including cellular uptake, tissue permeability and in vivo biodistribution[283]. Other direct approaches that can be utilized in various cell types include active chemical loading (such as the use of saponin surfactants[284]) and cellular-nanoparticle biochips (such as prepolymer moulds or predesigned microfluidic channels combined with electronic apertures)[285] or silicon nanochannel array devices modified to generate nanopores[237], which enable the production of EVs containing therapeutic nucleotides. This nanoporation process has been reported to enhance the endosomal secretion of EVs containing bioactive RNA transcripts, with mRNA cargoes resulting in restoration of tumour-suppressive functionality in orthotopic in vivo models[237].

The optimization of EV loading strategies for selective delivery to the lumen and/or surfaces of EVs offers the potential for the development of therapeutic applications, although several key challenges need to be considered (described in detail elsewhere[65,84,122]). These approaches include modifying EV cargoes to account for the effects of the cargo on donor cells (including implications for phenotype and viability) or the pharmacokinetic properties of the derived EVs (such as stability or bioavailability), the efficiency of encapsulation, extent of biocompatibility and programmability, the molecular orientation of the cargo and ultimately therapeutic activity[286].

Tuning donor cell sources for therapeutic EVs

Ensuring efficient loading of the therapeutic cargo into EVs is a key consideration for engineering of therapeutic EVs, regardless of the primary source of the EVs (Table 2). To this end, researchers are now considering modifications of proteins with a role in EV biogenesis[261,287,288]. Specific therapeutic cargoes can be expressed as fusions with EV sorting proteins (such as LAMP2B, PTGFRN, CD63 and CD81) in donor cells, thereby promoting endogenous EV cargo loading and overcoming challenges relating to the efficiency of EV cargo loading associated with non-selective genetic strategies[248,289] (Fig. 4a). Other methods for loading therapeutic RNAs into EVs include Targeted And Modular

EV Loading (TAMEL)[290] and EXOmotif recognition sequences[291]. These approaches are based on the existence of several RNA sequence motifs that are thought to enrich specific RNA species in EVs, including in a cell-type-specific manner. Two examples are provided by the RNA-binding proteins Alyref and Fus (which are involved in the export of cell-type-specific miRNAs) incorporating the EXOmotif CGGGAG[292]; this sequence links the type of EV miRNAs with their tissue of origin and modifying miRNAs to include these trafficking and targeting sequences improves the extent of miRNA uptake by small EVs[292]. Various proteins (associated with RNA-binding proteins) have been shown to mediate the loading of miRNAs into EVs, including various heterogeneous nuclear ribonucleoproteins (such as USP7-mediated loading of miR-522 (ref. 293)) and Y-box-binding protein-1 (YBX-1)[294].

EVs can also be engineered to display therapeutically active specific protein receptors or surface molecules, which are also considered to be therapeutic cargoes including surface signalling factors such as antibodies and peptides, immune cell receptors and lipids capable of cellular priming and promoting antitumour immunity as well as stabilizing surface molecular cargoes that extends the circulation half-life and limits the extent of protein turnover and membrane recycling. For example, glycosylphosphatidylinositol (GPI), platelet-derived growth factor receptors and lactadherin (via the C1 or C2 domain) can all localize to EV membranes and can be modified to display specific proteins of interest on the EV surface[295,296]. Donor cells can also be genetically engineered to promote the expression of oligomerized EV sorting domains as well as N-terminal fragments of syntenin[297], resulting in the display of specific cytokine decoys on the EV surface. This strategy provides a highly effective method of functionally ameliorating the inflammatory phenotype in mouse models of systemic or more-localized inflammation compared with clinically approved agents targeting these pathways, such as tocilizumab and etanercept[297]. The ability to display different receptor molecules simultaneously in multimeric form, thereby enhancing EV inhibitory activity, has led to advances in the development of engineered, combinatorial EV-based surface-displaying protein therapeutics and, potentially, the ability to target specific receptor ligands. Such strategies are being developed to engineer artificial nanovesicles that retain the membrane features, structure and surface antigens of the donor cells of origin[298–300] (an approach known as biointerfacing[301]). These EV-based cancer therapeutics can be engineered to incorporate variants of signalling proteins to extend circulation time and target specific cell membranes for tumour therapy[258]. Such hybrid EVs are typically synthesized using liposomes (to provide an underlying structure for the artificial EV) and the membranes and luminal contents of native EVs (to incorporate both luminal and surface contents) and thus create EV-like hybrids[302] (described in detail elsewhere[303]). These strategies can be adapted to engineer various EV membranes and core content features (such as proteins, lipids or RNAs) while systematically varying certain properties of the EV membrane, such as particle size and heterogeneity[304]. The scalability of these methods is a salient advantage that can overcome the inefficiencies and low yields associated with obtaining native EVs from cultured cells.

Alternative donor source systems are now being considered for EV-based cancer therapeutic applications, including plant cells, which have the inherent advantages of scalable generation, high yields[305] and limited toxicities[306]. As an antitumour therapeutic strategy, a pipeline enabling the delivery of miRNAs for cancer therapy using a selection of EVs derived from different plant cell sources was developed, resulting in a hybrid, EV-polymeric system combined with a dendrimer (micelle nanoparticle) bound to an miR-146 mimic[268]. This loading strategy resulted in a fivefold increase in miRNA uptake compared with either component alone and demonstrated successful cargo delivery as well as antitumour activity in mouse xenograft models of ovarian cancer[268]. The extent of cellular internalization of plant-derived hybrid EVs is reportedly similar to that of extant mammalian-derived EVs[307].

Engineering EVs for targeting and extended circulation

Therapeutic anticancer EVs provide an opportunity to selectively target cancer cells and avoid systemic clearance. In this regard, EV surface proteins have an important role[106,308], which includes regulating the interactions of native EVs with their extracellular environment including target cells and the extracellular matrix[309,310], thus determining biodistribution[80] and influencing various pharmacokinetic aspects including circulation half-life[310,311]. For these reasons, EV surface molecules are being investigated for targeted EV enrichment and capture strategies[184,185] including applications involving aptamer technology[312]. EV-based engineering strategies have focused on EV surface proteins to enhance EV targeting[251,254,313,314], expression of specific tumour antigens for immune recognition[233,245], to modify and extend circulation time[315], facilitate intracellular delivery and promote tumour penetrance and accumulation[316] (Fig. 4c).

For example, modifying EV membranes with the targeting heptapeptide PTHTRWA has been shown to enable selective interactions with lung cancer cells via α5β1 integrins in both cell lines and mouse xenograft models[254]. This functionalization process used click chemistry to conjugate the heptapeptides to the EV surface via the N or C termini, with activity of the modified EV assessed using an in silico model of lipid membrane dynamics. Importantly, modelling data suggest that orientation of this EV–heptapeptide conjugate directly regulates the targeting capacity and binding affinity for the α5β1 receptor and therefore the ability to transcytose the cancer cell membrane[254]. Elsewhere, investigators engineered EVs combining the integrin α5-targeting peptide RYYRITY, the antifibrotic agent pirfenidone and miR-138-5p and demonstrated that this combination perturbs tumour growth, progression and metastatic dissemination in mouse models of pancreatic ductal adenocarcinoma (PDAC) owing to reprogramming of the cancer-associated fibroblast phenotype and remodelling of the extracellular matrix composition[317]. These findings highlight the inhibitory effects of modified EVs in a multicellular tumour model that preserves both tumour heterogeneity and the stromal microenvironment, with evidence of an ability to reprogramme cancer-associated fibroblasts within the TME.

Recent biomimetic design strategies that use different cell types and engineered targeting features have been developed to enhance cellular uptake and overcome rapid clearance mediated by the mononuclear phagocyte system. A key example of this dual strategy (mononuclear phagocyte system escape and targetable delivery) was shown, in which EVs derived from DC2.4 cells with high levels of tropism for macrophages and therefore a propensity for mononuclear phagocytosis were functionalized using cationized mannan in an attempt to presaturate this 'eat me' signal[315]. Subsequently, EVs obtained from human serum and enriched for CD47, reflecting a 'don't eat me' strategy, were combined with a polyethylene glycol nanocarrier functionalized with an integrin-binding complex[315]. The resulting EV hybrid was less vulnerable to macrophage endocytosis and had a prolonged circulation time with increased target tissue accumulation in a mouse xenograft model of NSCLC[315]. As well as reducing systemic clearance and improving the circulation time in blood compared with non-CD47-enriched EVs, these hybrid vesicles retained their targeting ability, avoiding the

challenges associated with protein corona formation seen with various other nanomedicines[318].

EVs can be engineered with nucleus-targeting transactivator of transcription (TAT) peptides (V2C–TAT) plus RGD peptide target sequences to refine their intracellular delivery. TAT is a highly cationic 11-amino acid cell-penetrating peptide sequence (YGRKKRRQRRR), whereas RGD targets the vasculature and cell membrane via interactions with integrin receptors[319]. This dual V2C–TAT plus RGD peptide combination served as the therapeutic core nanoparticle, resulting in engineered EVs that act as vectors for cell and nuclear delivery, combined with photothermal therapy, with potent antitumour activity in vivo[319]. Various cell-penetrating polypeptides (such as R9 or synthetic arginine-rich peptides) have been engineered for presentation on EV surface membranes, resulting in improved tumour penetration, cellular delivery and intracellular release[316]. These modified EVs demonstrated more efficient cargo delivery owing to the ability to engage rapid clathrin-mediated endocytosis with selective suppression of BCL-2 mRNA in HepG2 cells in vitro[316]. Together, these approaches appear to overcome the challenges associated with the use of antisense oligonucleotides administered without a delivery system, including their negative charge and inability to cross cell membranes as well as rapid enzymatic degradation.

In an attempt to enhance therapeutic versatility and antigen recognition, EVs have been modified with antibody-binding moieties selected using a domain-binding screen designed to select for specificity for the Fc domains of therapeutic antibodies[251] (such as those targeting HER2 or PD-L1). Importantly, such modified EVs could potentially be adapted to display other antibodies or Fc-fused proteins, including bispecific antibodies and antibody–drug conjugates (Fig. 4b,c).

In an attempt to better understand the systemic communication networks of EVs, investigators developed a tagging reporter system enabling tissue-specific and cell-type-specific in vivo tracking of $CD63^+$ EVs[141]. This genetic modification approach (termed ExoBow[141]) enables comprehensive in vivo tracking of the spatiotemporal distribution of EVs to dissect their origin and distribution and has been applied in mouse models of PDAC. Other applications of this EV labelling approach include investigating the routes of communication between different EV subpopulations of the same cell of origin[87]. Knowledge of such processes might enable the development of EV-based therapeutics capable of addressing intratumour heterogeneity[320].

An alternative strategy for avoiding phagocytosis and enabling targeted cargo delivery involves incorporating biological membranes (such as those containing specific antigens or receptors, including CD47 and various adhesion proteins) from different cellular sources including red blood cells or cancer cells into synthetic phospholipid bilayers surrounding the EV[321]. Such bimodal-engineered EVs can be equipped with the ability to evade phagocytosis (similar to that of red blood cells), as well as the homologous targeting ability of cancer cells, enabling improved tumour-specific accumulation[321].

Engineering EVs for immunotherapy and cancer vaccines

Various engineered EVs have been designed to enhance their antitumour therapeutic delivery, penetrance[322] and immunoregulation[323,324]. Most studies test these agents in preclinical models with the precise effects in patients with cancer remaining largely unexplored[325].

Various bioengineering approaches have been applied to the development of artificial-EV-mimetic nanoparticles, hybrid-EV-mimetic nanovesicles and EV-camouflaged nanoparticles[326–328]. To simultaneously boost intratumoural immune infiltration and immune activation, photoactivatable silencing EVs have been generated as EV-camouflaged nanoparticles[323]. This approach localizes selective activation of therapies in proximity to the target site and thus minimizes systemic exposure, which might also reduce the risk of toxicities. In one study, investigators engineered a photoactivatable reactive oxygen species-sensitive nanocomplex core loaded with siRNAs targeting PAK4 followed by cloaking with EVs derived from M1 macrophages[323]. These photoactivatable silencing EVs had an extended circulation half-life (~4 h) and improved tumour accumulation compared with nanoparticles coated with M0-macrophage-derived EVs[323]. Furthermore, reactive oxygen species-responsive intracellular release of siPAK4 via 660 nm laser irradiation not only silenced PAK4 but also modulated the TME in mouse models of melanoma and colorectal cancer[323]. Such hybrid EV systems provide a novel approach that has potential for the selective delivery of therapeutic cargoes to tumours.

Current clinical studies testing cancer vaccines are challenging and have mostly failed to provide substantial improvements in clinical outcomes, especially in patients with advanced-stage disease. For example, the first FDA-approved autologous DC-based therapeutic cancer vaccine (Sipulecel-T) provided only a modest survival benefit in patients with metastatic castration-resistant prostate cancer compared with other therapies that became available around the same time, such as enzalutamide[329]. Importantly, innovative strategies and advances in technology might overcome these challenges and broaden the opportunities for clinical application[57]. A personalized EV-based engineered therapeutic cancer vaccine has been developed using these advances[233]. Here, modified EVs (termed IL2-ep13nsEV) from autologous dendritic cells (DCs) were pulsed with tumour cell lysates for enrichment of MHC-bound antigens inherited from primed DCs and T cell-promoting costimulatory factors at the EV surface and engineered to express membrane-bound IL-2 (ref. 233). This approach enabled the EVs to present antigens and thus promote cancer cell-specific T cell-mediated cytotoxicity in syngeneic mouse models and patient-derived xenograft (PDX) models of breast cancer[233] (Fig. 4c). The presence of surface-bound IL-2 supported the latter function by enabling these modified DC-EVs to migrate towards lymphoid organs and to activate IL-2 signalling on lymphocytes[233] (described in detail elsewhere[15]). This EV-based personalized therapeutic cancer vaccine strategy induced robust antitumour immunity capable of preventing tumour development following injection of patient-derived tumour material in 50% of humanized PDX mice and sensitizing pre-existing tumours to immune checkpoint inhibitors[233]; this approach might provide a novel treatment approach for patients with metastatic breast cancer.

In another approach, EVs generated from OVA-pulsed DCs were conjugated with an anti-CTLA4 antibody[330]. This EV-based therapeutic cancer vaccine was demonstrated to promote tumour infiltration by T cells leading to antitumour activity in a mouse model of melanoma[330]. Elsewhere, researchers engineered antibody-decorated EVs from tumour antigen-pulsed DCs equipped with antibodies targeting CD3 or EGFR that are able to activate T cells directly and to promote T cell–cancer cell interactions, resulting in cell crosslinking and enhanced tumour cell killing in a B16-OVA mouse model[231]. This cancer vaccine also elevated PD-L1 expression in tumour tissues in vivo and combination therapy with anti-PD-L1 antibodies further enhanced the efficacy of the tumour-targeting EVs[231].

Accumulating evidence indicates that immune cell-derived EVs can mediate communications between various immune cell types to dynamically regulate the immune response[331], providing a new platform

for cancer treatment focusing on immune cell regulation[15,234] (Fig. 4c). In a seminal study, researchers demonstrated that cancer-derived EVs (from preclinical models of lung or breast cancer or melanoma) containing PD-L1 can induce T cell senescence owing to alterations in lipid metabolism and activation of cAMP-response element binding protein (CREB) signalling[332]. Inhibition of EV formation, lipid metabolism and/or cholesterol synthesis or CREB signalling reversed this T cell senescence and sensitized mouse xenograft models to anti-PD-L1 antibodies in vivo[332]. These preclinical data reveal a novel onco-EV-mediated immunosuppressive mechanism of T cell senescence with dysfunction and the potential for resistance to immune checkpoint inhibitors.

Elsewhere, a therapeutic strategy involving a combination of EVs derived from induced pluripotent stem cells (iPSCs) exposed to tumour-associated antigens alongside DCs was investigated, resulting in antitumour activity following injection into syngeneic mouse models of various solid tumours[229]. These human iPSC-EVs (hiPSC-EVs) can be recognized by the mouse immune system and are able to elicit specific antitumour activity against various isolated tumour cells and in mouse models of lung metastasis and melanoma[229]; such findings provide a solid theoretical basis for clinical trials with hiPSC EV-based prophylactic and therapeutic cancer vaccines (Fig. 4c). These iPSC-derived EV-based vaccination strategies might overcome the challenges associated with current iPSC vaccines (such as autoimmune rejection and potential tumorigenicity) that limited antitumour activity, particularly for metastatic cancers[333].

A further EV engineering strategy includes the development of functional gasdermin D mRNA-encapsulating EVs coated with the hydrophilic photosensitizer chlorin e6 and an anti-HER2 antibody, which engage pyroptosis in both mouse HER2-overexpressing 4T1 syngeneic and SKBR3 xenograft models[334]. Elsewhere, various approaches involving activation of the STING signalling pathway have been developed[335,336], primarily focusing on cytosolic DNA to stimulate DCs and activate cytotoxic immune cells. In one study, HEK293 cell-derived EVs externally loaded with the STING agonist cGMP–cAMP were efficiently internalized by DCs with subsequent activation of $CD8^+$ T cells and suppression of tumour growth in syngeneic mouse models of melanoma. Following intratumoural injection, cyclic dinucleotide (CDN)-loaded EVs enhanced local T cell responses with >100–200-fold potent inhibition of tumour growth compared with doses of free CDN, or co-administered unloaded[335]. Importantly, such tumour localization and retention of CDN-loaded EVs resulted in systemic antitumour immunity without evidence of systemic inflammatory cytokine storm in the mouse model[335].

Improving our understanding of the expression of specific tumour-targeting EV surface proteins and their implications for retention in the tumour vasculature remain key areas of research. In this regard, developments in EV engineering are delivering substantial advances in the ability to generate tumour-specific T cell responses[337]. Multifunctional DC-derived EVs provide several advantages over existing immunotherapies such as the ability to stimulate both innate and adaptive immune responses, as well as their ease of manipulation for the delivery of broad-spectrum tumour antigen complexes for T cell activation, including cytosolic delivery of STING agonists for enhanced antigen presentation as well as an improved capacity to transverse tissue barriers.

Collectively, these therapeutic approaches, using complementary EV-based strategies (Box 1 and Fig. 4c), have demonstrated potent preclinical activity in various models, including humanized models[233]. Such versatile EV-based systems provide a biotechnology tool for clinical development across multiple delivery strategies with the potential for long-term stability. In the early phases of clinical development (such as in NCT01159288, NCT01550523 and NCT02507583), bioengineered EV-based vaccines have primarily been tested in patients with advanced-stage cancers — with salient findings in terms of safety, immunomodulatory properties and in some cases ease of adaptation. However, efficacy in terms of both stimulating adaptive antitumour activity and clinical activity has been underwhelming in many of the early trials.

In summary, much of the current focus has shifted towards using EVs for the delivery of targeted therapies and as cancer vaccines. Selection of the most appropriate EV precursor cells is a critical point to be considered when designing an EV vaccine owing to the implications of the intrinsic properties of the EVs for activity and potency, surface antigen display, antigen loading and EV integrity, pharmacokinetic profiling and the feasibility of large-scale production and purification, as well as the ability to achieve consistent immune responses across experimental models and in patients.

EVs as a source of biomarkers

Roles in detection and clinical management

Over the past decade, onco-EVs have attracted considerable clinical interest as diagnostic platforms (described in detail elsewhere[338]). This interest primarily reflects their release directly from tumour cells as well as the ability to obtain samples in a minimally invasive manner from the systemic circulation and other bodily fluids[78,248,339–341]. EVs have the potential to provide clinical applications in the areas of cancer diagnosis[342], including disease staging[78,152,343], early cancer stage risk factors[344], dissemination and/or metastasis loci[152,344] and prognosis[13,341,345,346] (Table 3 and Fig. 5a). For example, in a groundbreaking study, investigators identified onco-EV-associated GPC1 (encoding glypican-1) as a prognostic biomarker enabling the detection of early-stage PDAC, including the ability to distinguish between patients with early-stage and advanced-stage disease[342]. GPC1 (mRNA and protein) from onco-EVs was subsequently combined with the serum CA19-9 test for early PDAC detection and demonstrated encouraging specificity (91.3%) and sensitivity (~81%) in comparison to measures of either biomarker alone in a retrospective analysis[345]. This approach provides a promising non-invasive method of early diagnosis and treatment decision-making in patients with potentially curable PDAC. In this regard, the ExoDx Prostate (IntelliScore) (EPI) test is a non-invasive urine-based EV gene expression assay designed to inform about the need for biopsy sampling in patients with prostate cancer[216,346,347]. In a randomized multicentre trial, all 1,094 patients had an EPI test, although only the intervention group received results during the biopsy decision-making process. A total of 833 patients had follow-up data available at 2.5 years and those with low-risk ExoDx scores (<15.6) had fewer and more-delayed biopsy procedures compared with those with high-risk disease[348]. The EPI test also leads to a 21.8% increase in the detection of high-grade prostate cancer in the EPI arm compared with those managed according to standard-of-care approaches while reducing the incidence of unnecessary biopsy sampling (44.6% of patients with low-risk disease in the EPI arm underwent biopsy sampling versus 79.0% in the control arm; $P < 0.001$)[348]. However, the prostate cancer is often slow to progress, and median survival outcomes from this study are thus far unavailable.

Challenges remain in determining the most suitable cancer biomarkers and, importantly, the type of molecule or molecules best suited specifically for the purpose of EV-based cancer detection or monitoring. In one

Table 3 | Extracellular vesicle-based liquid biopsy biomarkers in patients with cancer

Cancer	Biomarkers and detection methods	Clinical efficiency and feasibility	Refs.
Stage I PDAC (*n*=16)	EVs present in frozen plasma samples are isolated using ultracentrifugation followed by single-EV analysis (combining pan EV lipophilic labelling, size exclusion, high-resolution microscopy imaging) for the detection of various cancer-associated proteins, including mutant *KRAS*, *P53*, *MUC1*, *EGFR* and *PGP4OH* detected in frozen plasma samples using ultracentrifugation	Mutant *KRAS* and/or *P53* detected in samples from 15 of 16 patients with stage I PDAC. Estimates suggest that tumours with a volume of ~0.1 cm^3 could be detected at 80% specificity using the current approach and that detection limits as low as <0.03 cm^3 might be feasible at similar levels of specificity with larger panels	124
mCRPC (*n*=76)	EVs present in frozen plasma samples were isolated using ultracentrifugation followed by low-association WGS with differential gene expression and gene-set enrichment analysis	Detectable tumour-derived DNA in ≥4% of EVs (EV-DNA TF ≥4%) associated with levels of clinical biomarkers such as serum PSA, LDH and ALP as well as shorter time to disease progression among patients receiving androgen receptor signalling inhibitors or taxanes (HR 2.76, 95% CI 1.45–5.25; log rank *P*=0.001)	157
HGSC (*n*=935)[a]	EVs present in plasma or serum samples were isolated using size-exclusion chromatography and immunoaffinity purification followed by proximity-ligation qPCR-based quantification and development of an EV-based test comprising BST2, FRα, MUC-1, MUC-16 and sTn	Specificity 97.0% (128 of 132 individuals), HGSC sensitivity of 97.0% (64 of 66 patients) and also detected 73.5% (61 of 83 patients) of non-high-grade ovarian cancers compared with serum CA-125	396
Breast cancer (*n*=196)	EVs present in serum samples were isolated using ultracentrifugation and size-exclusion chromatography followed by proteomic analysis	A panel comprising 12 EV proteins (TTYH3, KPNB1, RANBP2, PEPD, NCL, PARP1, ACTA2, ACTG2, TBCA, MATR3, KRT16, and CCT6A) was able to discriminate between patients with vs without lymph-node metastases at 93.8% sensitivity and 81.3% specificity; TALDO1 associated with distant metastases	152
Prostate cancer (*n*=1,563)	EVs present in urine samples (25–50 ml total volume) were isolated using a urine sample concentrator kit followed by qRT-PCR and analysis using the ExoDx Prostate IntelliScore panel (comprising ERG levels and fusion products plus, TMPRSS2) to calculate an EPI score	At 2.5 years of follow-up, patients with EPI scores suggesting low-risk disease were less likely to have high-grade prostate cancer (7.9% vs 26.8%, *P*<0.001). Furthermore, patients with low-risk EPI scores were less likely, and those with high-risk EPI scores were more likely to undergo biopsy sampling (44.6% and 79.0%) compared with those in the control group (59.6% and 58.8%)	216,347

ALP, alkaline phosphatase; EPI, ExoDx prostate; EV, extracellular vesicle; HGSC, high-grade squamous ovarian cancer; LDH, lactate dehydrogenase; mCRPC, metastatic castration-resistant prostate cancer; PDAC, pancreatic ductal adenocarcinoma; PSA, prostate-specific antigen; qRT-PCR, quantitative real-time PCR; sTn, sialylated Thomsen–nouveau antigen; TF, transcription factor; WGS, whole-genome sequencing. [a]Includes 156 patients with HGSC as well as various control groups in both the training and validation cohorts.

EV-based diagnostic approach, the lipid composition of cancer-derived EVs was investigated[151]. This study used a mass spectrometry-based detection platform focusing on the presence of sphingolipids and glycerophospholipids in EV membranes, with an overall accuracy for the detection of breast cancer of 93.1% by logistic regression classification with recursive feature elimination analysis[151]. Furthermore, assays designed to detect cancer-associated mutations in DNA from EVs (such as those in *KRAS*[349] or *EGFR*[350]) demonstrated superior sensitivity and specificity for the early detection of pancreatic or lung cancer, respectively, compared with the equivalent methods involving cell-free DNA (cfDNA). For example, sensitivity for activating *EGFR* mutations was 98% versus 82% and for $EGFR^{T790M}$ was 90% versus 84% in prospectively obtained samples from the TIGER-X cohort, in which patients with *EGFR*-mutant NSCLC (determined based on the analysis of a tumour biopsy sample) received the EGFR tyrosine-kinase inhibitor rociletinib[351].

Immune cell-derived EVs harbouring integrin $\alpha_x\beta_2$ can serve as novel, blood-based biomarkers of thrombosis risk, specifically in the context of lung cancer[82]. Circulating tumour-derived CXCL13 was shown to reprogramme lung interstitial macrophages to produce EVs carrying activated integrin $\alpha_x\beta_2$, which was able to induce systemic platelet activation and aggregation responses via interactions with the platelet-bound glycoprotein GPIb. This EV-mediated phenotype was suggested to contribute to a hypercoagulable state that might predispose to organ dysfunction and failure in patients with cancer[82]. Importantly, EVs containing surface integrin β_2 (ITGB1) were also identified as an initiator of intravascular thrombosis, with selective inhibition preventing thrombosis and reducing the extent of metastatic dissemination in preclinical models of both early-stage and advanced-stage cancers[82]. Other cell types might further contribute to the pool of circulating EV-associated ITGB1; nonetheless, this study demonstrates the potential of integrin β_2 as a biomarker of various cancer types associated with a high risk of thrombosis (including pancreatic and lung cancers and Hodgkin lymphoma)[82]. These findings highlight the complex interplay between EV signalling and metastasis-promoting factors released by activated platelets and a potential role of these factors as predictive/prognostic biomarkers for tumour-associated thrombosis and potential targets for the treatment of advanced-stage cancers.

In a seminal study[352], the researchers investigated circulating exosomes expressing PD-L1 on the vesicle surface as a prognostic biomarker in patients with metastatic melanoma receiving these agents. This study identified correlations between maximum fold changes in exosomal PD-L1 level after 3–6 weeks of pembrolizumab and an improved overall response rate (80% sensitivity and 89.7% specificity), whereas fold increase in total circulating PD-L1, MP-PD-L1 and EV-excluded PD-L1 levels was associated with an inferior overall response rate, suggesting a potential of exosomal PD-L1 as a blood-based prognostic biomarker. Such insights raise important clinical considerations for biomarker discovery studies, in which sensitivity and specificity in specific settings dictate the ideal tumour biomarker. Circulating exosomal PD-L1 levels have also been associated with prognosis in patients with various solid tumours including head and neck squamous cell carcinomas[353], gastric cancer[354] and NSCLC[355]. The suggestion that exosomal PD-L1 inhibits the activity of $CD8^+$ T cells in mouse models supports EV elimination as an effective adjunct anticancer therapy might improve the effectiveness of anti-PD-L1 antibodies in patients[352]. Elsewhere, Melac-Chip, a microfluidic metabolic

glycan-labelling technology enabling temporal-selective labelling of newly released EVs, was used to demonstrate a positive correlation between PD-L1$^+$ EV levels and tumour volume in mouse xenograft models of breast cancer or melanoma[356]. Collectively, these findings suggest that EV monitoring approaches could provide novel assays for early cancer diagnosis and for informing treatment-related decision-making.

Other strategies associated with EVs and immunoregulation are being directed towards prediction of immunotherapy response in patients[357,358]. For example, a clinically relevant voluntary exercise regimen was found to promote the release of EVs containing miR-29a-3p from the myocytes of patients with various solid tumours[357]. miR-29a-3p released in this way was found to correlate positively with immune cell infiltration and survival outcomes, suggesting potential relevance as a biomarker[357].

Similarly, typically expressed EV-associated proteins such as CD9, HSPA8, ALIX, HSP90AB1, ACTN, MSN, RAP1B and SDCBP have been tested clinically as universal biomarkers for early cancer detection[78,121,210]. Advances in technology and artificial intelligence (AI)-based refinement of EV-based, specific[124,359] and multicancer tests[210] have the potential to optimize the performance of early cancer detection tests and warrant further testing in prospective studies. For example, ExoLuminate is a prospective, multicentre, observational registry study (NCT05625529), which is designed to evaluate the ExoVerita assay for the early detection of pancreatic cancer, which has already demonstrated 93% sensitivity and 91% specificity for PDAC in the training data set[57]. In April 2025, the EvoLiver test was designated as an FDA Breakthrough Device for early detection of hepatocellular carcinoma (HCC) in patients with cirrhosis and therefore a high risk of HCC. In a multicentre trial, this assay demonstrated 86% sensitivity and 88% specificity for the detection of early-stage liver cancer, surpassing the performance of methods such as ultrasonography and α-fetoprotein testing.

Bulk EVs versus onco-EVs

The vesicular population of blood comprises EVs originating potentially from any tissues or cell types (Fig. 5b). Within this complex EV

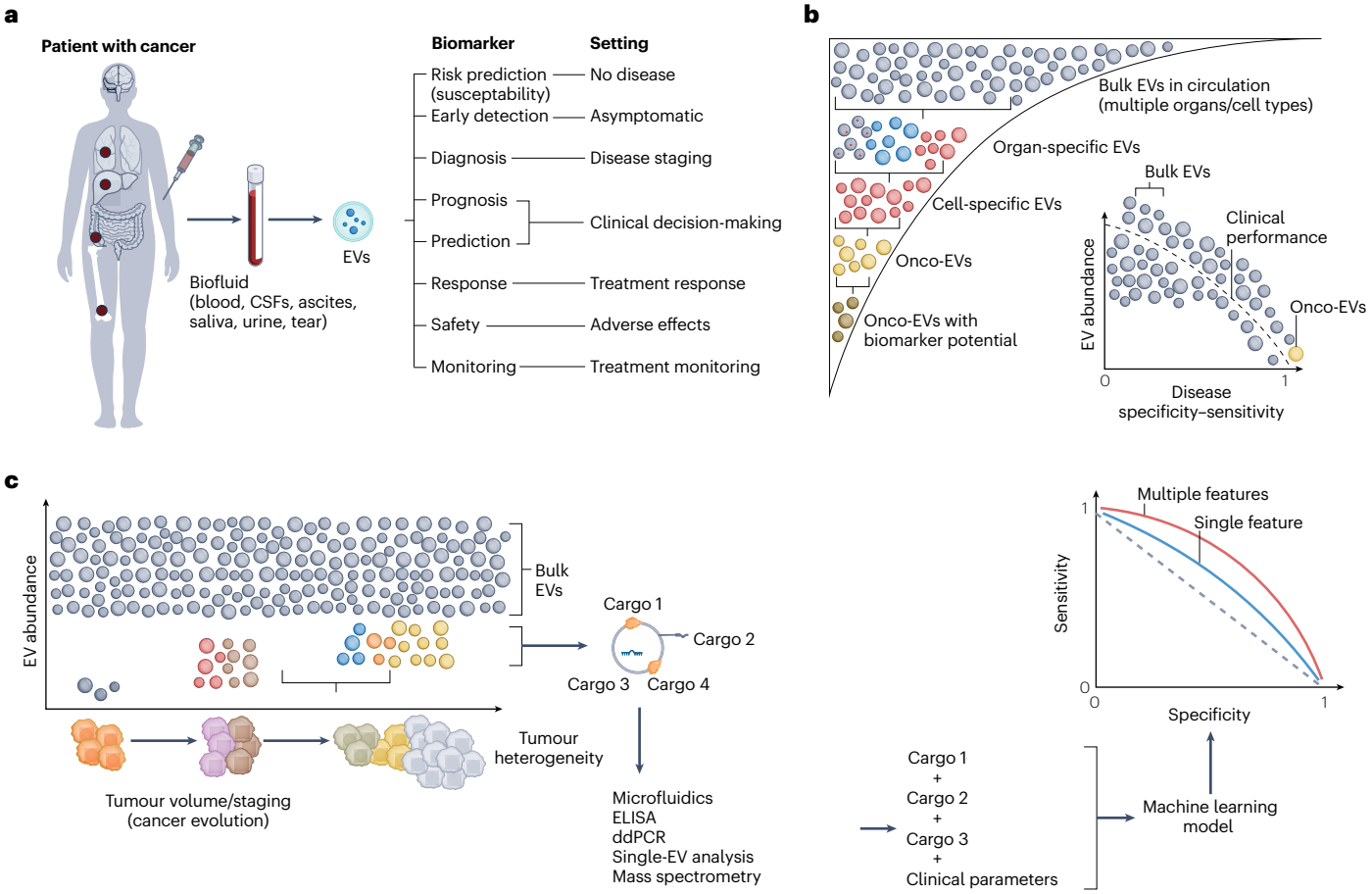

Fig. 5 | Cancer extracellular vesicles as diagnostic–prognostic platforms. **a**, Extracellular vesicles (EVs) containing complex biomolecules reflective of their cells and tissues of origin have the potential for clinical utility as diagnostic and/or prognostic biomarkers with the potential for minimally invasive sampling via blood plasma or other bodily fluids. **b**,**c**, Advances in the sensitivity of detection strategies enable the identification and monitoring of cancers using EV-bound biomarkers and/or molecular cargoes (**b**) including both specific cancers and multiple cancer types (pan-cancer biomarkers) using methodologies, such as genome sequencing (for the detection of mutations, or potentially changes in DNA methylation or fragmentation)[129,373,417,418], mass spectrometry[78], lab-on-a-chip platforms[210], label-free and fluorescent and/or metabolic labelling combined with microfluidic isolation[356,419], machine learning[147], interaction mapping (via ligand–receptor networks[420] or cellular interaction interfaces[421]), single-EV-based analyses[124] and antitumour therapy response[352,356] (**c**). CSF, cerebrospinal fluid; ddPCR, digital droplet PCR; ELISA, enzyme-linked immunosorbent assay.

mixture exists a small subset of organ-specific EVs arising from different cells within each organ. Cancer cells within organs are known to release onco-EVs that typically comprise a tiny subset of the vesicular population. Nonetheless, these EVs might have potential as cancer biomarkers owing to the presence of tumour-specific features reflecting their cell type of origin. Onco-EVs have a limited abundance in the systemic circulation (estimated to be <0.1% of the total EV population using single-EV analysis-based fluorescence detection[124]). To address this limited abundance, high-throughput analysis of single-EV surface markers has been used to measure the kinetics of EV interactions with an affinity-labelled surface as specific and non-specific EV interactions, with high sensitivity (89%) and specificity (100%) detection of a protein signature (comprising EpCAM, GPC1 and HER2) in pancreatic cancer-derived onco-EVs against a background of a 350-fold excess of non-specific plasma EVs[359].

The ability to selectively isolate onco-EVs from blood samples or other bodily fluids containing populations of circulating EVs originating from potentially any organ or cell type (typically 10^{10}–10^{12} EVs/ml in blood)[360] remains a major challenge for both basic researchers and clinical translation. Nonetheless, several studies have demonstrated the feasibility of capturing circulating onco-EVs originating from specific cancer cell types[124,129,157,361] (Fig. 5c). Recently, a transcriptomic-based approach was developed to specifically enrich circulating EVs with RNA or DNA cargoes[157]. This study demonstrated enrichment for DNAs and RNAs encoding epithelial markers commonly amplified or deleted in bulk EVs derived from patients with cancer, both globally and at individual patient levels[157]. Further analysis of these EV cargoes revealed the presence of tumour-specific mutations, copy-number alterations and transcriptional programmes that largely correlated with gene expression patterns in EV-RNAs derived from matched tumour biopsy samples. Evaluation of plasma EV-RNAs in patients receiving systemic androgen receptor inhibitors after 4 weeks of treatment revealed downregulation of cancer-related transcription programmes including those regulating epithelial development, keratinization and proliferation (E2F, MYC), PI3K–mTOR and AR signalling, with upregulation of resistance-associated signatures including those involved in neuroendocrine differentiation, basal-like programmes and RB1 loss. This EV isolation strategy (combining ultracentrifugation, density gradient purification and column-based capture), while showcasing the potential of onco-EV isolation based on specific molecular cargoes, raises questions concerning the number of EVs released by tumour cells and other cells populating the TME and whether certain EV populations (such as those associated with cell death) might dominate in terms of the RNA or DNA cargoes detected and thus provide a biased readout of tumour biology[362].

Elsewhere, a comprehensive analysis of EV-DNA including sequences, structure, biogenesis and function in cancer progression demonstrated that EV-associated genomic DNA can inhibit liver metastasis by reprogramming the immune microenvironment[363]. In this study, EV-associated DNA was predominantly detected at the EV surface and derived from nuclear DNA, while its chromatin structure differed strikingly from that of cellular DNA. Importantly, the EV-associated histones were identified and differed markedly from those of their cell source-derived nuclei in that the EV-derived histones had signs of DNA fragmentation and increased methylation, suggesting a puzzling and non-random mechanism of extracellular export[364]. This study used advanced microscopy and genome-wide CRISPR–Cas9 screens to identify candidate genes associated with the packaging of DNA into EVs[363]. Importantly, these EVs demonstrated an anti-metastatic function in a mouse model of liver metastases, without any obvious effects on the growth of the primary tumour. Furthermore, EV-associated DNA triggered Kupffer cell-mediated suppression of the immune microenvironment as well as generated transcriptional signatures associated with DNA damage response pathways, in parallel with the release of immunostimulatory cytokines and chemokines (such as tumour necrosis factor, CCL5 and CXCL1/10). This study also provided evidence of a potential prognostic role of onco-EV-DNA, with reduced amounts of onco-EV-associated DNA correlating with an increased risk of post-operative metastases in patients with various cancer types, with 87% sensitivity and 56% specificity for metastatic recurrence at a cut-off of 49.2 ng/μg (ref. 363).

A large-scale comprehensive analysis of the proteomes of EVs isolated (using ultracentrifugation and size exclusion) from serum samples from 196 patients with breast cancer or those without cancer identified several tumour-associated EV proteins, which could serve as potential biomarkers for the detection of early-stage breast cancer (suggesting a 7-marker panel) and metastatic disease (suggesting a 12-marker panel)[152]. These findings could be complemented by advances in our understanding of circulating EVs, for example, by using multi-omics to identify onco-EV markers that are conserved across different clinical cohorts that precisely differentiate EVs[365] — such knowledge would be crucial in enabling the clinical potential of circulating EVs to be fully realized.

Evolving technologies

Advances in detection and capture and the ability to obtain multiplexed measurements from onco-EVs are beginning to address the inherent heterogeneity of putative cancer cell-associated proteins and the frequency of specific mutated oncogenes or tumour suppressor proteins in single EVs[87,88,124]. Various methodologies, including digital detection assays[124] and single-cell EV nanoscopy[366] (described in detail elsewhere[367]), have been applied for this purpose. Current EV biomarker strategies capable of sensitive capture and digital detection (such as enzyme-linked immunosorbent assay, digital flow cytometry, digital surface-enhanced Raman scattering and other emerging digital strategies[366]) and microfluidics technologies to analyse individual molecules from single EVs[150], including double-stranded DNA and single-stranded DNA[368], are highlighted (Box 2).

The ability to define transcriptional profiles of tumours from liquid biopsies has focused on methylation changes in ctDNA[369], and fragmentomics[370,371] (reviewed elsewhere[372]), in which highly sensitive evaluation of fragmentation patterns of cfDNA across the genome have demonstrated 80–98% cancer detection specificity[370,371]. Given that fragmentation profiles reveal regional differences between tissues, their profiles can be matched and analysed to identify the tissue of origin of ctDNA. Although this approach highlights the clinical importance of combining assessments of characteristics such as DNA fragmentation patterns across the genome for screening, early detection and monitoring of cancer in patients[371,373], fragmentomics analysis has not yet been applied to the interrogation of EV-derived DNA.

The availability of sensitive assay platforms and alternative technologies to determine the origin and molecular features of EVs is expected to provide a key step forward in the absolute quantification of biomarker expression levels and their characterization at the single-EV and single-molecule levels of resolution[124,366]. In theory, patient-derived onco-EVs can, in essence, capture the neoantigen landscape[124,362] and might therefore facilitate the development of comprehensive, adaptable and highly personalized vaccines targeting a broad spectrum of

Box 2 | Emerging technologies for extracellular vesicle-based cancer diagnostics

Emerging new technologies and approaches for more sensitive and more specific extracellular vesicle (EV)-based cancer diagnosis include the following.

Microfluidics

Microfluidic platforms for label-free EV isolation and/or capture from biofluids or tissue samples have the advantages of high throughput, purity in isolating EV subpopulations, sensitivity in their capacity to derive cell-specific features on EVs as well as multiplexing capabilities[356,430]. Microfluidic platforms such as functionalized magnetic beads for rapid isolation of EV subtypes have demonstrated superior performance relative to other EV isolation methods including at the single-cell and single-vesicle levels[431]. For example, the detection of biotin-linked metabolic glycan labelling $CD63^+$ EVs using a microfluidic-assisted enrichment strategy based on metabolic labelling and click chemistry (Melac-Chip) demonstrated the ability to distinguish nascent from pre-existing EVs[356]. Microfluidic techniques also enable the controlled production of synthetic EVs and effective cancer drug loading for EV-based therapies[432]. However, maintaining precise flow control within microchannels, which requires complex and multilayered designs within nanoscale structures[433], and impurities and a limited ability to isolate EVs from whole blood remain challenges in this area. Nonetheless, a viscoelastic-based microfluidics platform equipped with a cell-depletion module and a single-EV isolation module has demonstrated 97% purity and an 87% recovery rate for small (<200 nm) EVs[419].

Multi-omics profiling of EVs and other biomarkers

Integrative multi-omics analysis enables the identification of multiple biomarkers with the potential for an improved understanding of the roles of such molecules in cancer[434,435]. For example, a liquid biopsy signature comprising EV-derived mRNAs, microRNAs and long non-coding RNAs has been shown to distinguish between patients with advanced-stage gastric cancer with a response and those without a response to neoadjuvant fluorouracil-based chemotherapy in a retrospective analysis (AUC 0.77)[436]. In another study, investigators analysed 52 omics data sets (comprising 481 exosome-derived molecules including miRNAs, mRNAs, lipids and proteins) and identified HLA-DQB2 and COL17A1 as high-performance predictors of breast cancer prognosis (AUC 85.4%)[437]. Further applications of EV-associated small DNA fragments are emerging, given that EVs typically provide large fragments of intact DNA[438], typically of ~150–500 bp (refs. 438,439) and up to 2,000 kb in length. Fragmentomics presents an orthogonal approach, providing additional information about the biological context of the DNA and the cell from which it originated, including vesicle biogenesis and cargo loading. Elsewhere, a dual mass spectrometry-based proteomic approach was applied to circulating EVs obtained from patients with breast cancer (n=126) or those without cancer (n=70). Both targeted and label-free proteomics enabled the identification of a panel of 7 proteins as a potential diagnostic signature for breast cancer, increasing to 12 proteins for the detection of lymph node metastases with validation in five independent cohorts demonstrating a sensitivity of 93.8% and a specificity of 81.3% (ref. 152). Translational potential was further established using high-throughput molecular docking and virtual inhibitor library screening, which identified inhibition of the pentose phosphate pathway enzyme transaldolase 1 as a method of delaying tumour progression in vivo[152].

Nanocapture technologies for EV-derived protein and/or gene detection

Several widely used and novel technologies are available for EV-based liquid biopsy analysis that is suitable for multitarget quantification and high-sensitivity detection such as various antibody-based assays[213], digital droplet PCR (ddPCR)[212,440,441], single-molecule resolution digital sEV counting detection (DECODE) chips[442] and droplet-barcode sequencing[443]. However, the limited abundance of most protein cargoes in EVs remains a challenge. Excitingly, single-EV and single-particle analysis[124] for high-sensitivity EV protein detection has been developed and enables the quantification of individual molecules from a single EV[124,150,366,444]; this approach can be used to detect stage I pancreatic ductal adenocarcinoma using a low-volume (100 µl) plasma sample[124].

Artificial intelligence-assisted EV-based early cancer prediction

Integrating EV multi-omics data and artificial intelligence (AI)-assisted analysis (for example, omics screens, imaging and predictive modelling) could provide powerful tools for early cancer prediction[210]. In one approach, investigators developed an explainable AI-based prostate cancer screening system combining AI (to identify the key role of each biomarker in decision-making and provided specific clinicopathological data supporting its decision for individuals) with a dual-gate field-effect transistor biosensor[445].

cancer antigens[124]. Technologies and machine-learning approaches enabling refinements in how we identify early drivers of tumorigenesis are rapidly evolving[374–377] (Fig. 5c and Box 2). Strategies involving the profiling of hundreds of millions of single analytes to identify very rare circulating EV-centric biomarkers (for example, less than ~0.01–0.05% of bulk EVs for early-stage solid tumours, ~0.1–1% for advanced-stage metastatic disease[241] and 1–10% for in vivo xenograft models[248]) are currently emerging[378]. EV profiling and modelling studies have begun to correlate tumour size with the presence and concentration of circulating EVs[124]. Such estimates of the number of EVs released per unit time and unit tumour volume highlight the challenges in developing bulk EV-based platforms for the detection of early-stage, small tumours and how single-EV detection strategies might resolve these challenges in detection sensitivity (~1,000-fold or more over bulk methods) and potentially enable the detection of early-stage cancers in humans <1 mm^3.

Alternatively, applying multiplex biomarker panels to the detection of onco-EVs might provide improved analytical specificity and sensitivity, therefore reducing tumour detection limits (Fig. 5c). Indeed, a framework model for the detection of stage I PDAC integrating the amount of circulating onco-EVs detected in PDX models of PDAC, analysis with multiplex marker panels and the effects of tumour volume predicted that such an approach would enable the diagnosis of 68% of patients with tumour volumes of 0.1 cm^3 at a specificity of 80%,

suggesting the potential for early cancer detection[124]. Such multiplex assays also have the potential to substantially contribute to EV initiatives aiming to standardize the definition of EVs by providing multilayer biomarker profiles.

Clinical translation

Despite promising data from preclinical studies and ongoing and completed clinical trials including studies testing promising early-stage cancer assay platforms (NCT05625529), prognostic biomarkers in patients with NSCLC (NCT05424029), the EvoLiver test for HCC surveillance in patients with cirrhosis, ExoDx prostate and OverC, a high-specificity multicancer detection blood test[79,127,198,332,379], major challenges remain that have limited the translation of knowledge of the role of EVs in cancer biology into clinically effective therapeutics or other clinical applications. In this regard, comprehensive investigations of fundamental questions regarding the functions and biological significance of EVs in the initiation and evolution of cancer[17] and stromal and immune cell regulation[15] are required and would probably support and expand the clinical application of EVs. Some generalized fundamental questions relate to the specific roles of EVs in cancer initiation and the identity of the molecular cargoes that drive these oncogenic programmes as well as the types of EV cargo that mediate immune checkpoint modulation or immunosuppressive signalling. Could these mechanisms be targeted using selective stage-specific inhibitors, or leveraged as biomarkers for cancer staging and prognosis? Furthermore, how can the signatures of tumour-specific EVs be leveraged to improve the sensitivity and specificity of liquid biopsy assays? Furthermore, knowledge of the interactions between EVs and anticancer and immunoregulatory therapeutic agents and how these affect treatment outcomes remains limited, as does our ability to address the fundamental biological and technical challenges created by heterogeneity in EV populations, which will need to be overcome to enable more precise functional studies.

EVs are highly heterogeneous and arise from complex biogenesis pathways with variable targeting mechanisms, all of which are not yet fully understood. Notably, the challenges created by the existence of many diverse subpopulations capable of producing and receiving a diverse range of signals are not unique to research into EVs, and in this regard single-EV studies will probably provide a deeper understanding of EV biology with important implications for biomarker discovery[380]. Single-cell studies of tumour-infiltrating immune cells have revolutionized immunology and cancer biology by revealing cellular heterogeneity, states and dynamics in an unprecedented level of detail. Yet, single-EV studies — although conceptually similar in aiming to dissect heterogeneity — have been challenging to execute owing to several limitations. These include the inherent technical limitations in resolving and specifically detecting and profiling single EVs, the need for standardized single-EV profiling strategies and the requirement for comprehensive, refined databases and standards for interpreting molecular profiles at the single-vesicle level (reference atlases). In this regard, advances in nanotechnology and microfluidic systems, and in combining multimodal approaches (imaging and molecular profiling), might reveal a level of EV heterogeneity akin to that demonstrated in single-cell studies[113] (Box 2). The regulatory machinery that controls the production and cellular uptake of EVs remains largely unknown, including the regulation of biodistribution and cell-specific interactions. The EV corona has an established role as a part of the EV interactome, with the potential to contribute to and expands EV functionality and influence EV hydrodynamic size and mobility[168,171–173,381]. The implications of this bioactive medium, which is known to affect and in certain scenarios enhance vaccine delivery and immune responses in nanostructures[382], require consideration when using EV delivery systems for immunoregulation and activation including in antigen-specific adaptive immune responses[383]. Overall, such advances in our understanding of the protein corona are expected to improve rational EV-inspired nano-therapy design.

In terms of clinical utility as therapeutics, substantial manufacturing challenges exist including the scalability of production of clinical-grade EVs, selection of the most appropriate cellular sources as well as the most appropriate standardized internal controls to monitor batch variance and ensure that EV integrity, yield, composition, function and ultimately clinical effectiveness remain consistent. Considerable improvements in EV isolation strategies have been made, although the optimal method of scalable isolation of pure, clinical-grade EVs remains unknown and attempts to define this are still ongoing[143]. In this regard, the mechanisms and kinetics involved in the loading of tumour-associated DNA[143,144] and RNA[245,384] into EVs remain an area of active research. For regulatory approvals, regulatory agencies such as the FDA and EMA have stringent standards for cell-derived products[385]; such requirements include ensuring the authenticity of the EV sources, maintaining strict biological control over parental cells, understanding the effects of such processes on EV corona composition and/or cell targeting and accumulation[172], utilizing standardized and reproducible production methods and implementing standardized testing protocols[143,385] (including protocols for the evaluation of particle biophysical characterization, membrane stability, biochemical composition as well as the development of in vitro–in vivo-correlated assays[367,386]), models that mimic the composition and architecture of specific cellular environments[387,388] and assessments of pharmacokinetics and therapeutic efficacy[183,385,387]. Innovations in strategies to extend the circulation time of modified EVs including membrane camouflage[388], cell target tethering[389] or biomaterial-based tissue-specific controlled release or retention strategies[390–393] are emerging. Despite these caveats, efforts for large-scale production of EVs (or hybrid EVs[303]) from various cell types under good manufacturing process-like conditions[394] for their development as therapeutic tools are ongoing[65,183]. The future characterization of therapeutic EVs should involve multi-omics data integrated with advances in digital and machine-learning-based models to predict product quality and therapeutic consistency[65,395].

Substantial technical challenges also exist when considering circulating onco-EVs as diagnostic tools — most notably, the development, and application of highly sensitive tumour cell-specific EV assays, including the EvoLiver test and ExoDx prostate. ExoDx prostate, an EV-based liquid biopsy assay designed to guide cancer diagnosis and treatment decisions using a non-invasive multiparameter test, has been designated as an FDA Breakthrough Device[347]. Importantly, this designation now also applies to the Mercy Halo ovarian cancer screening test, an EV-based test designed for ovarian cancer screening of asymptomatic postmenopausal women[396], with detection based on a panel of biomarkers (BST-2, FOLR1, MUC-1, MUC-16 and STN) colocalized on the surface of individual EVs[380]. The recent concomitant analysis of cfDNA, EV-DNA and EV-RNA from longitudinal cohorts of patients with prostate cancer links comprehensive analyses combining multi-omics and multi-analyte liquid biopsy — highlighting the salient fragmentation features of EV-RNA cargo as promising biomarkers for diagnostic and monitoring treatment response. This approach correlates DNA copy number and EV-RNA expression levels, globally and at an individual patient level, for regions commonly amplified

or deleted in prostate cancer, demonstrating the ability to detect individual tumour-specific features[157]. As nanotechnology platforms, microfluidics could be used to quantify EVs for diagnosis[215]. Nanosensor arrays and fluidic devices can measure specific protein and nucleic acid biomarkers and thus enable in-depth liquid biopsy assessments[359]. However, such strategies are still not widely used in clinical diagnosis owing to challenges related to reproducibility, standardization and a lack of robust assessment workflows. Most importantly, no standardized protocols and analyte references exist that would enable uniform evaluations of the performance of the various isolation and detection methods for EV analysis, especially at the low levels of abundance of most EVs. In this regard, highly specific EV detection and surface protein profiling strategies will be needed for effective early cancer detection assays. These assays could include strategies such as dynamic immunoassay surface profiling, which is capable of detecting multiple marker signatures from rare, ultra-low abundance tumour-specific sources in plasma and has already demonstrated an ability to discriminate between patients with PDAC and those without in a small retrospective cohort[359]. Although still rather preliminary, an understanding of the differences in EVs derived from different organs or tissues is emerging — which adds a level of complexity and variability in defining specific changes in EV composition. Indeed, applications of highly sensitive single-EV detection assays reflect considerable improvements in the limits of detection of CTC-derived EVs from early-stage cancers[149]. This high sensitivity presents a seminal challenge in applying knowledge of circulating EV origins to the isolation of heterogeneous EVs, with certain biomarkers found to be much less prevalent than initially thought[213]. Finally, distinct differences in sample collection criteria, storage, pre-handling, separation and detection methods often exist across different laboratories, making direct interlaboratory comparisons of results difficult.

Despite these various challenges, clinical implementation of EV-based biomarkers will require a method capable of specifically capturing, reproducibly separating and detecting EV-based biomarkers and for this approach to demonstrate superior diagnostic performance in large cohorts of patients[359,380] (such as the ExoLuminate trial (NCT05625529)). Considering these challenges, various studies have explored isolation methods based on the detection of tumour-specific membrane protein markers on EV surfaces to enrich for onco-EVs, or aiming for single-EV characterization[149]. Salient EV-based marker profiling studies focused on other cancer-related effects, such as linking liver metabolic reprograming with adverse effects of chemotherapy in mouse models, might be suitable for development into clinical assays[160]. Profiling of circulating EVs from 20 patients with ovarian cancer and 10 age-matched individuals without cancer highlighted EV FRα as a potential biomarker for the early detection and monitoring of ovarian cancer[211]. Elsewhere, an EV-based molecular profiling analysis referred to as RExCuE was found to enable longitudinal assessments of ongoing responses to anticancer therapies, including androgen receptor signalling inhibitors and taxanes[157], highlighting the clinical feasibility of comprehensive EV profiling to inform the management of patients with advanced-stage cancer.

Conclusions

Knowledge of the potential role of EVs as biomarkers enabling early cancer detection, monitoring of disease progression or responses to treatment and the development of metastasis has made tremendous leaps in the past decade. This progress is evident from our understanding of onco-EVs and the development of various high-sensitivity, high-throughput and high-resolution techniques appropriate for the isolation of onco-EVs[397,398] (comprising, for example, ~1–0.01% of total EVs in blood[124,355]) as well as various high-sensitivity methods for analysing EV cargoes[78,148,157,158,160,165,215,345,363] and for optimizing the loading of cancer drugs and EV bioengineering strategies for targeted therapies.

A diverse range of strategies for engineering EVs for anticancer therapy are emerging. These approaches might overcome the challenges associated with the use of natural EVs as engineered cancer-based therapeutics, including their scalable generation, heterogeneity, cellular uptake efficiency and deficient loading, with hybrid engineered

Glossary

Bulk EVs
The entire vesicular population (including onco-extracellular vesicles and all other subtypes) present in biofluids, originating from multiple organs, tissues and cell types.

Exosomes
A major class of extracellular vesicle (typically 30–150 nm in diameter) of endocytic origin released by all cell types following fusion of multivesicular bodies with the plasma membrane.

Extracellular vesicles
(EVs). Lipid membrane-encapsulated particles released by cells into the intercellular space and/or circulation that enable bidirectional cell–cell communication. EVs comprise various subclasses based on their molecular cargo, biogenesis and biophysical properties.

Liquid biopsy
Analysis of blood samples to identify circulating cancer biomarkers that can aid in clinical diagnosis and disease prognosis.

Microparticles
A major class of membranous extracellular vesicles (typically, 50–1,500 nm in diameter) formed by direct budding from the plasma membrane; microparticles can also be known as microvesicles and ectosomes.

Nanoparticles
Synthetic, lipid-based, polymeric and inorganic nanostructures (typically <100 nm in diameter) that determine their functionality, activity and utility for drug delivery applications.

Nanoscopy
Use of light microscopy technique with diffraction-unlimited spatial resolution, which produces high-resolution images at the nanometre scale (also known as single-molecule localization microscopy or super-resolution microscopy).

Nanotherapeutics
The use of nanotechnology to design and deliver drugs and devices to treat a wide range of diseases, improve drug delivery and reduce toxicity.

Non-vesicular extracellular particles
(NVEPs). Non-extracellular vesicle particles include plasma lipoproteins, protein aggregates, supermeres, exomeres and even viruses.

Onco-EVs
Cancer cell-derived extracellular vesicles (EVs) that contain oncogenic cargoes (such as EV surface proteins, antigens, intracellular proteins, lipids, metabolites, RNAs, DNA fragments and others) that might augment cancer progression.

Pre-metastatic niche
A microenvironment induced by factors released from the primary tumour in a distant organ that supports metastatic cell seeding, survival and outgrowth. Such factors include tumour cell-derived exosomes and extracellular vesicles.

EVs having improved therapeutic delivery, tumour penetrance[322] and immunoregulation[323,324]. EV cargo modification approaches such as indirect passive loading, chemical transfection and genetic engineering have also all been widely used[399], in addition to the integration of other approaches for cargo loading including click chemistry, cloaking and biocoupling[173]. Bioengineering techniques that alter the surfaces of EVs, such as aptamers, click chemistry and covalent binding interactions, enzymatic conjugation, ligand–receptor binding or membrane fusion have all been used to improve EV delivery to targeted cells and to regulate immunoreactivity, intracellular uptake and to enhance tissue and/or tumour penetrance[251,297]. Bioengineering strategies to overcome challenges such as drug toxicity and that enable more specific tumour targeting for therapeutic applications warrant further attention. Multidisciplinary collaboration in areas such as enhancing bioengineering strategies, modelling cellular interactions and strategies such as AI virtual cell as multiscale, multimodal large-neural-network-based models that can represent and simulate the behaviour of molecules, cells and tissues — and potentially EVs — across diverse states will probably be required to facilitate the development and clinical translation of EV-based approaches.

Onco-EVs, which are released directly from tumour cells, have immense clinical potential as cancer biomarkers[150]. Onco-EVs can provide information on tumour biochemical features and origins as well as enabling detection at early disease stages[124,150,363,366]. Encouragingly, several studies from the past few years have demonstrated the feasibility of capturing and identifying circulating onco-EVs from specific cancer types, with the potential to leverage these EVs as biomarkers[129,150,158,356] as well as the potential for EV capture and/or analysis using a growing range of physical and/or biological characteristics. Together, these technological advances now enable the profiling of multiple analytes from EVs, which in turn enable the identification of rare cancer biomarkers in bodily fluids, including blood and urine[78,210,400,401]. These advances, coupled with evolving AI-enhanced and machine learning technologies[147,376,401] that integrate different marker types in multimodal tests, are accelerating progress in EV-based diagnostics[210,362,402]. This field is now at an exciting juncture in terms of addressing how we apply EV-based tumour-targeted therapies and in the development of EV vaccines. The availability of EV-based diagnostic platforms is likely to change how we detect, monitor and manage cancer, and, importantly, our understanding of how EVs regulate the oncogenic programmes that drive and influence the development of cancer. The numerous trials underway or already completed as well as ever-increasing amounts of preclinical data accompanied by technological advances as well as expanding investment interest promise to usher in EV-based approaches as clinically translatable platforms in the foreseeable future.

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