Book Chapter

The Role of Extracellular Vesicles in the Intercellular Transfer of Cancer Hallmarks and Drug Resistance Traits

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Abstract

Extracellular Vesicles (EVs) are nanosized particles enclosed by a lipid bilayer, that are secreted by almost all cell types within the body into the extracellular space, both in physiological and pathological conditions. EVs carry complex molecular cargos, such as proteins, nucleic acids and lipids, which can be delivered to recipient cells, thus mediating intercellular signaling and communication between cells. Importantly, the cargo of EVs reflects the nature and status of the cells of origin.

As a result of the exponential interest in this field of science, various techniques for isolating and characterizing EVs have been introduced. Each methodology has distinct advantages and disadvantages, and the selection of the most appropriate one depends on the type of sample used and the purpose of the study.

Nevertheless, different techniques and procedures have been implemented in different laboratories, which makes it difficult to make comparisons between studies. For this reason, the international scientific community has been making efforts to standardize the methodologies for the isolation and characterization of EVs, as well as the terminology used.

Several studies highlighted the role of EVs released by tumor cells in promoting proliferation, survival, angiogenesis, migration and metastasis of malignant cells, as well as in the intercellular transfer of cancer drug resistance traits. Indeed, these small particles were demonstrated to interfere with different "Hallmarks of Cancer".

Due to the high abundance of EVs in various biological fluids and their ability to protect the molecular cargo from external enzymatic degradation, EVs have gained great prominence as a novel source of cancer biomarkers in liquid biopsies. Many reports revealed the importance of plasma EVs to predict disease progression as well as the emergence of drug resistance. In fact, the biological properties of EVs have recently raised the possibility of developing either EV-based diagnostic or therapeutic tools.

Keywords

Extracellular Vesicles; Extracellular Vesicles Cargo; Methods of Isolation and Characterization of Extracellular Vesicles; Cancer; Intercellular Transfer of Cancer Hallmarks and Drug Resistance

List of Abbreviations

ABC- ATP-binding Cassette; AKT- Protein Kinase B; CAFs- Cancer-associated Fibroblasts; cfDNA- Circulating Cell-Free DNA; circRNA- Circular RNA; CTCs- Circulating Tumor Cells; ctDNA- Circulating Tumor DNA; ctRNA- Circulating Tumor RNA; DCs- Dendritic Cells; DgUC- Density Gradient Ultracentrifugation; DLS- Dynamic Light Scattering; DNMT- DNA Methyltransferase; ECM- Extracellular Matrix; ECs- Endothelial Cells; EGFR- Epidermal Growth Factor Receptor; EM- Electron Microscopy; EMT- Epithelial-to-Mesenchymal Transition; Enos- Endothelial Nitric Oxide Synthase; ESCRT-

Endosomal Sorting Complex required for Transport; EVs- Extracellular Vesicles; FBS- Fetal Bovine Serum; FGF-2- Fibroblast Growth Factor 2; FOXO- Forkhead Box O; hnRNPA2B1- RNA Binding Protein; HSP- Heat-Shock Proteins; PD-L1- Programmed Death-Ligand 1; P-gp- P-Glycoprotein; PTEN- Phosphatase and Tensin Homolog; PKM2- Pyruvate Kinase M2; S100A4- S100 Calcium-Binding Protein A4; SEC- Size-Exclusion Chromatography; SMAD3- SMAD Family Member 3; SOCS3- Suppressor of Cytokine Signaling 3; SUMO- Small Ubiquitin-Related Modifiers; TAMs- M2-like Tumor-Associated Macrophages; TEM- Transmission Electron Microscopy; TGF-β- Transforming Growth Factor-β; TME- Tumor Microenvironment; TNF-α- Tumor Necrosis Factor α; UC- Ultracentrifugation; UF- Ultrafiltration;VEGF- Vascular Endothelial Growth Factor; WB- Western Blotting

Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) are cell-released nanosized particles ranging in size from 30 to 5000 nm, enclosed by a lipid bilayer, which do not contain a functional nucleus and therefore cannot replicate [1-3]. The first evidence for the existence and potential function of EVs emerged from research in coagulation. Indeed, EVs were initially described in 1946 by Chargaff and West as platelet-derived procoagulant particles and in 1967 by Wolf, as “platelet dust” [4-6]. Nevertheless, the term “extracellular vesicles” was only introduced in 1971, when Aaronson first recognized EVs biogenesis as a biological phenomenon [7]. Since then, our understanding of the physiological and pathological roles of these small structures has grown at an exponential rate. In fact, beyond the accumulating evidence regarding the involvement of EVs in cellular waste removal, these particles are now recognized as key mediators of intercellular communication by transferring biological molecules – proteins, lipids, metabolites or nucleic acids – from donor cells to recipient cells [8-11]. Accordingly, it is now widely accepted that EVs play a critical role in the regulation of several physiological processes, including blood coagulation, tissue repair, immune response, neuronal response and reproduction [8]. Moreover, their role in the development of specific pathologies, such as cancer, has been extensively studied [12].

Indeed, it is widely accepted that EVs participate in a variety of oncological processes, including cancer initiation, promotion and progression, through intervention in the different "Hallmarks of Cancer" [13,14].

Importantly, EVs are secreted into the extracellular space by nearly all cell types within the body, and can be recovered from a wide range of biological fluids, including blood plasma, saliva, cerebrospinal fluid, breast milk, urine and feces [15-21]. Furthermore, the lipid bilayer surrounding EVs protects their cargo from external enzymatic degradation, increasing their ability to travel long distances when compared to free proteins, nucleic acids and lipids [14,22]. These characteristics, along with their intrinsic cell targeting properties and ability to cross physiological barriers, have raised the possibility of using these small structures as drug delivery systems. Furthermore and interestingly, EVs' cargo may reflect the molecular signature of their cell of origin, which contributed to the recognition of EVs as attractive sources of disease-related biomarkers [23-25].

Altogether, the particular biological properties of these cell-released particles suggest the potential of EV-based therapies and diagnostic tools, which has been reflected in the exponential increase in the number of studies conducted in the field over the last decade [14,26,27].

EVs Biogenesis

EVs can be broadly classified into three main classes, namely exosomes, microvesicles (MVs) and apoptotic bodies, based on their sizes and biogenesis [2,15]. Notably, EVs are highly heterogeneous, since different cell types are capable of producing diverse subtypes of these vesicles, under normal conditions or under different stimuli [14,28].

Exosomes have a size range between 30 to 150 nm, being formed by inward budding of the limiting membrane of early endosomes, which mature into multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) [15,29,30]. Ultimately, ILVs are secreted into the extracellular milieu as exosomes, upon MVBs fusion with the cell's plasma membrane (Figure 1) [15,29,30]. Alternatively, MVBs fuse with lysosomes for

degradation [15,30]. The mechanisms underlying the balance between the fusion of MVBs with the plasma membrane or with lysozymes remain poorly understood, but Rab GTPase and tetraspanins are thought to be involved [30,31]. In addition, it has been reported that the formation of MVBs and ILVs, together with their release, are usually controlled by the endosomal sorting complex required for transport (ESCRT), a series of four protein complexes (ESCRT-0, I, II, III) and other accessory proteins [31,32]. Some cells also secrete exosomes through ESCRT independent pathways, which involve the sphingomyelinase enzyme, cholesterol, or tetraspanins (such as CD9 and CD81) [15,28].

As for MVs, also termed ectosomes, shedding vesicles or microparticles, their size typically ranges from 100 to 1000 nm in diameter and they are released through direct outward budding and shedding of the plasma membrane (Figure 1) [15,29,30]. This separation process is promoted by actin-myosin interactions with subsequent ATP-dependent contractions [30,33]. The shedding of MVs includes rearrangements in the asymmetry of phospholipids within the plasma membrane, due to an increased level of intracellular Ca^{2+} and cytoskeleton reorganization [28,31]. Interestingly, several molecular complexes participate in the biogenesis of both MVs and exosomes, including the ESCRT proteins and the conversion of sphingomyelin to ceramide, even though MVs and exosomes are originated at different sites within the cell [30].

Although both exosomes and MVs were originally considered structures dedicated solely to cellular waste disposal, i.e., ways for cells to dispose of unneeded or unwanted material, they have more recently been recognized as vital mediators of intercellular communication, participating in numerous essential physiological and pathological processes [8,15,27,28].

Finally, apoptotic bodies are the largest class of EVs, with the majority of them ranging in size from 100 to 5000 nm in diameter [29,34]. In contrast to exosomes and MVs, which are generated mostly by live cells, apoptotic bodies are originated from dying cells, more specifically via the outward blebbing and decomposition of the cell membrane during the late phase of

apoptotic programmed cell death (Figure 1) [14,29,34]. Of the three EV subtypes, apoptotic bodies are the ones less relevant for cell-cell communication [14].

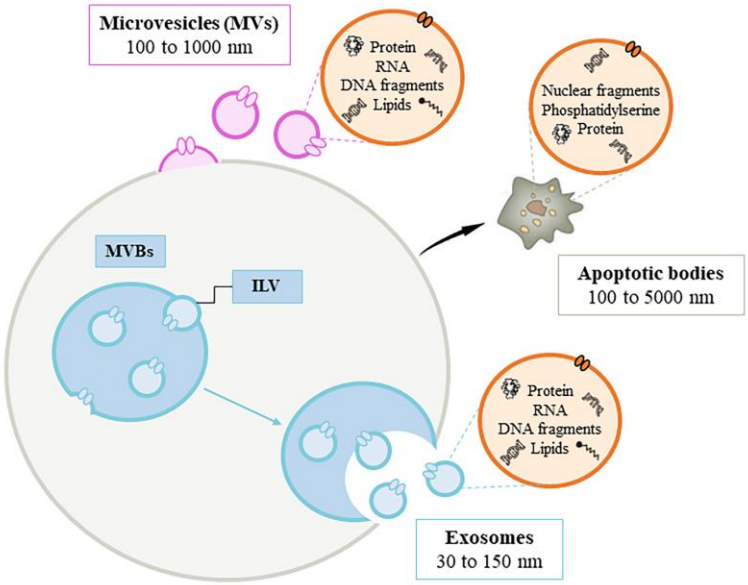

Figure 1: Biogenesis of the different subtypes of extracellular vesicles (EVs). Exosomes are released via exocytosis of intraluminal vesicles (ILVs), which are contained within multivesicular bodies (MVBs). Microvesicles (MVs) are formed via budding of the plasma membrane. During apoptosis, dying cells can also release apoptotic bodies. EVs contain proteins, nucleic acids, and lipids in their cargo.

It is noteworthy that the similar features of exosomes and MVs, such as the overlapping sizes of the smallest MVs with exosomes and the sharing of many surface markers, hinders the accurate discrimination of their origin [14,27]. Moreover, currently available isolation methods do not allow single EV analysis with a high degree of purity [27,32]. Importantly, given the absence of consensus on the nomenclature and the difficulty in understanding their molecular mechanisms of biogenesis and release, the *International Society for Extracellular Vesicles* (ISEV) published the MISEV2018 guidelines that advises researchers to name all subtypes of extracellular vesicles as "general" EVs, and classifying them taking into consideration

their size, density, presence of specific surface markers, biochemical composition, or cell of origin, unless their biogenesis pathway is well established [14,25,35].

Cargo of EVs

Due to the mechanisms through which exosomes and MVs are generated, it is easy to understand that their cargo reflects protein, nucleic acid and lipid signatures of their parental cells [2,36]. Furthermore, the molecular content of EVs and their biological function will depend on the originating cell type [37]. For instance, exosomes released by dendritic cells contain co-stimulatory proteins required for T-cell activation, but those released by tumor cells do not [37]. Importantly, the encapsulation of the cargo in a lipid bilayer membrane confers to this cargo: 1) higher stability, 2) higher resistance to external enzymatic degradation and 3) greater ability to travel long distances when compared to free proteins, nucleic acids and lipids in the plasma [14,22]. Additionally, EVs can cross physiological barriers within the body, such as the blood-brain barrier, thus delivering their cargo to recipient organs and cells protected by them [14]. Currently, the sorting of molecular cargo into EVs is still poorly understood [31]. However, according to recent studies, this is a regulated process that leads to the enrichment or depletion of EVs in specific cargo types, and a few selective mechanisms have been described [25,31].

Proteins

Accordingly to the ExoCarta and Vesiclepedia databases, more than 10 000 unique proteins were detected in EVs from various sources, representing almost half of the human proteome [31,38]. The protein composition of EVs is related to the cell type of origin and mode of biogenesis [39]. In fact, small EVs tend to be enriched in tetraspanins CD9, CD63 and CD81, ESCRT accessory proteins (such as Alix and tumor susceptibility gene protein 101), syntenin-1 and ADAM10, while medium and larger EVs are enriched in actinin-4 and mitofilin [39,40]. In general, MVs tend to be more enriched than exosomes in integrins, glycoprotein Ib and P-selectin, for example, due to their plasma membrane origin, whereas apoptotic bodies contain DNA-

binding histones and are depleted in glycoproteins [35,39]. Like exosomes, MVs contain tetraspanins CD9, CD63 and CD81, as well as proteins with post-translational modifications [15,31]. Moreover, other proteins like major histocompatibility complex class II (MHC-II) proteins, flotillin-1 and heat-shock 70-kDa proteins (HSP70) are similarly present in all types of EVs and can, therefore, be used as general EV markers [39,40].

Emerging evidence indicates that several post-translational modifications are implicated in protein sorting into EVs [31,41]. Ubiquitination is one of the most essential signals for sorting various proteins into exosomes, as for example the oncogenic protein EGFR (epidermal growth factor receptor) [31,42]. In this process, ubiquitin is added to the lysine residues on the target proteins through isopeptide bonds, recruiting the ESCRT machinery and thus facilitating the sorting of transmembrane cargos into ILVs [42,43]. SUMOylation, which is the biding of small ubiquitin-related modifiers (SUMO), alters protein localization in the cell, representing another post-translational modification crucial for EV packaging [31,41]. Recently, sumoylated RNA binding protein (hnRNPA2B1) has been reported to recognize and sort specific microRNA (miRNA) motifs into EVs [31,44]. Similarly, α-Synuclein, a neurotoxic protein that plays a role in the pathogenesis of Parkinson's disease, was demonstrated to be sumoylated and loaded into EVs [31,44].

Furthermore, phosphorylation also participates in the selective targeting of proteins to EVs, as demonstrated by the selective incorporation of Annexin A2, a Ca^{2+}-dependent phospholipid-binding protein, into the luminal membranes of the endosomes, which requires tyrosine 23 phosphorylation in order to escape the endosomal degradation pathway [43,45]. Similarly, phosphorylation at tyrosine 14 of the cell surface protein caveolin-1 leads to the interaction with hnRNPA2B1, and the complex is subsequently incorporated into EVs, along with the hnRNPA2B1-bound miRNAs [43,46]. Furthermore, it has been shown that EVs are enriched in carbohydrate modifications, such as high mannose, polylactosamine and other complex *N*-linked glycans, when compared with their parental cells, thus implying

a role for glycosylation in EVs recruitment through oligomerization of specific glycoproteins [47].

Another mechanism of protein sorting involves citrullination, a type of post-translational modification that leads to structural and functional changes on a protein by altering their positively charged amino acid arginine into citrulline, a conversion that is catalyzed by the peptidyl arginine deiminase family of enzymes [43,48]. Certain citrullinated proteins, such as fibronectin, α2-macroglobulin and fibrinogen fragment D were detected in EVs isolated from synovial fluid of rheumatoid arthritis patients; however, it remains unclear whether there is a selective sorting of citrullinated proteins into EVs [31,49].

Moreover, some studies have highlighted the possible role of oxidation in EV cargo sorting [31,50]. For instance, α-Synuclein in its oxidized state was shown to be sorted into EVs, however, oxidation can also occur during sample processing, so further investigation is needed [31,50].

Apart from post-translational modifications, other mechanisms that involve protein domains, like the WW domain and coiled-coil domain, have been implicated in the sorting of proteins into EVs [31].

RNA

EVs can also carry a variety of different types of RNAs, such as mRNA, miRNA, and other non-coding RNAs [51,52]. Of note, the RNA content of EVs differs substantially from the ones found in their cells of origin, which suggests that the packaging of RNA into EVs is actively regulated and that RNA is selectively incorporated, since specific sequences are either preferentially retained or sorted inside the cells [51,52]. Multiple mechanisms have been described to be implicated in the specific loading of RNA into EVs, including recognizing the GGAG motif in the 3' portion of miRNA sequences and subsequent sorting into EVs by RNA-binding proteins, such as sumoylated hnRNPA2B1[52,53].

There is also some evidence that miRNA sorting is dependent on the 3'-end of the miRNA, as shown by the 3'-end adenylated

miRNAs that are relatively enriched in cells, while 3'-end uridylated isoforms are enriched in EVs from human B cells or urine [53,54]. In addition, a study using macrophages demonstrated that there is a dependency on the levels of miRNA targets in the cells cytoplasm, with a negative correlation between miRNA/target interactions in the producer cells and miRNA enrichment in EVs [53,55]. Argonaute 2, a miRNA-induced silencing complex protein, has been reported to exert some control in miRNA sorting [51]. Moreover, the inhibition of neutral sphingomyelinase-2, a protein responsible for the synthesis of ceramide, leads to a decrease in EVs production and also a decrease in the miRNA levels inside EVs, which suggests that the ceramide pathway may be involved in miRNA loading [56]. Furthermore, it has been demonstrated that many colorectal cancer cells have mutations in the oncoprotein KRAS (Kirsten rat sarcoma viral oncogene homolog), and those cells released EVs with distinct miRNA profiles depending on the KRAS mutation status, thus suggesting the existence of a KRAS-dependent miRNA sorting [57].

DNA

Recent studies have reported that EVs contain multiple types of DNA fragments, including genomic and mitochondrial DNA, with these fragments representing all chromosomes of parental cells and reflecting their mutational status [58,59]. Even though the localization of this DNA is not fully understood, some evidence support that it can be found on the EVs surface, where it facilitates EVs binding to the extracellular matrix protein fibronectin, or in their lumen [60-62]. In addition, different amounts and types of DNA are found in various subsets of EVs, even in those from the same cellular source [58]. Currently, it remains elusive whether or not there is a sequence-specific mechanism of loading DNA into EVs [63].

Lipids

The lipidomic analysis of EVs cargo has shown that EVs are often enriched in cholesterol, sphingomyelin, glycosphingolipids and phosphatidylserine, whereas phosphatidylcholine and diacylglycerol are less abundant in EVs than in their cells of

origin [64]. Nevertheless, there is little knowledge about how lipids are targeted to the EVs, being reported that protein and lipid loading into EVs are done independently of each other [65]. Moreover, differences in lipid composition are thought to be related to the biogenesis of the different types of EVs [65,66].

Isolation and Characterization of EVs

Despite emerging evidence that suggests the potential usefulness of EVs as biomarkers in the context of liquid biopsies, the protocols used for isolation and characterization of EVs need to be optimized and standardized. At present, the use of distinct procedures between laboratories is generating controversial data, with uncertain biological relevance [41,67].

Methods to Isolate EVs

Currently, there are diverse techniques for isolating EVs from cell culture conditioned media or physiological fluids, such as differential ultracentrifugation (UC), density gradient ultracentrifugation (dgUC), ultrafiltration (UF), size-exclusion chromatography (SEC), precipitation-based methods, immunoaffinity capture and microfluidics. Each methodology has its own advantages and disadvantages, and therefore, depending on the objective, it is important to select the most appropriate methodology (Table 1) [41,68].

Regarding the differential UC methodology, this method consists of a series of centrifugations with increased forces and durations, intended to remove contaminating material at lower speeds and to pellet the EVs at higher speeds [41,68,69]. Accordingly, larger EVs are first separated by gradual centrifugal forces between 200 to 10000 × g and afterwards a final ultracentrifugation step at 100000 × g is applied to isolate small EVs [33,68,70]. Fetal bovine serum (FBS) is frequently used in cell culture experiments, and since FBS-derived EVs may influence experimental results, those EVs are often removed from the FBS by using an UC-based depletion protocol [71,72]. Of note, there are discrepancies in the efficiency of EVs isolation by UC between different research groups due to the use of different types of rotors, acceleration (g) and the viscosity of the samples

[2,69]. Some advantages of UC are: 1) the capability to process large sample volumes, 2) ease of manipulation; 3) affordability, and 4) the non-use of reagents or additional chemicals that might affect the subsequent EVs analysis [2,41]. Nevertheless, this technique also has several disadvantages [68]. The use of differential ultracentrifugation requires higher volumes, which is only possible when isolating EVs from cell culture conditioned media, becoming a problem when the source of EVs is biofluids [68]. This technique is also time-consuming and a major downside is the impossibility to obtain pure EVs, free of contaminants, and subsequently, EVs tend to form aggregates with different protein complexes [2,68,73].

Another methodology to isolate EVs is the dgUC, which can be employed after differential centrifugation, typically using sucrose as a density gradient medium, to separate EVs from protein aggregates and non-membranous particles, thus improving EVs preparations purity [15,41,73]. Studies have demonstrated that this method enhances the yield of EVs proteins and nucleic acids after isolation, when compared to differential UC [2]. However, dgUC is more complex and requires longer processing time. The considerable EVs losses that may occur during the isolation process is also a problem [2,15]. Moreover, some contaminants that have the same density of EVs, such as some viruses and albumin, may be co-isolated with EVs [71].

Another approach used is the combination of differential ultracentrifugation with ultrafiltration, to improve the purity of the EVs preparations [41,71,73]. Ultrafiltration relies on the use of membranes with specific pore sizes to filter out unwanted particles, leaving a relatively concentrated filtrate of EVs [2,73]. This option is frequently used to isolate EVs from relatively dilute samples, such as urine and cell cultures, and presents several advantages, such as 1) the simplicity of the procedure, 2) low cost, and 3) the relatively lack of limitations on sample volume [2,70,73,74]. However, significant sample losses due to the irreversible binding of EVs to filtration membranes, resulting in a lower yield, might occur [2,73]. Moreover, non-EVs proteins and biopolymers can block the pores, which not only slows down the filtration process but also leads to the

accumulation of contaminants and decreases the efficacy of EVs isolation [2]. In addition, EVs may become deformed due to the pressure and contact with the filter membrane [2].

More recently, due to an increase in interest in EVs research, the use of alternative techniques, such as SEC, precipitation-based methods, immunoaffinity capture, and microfluidics has been documented [41,68]. For instance, SEC makes use of porous beads to selectively separate fractions containing EVs of different sizes [68,70]. Some studies have shown that this technique yielded EVs derived from plasma with better functionality compared to ultracentrifugation [71,74]. In addition, SEC has been regularly applied following differential ultracentrifugation and ultrafiltration, resulting in higher EVs purity, preventing EVs aggregation, and preserving EVs structural integrity [41,71,73]. This approach is suitable for EVs cargo characterization in hematological malignancies in the context of liquid biopsy [73]. A downside of SEC is that it dilutes samples, hence requires EVs to be re-concentrated following isolation, which may reduce the EVs yield [2,41]. In addition, it can be very time consuming and the yield of recovery is low [73].

On the other hand, precipitation methods, which use a hydrophilic polymer solution or organic solvents, tend to result in a higher yield of EVs recovery but relatively low purity because of coprecipitation with contaminants, such as non-EVs proteins, viruses, and other particles [2,41,73,75]. Nevertheless, this isolation method is quick, simple, and cost-effective, but lacks selectivity [15,68].

The immunoaffinity-based methods, which rely on specific EVs surface proteins that can be captured by their corresponding antibodies, present greater purity but isolate a particular sub-set of EVs, which present the selected surface proteins [2,15,68]. This process aids in the isolation of specific EVs subpopulations, but it is not suitable for large sample volumes, is expensive, and EVs elution can be difficult, potentially impacting EVs structural integrity [2,15,68].

Finally, microfluidics methodology allows the separation of EVs based on their physical and biochemical properties simultaneously by employing multiple EVs isolation techniques into a single compact chip device [2,15]. Of note, additional off-chip processes can be needed for sample preparation and reagent mixing [68]. Despite many advantages, such as effectiveness, speed, high throughput and the fact that it requires smaller volumes, this approach also has several disadvantages, including complexity of devices and shear stress on EVs, which must be addressed before being translated to clinical use [2,68].

Table 1: Summary of advantages and limitations of EVs isolation methods [2,41,68,73].

Methods to isolate EVs	Advantages	Limitations
Differential ultracentrifugation (UC)	- Large sample capacity - Ease of manipulation - No additional chemicals	- Requires high volumes - Time-consuming - Contamination with protein aggregates
Density gradient ultracentrifugation (dgUC)	- High purity - Enhanced yield comparatively to UC - No additional chemicals	- Complex technique - Time-consuming - Sample loss - Contamination by particles with similar density of EVs
Ultrafiltration	- Simple procedure - Relatively lack of limitations on sample volume - Low cost	- Limited filter lifetime - Lower yields due to sample loss - Contamination by non-EVs proteins - Distortion of EVs
Size-exclusion chromatography (SEC)	- High purity - Prevents EVs aggregation - Preserves EVs structure	- Dilution of samples - Lower yield - Potential contamination with non-EV particles (as lipoproteins)
Precipitation-	- High yield	- Low purity

based methods	- Simple technique - Rapid - Low cost	- Coprecipitation with non-EVs proteins, viruses and other particles
Immunoaffinity capture	- High purity - High specificity for the selected target proteins	- Difficult to isolate EVs from large sample volumes - High cost - Limited yield - Possible loss of EVs structural integrity
Microfluidics	- Effectiveness - Replaces bulky equipment - Requires small sample volumes - Rapid, automatized	- Complexity of devices - Stress on EVs - Requires off-chip processes

Methods to Characterize EVs

The characterization of isolated EVs is an important step to confirm their nature and purity [68,70]. Several methods are applied to characterize the EVs isolated: transmission electron microscopy (TEM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), Western blotting (WB) with appropriate markers, and flow cytometry following the immobilization of EVs on the surface of beads [68,70].

TEM is effective in determining the size and morphology of individual EVs [2,15]. Usually, the rounded structure of EVs is analyzed by cryogenic electron microscopy techniques to surpass limitations, such as the need for fixation and dehydration of the sample, which can result in the deformation of EVs [2,15,68]. Currently, cryogenic TEM is considered to be the gold standard technique as it has significantly higher resolution, allowing the distinction between EVs and similarly-sized non-EVs particles that may reside in the sample after isolation, and providing visualization of the spatial arrangements within EVs [2,70]. Moreover, combining TEM and immunolabeling allows the characterization of EVs surface proteins [2,68].

DLS measures the size of particles according to their Brownian motion in solution, which is based on the fact that lighter particles diffuse faster [2,70]. DLS analyzes the different wavelengths and intensities of scattered light from EVs, providing information on the size distribution of EVs [2,70]. This method is also used to determine the zeta potential of EVs, which refers to the relative stability of EVs in solution and is based on how fast the particles move during electrophoresis [2,70]. Although DLS presents simplicity and speed as advantages, its applicability is limited by its lack of specificity and lower accuracy for heterogeneous mixtures of EVs [2,70].

NTA enables efficient and reliable estimation of both particle size distribution and concentration of EVs also based on the Brownian motion [15,68,70]. In NTA, a laser beam illuminates particles in suspension and a camera captures the scattered light produced [2,15]. Afterwards, NTA software generates a high resolution size distribution on a particle-by-particle basis using the Stokes-Einstein equation, which determines the mean square displacement for each particle, and calculates the total concentration [2,15,70]. Notably, NTA can give the percentage of EVs by number of particles [15,70]. A major drawback is that this method cannot distinguish between EVs and a different particle, which warrants the need to subject the sample to prior isolation and purification [2,70]. Furthermore, NTA only has the highest precision for EVs that have a size range between 10 to 1000 nm in diameter [15,70].

Importantly, EVs protein content comprises both surface protein markers and cytosolic proteins [2,15]. The EVs surface marker phenotype gives insight into the cellular origin of EVs, thus allowing presumption of whether aberrant cellular processes are occurring even before the manifestation of clinical signs [2,15]. In WB analysis, EVs are first purified and lysed to release their cargoes, which are then detected using specific antibodies that bind to the target protein [2,15,73]. Although this methodology is simple and widely accessible, it requires a large amount of protein, it is only semi-quantitative, and is unable to deduce the heterogeneity within the EVs population [2,15].

The flow-cytometry methodology is an indirect optical detection method that performs multiparametric analysis of individual EVs, which are labeled with fluorophores to determine their quantity and phenotype [2,68,73]. Even though modern digital flow cytometers with enhanced sensitivity have detection limits between 200 nm to 1.0 μm, most instruments have a larger limit than the size of small EVs [2,15]. In addition, this method requires the immobilization of EVs on the surface of beads to avoid aggregation of vesicles during the isolation process, which leads to unreliable data because several particles are observed at once, as well as to increase their size in order to enable the detection by the flow cytometer [2,15].

Interestingly, proteomic analyses are also used to characterize EV-associated proteins [2,15]. However, it should be noted that the type of isolation method used changes the proteomic profiles of EVs [2,15,72]. The use of mass spectrometry (MS)-based proteomic tools in the field of EVs has been aiding in the development of biomarkers for different diseases including distinct cancers, based on the signature of the originating cells [15]. Moreover, high resolution, accurate and sensitive MS-based analyses allow the identification and quantification of thousands of EV's proteins [71,72]. Currently, the bottom-up MS is used worldwide as the strategy of choice, being combined with prior separation processes, such as 1D or 2D gel electrophoresis and liquid chromatography [71,72]. In this approach, proteins are digested with a protease, then the peptide ions are fragmented in the gas phase and their sequence and post-translational modifications can be deduced [15,71]. Regarding protein quantification, different strategies can be used, including 1) shotgun proteomics, in which peptide ions are heuristically selected for fragmentation using a data-dependent model, and 2) targeted proteomics, in which only predefined peptide ions are selected for fragmentation [15,72]. In fact, proteomic analysis has been used in several studies [71]. For instance, its application in a recent study revealed the presence of chitinase 3-like-1 and fibronectin in the cargo of EVs released by macrophages [76]. These two proteins were shown to increase pancreatic ductal adenocarcinoma cells resistance to the anticancer drug gemcitabine, being suggested as promising molecular targets for therapeutic intervention [76]. In another study, using EVs

proteomic analysis, the phagocytic glycoprotein-1 (CD44) was identified as a novel marker of overall survival associated with multiple myeloma [73]. Moreover, a recent study used a proteomic approach to analyze the protein cargo of EVs shed by acute myeloid leukemia cell lines in an attempt to identify new biomarkers of disease [77]. Many other similar studies on proteomic analysis of the EVs cargo from different cell types have been published in recent years [71].

The Role of EVs Released by Tumor Cells in the "Hallmarks of Cancer"

Cancer is classically defined as a complex and heterogeneous group of diseases in which cells acquire a specific set of characteristics, the so-called "Hallmarks of Cancer", dictating their progressive transformation into malignant neoplastic cells. The "Hallmarks of Cancer", initially described by Hanahan and Weinberg (2000) and recently updated by Hanahan (2022), include sustained proliferative signaling, evading growth suppressors, resisting cell death, inducing and/or accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, avoiding immune destruction, unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes and senescent cells [78-80]. The development of these malignant features is dependent on cell-to-cell communication, within the tumor and between the tumor and its extracellular matrix or surrounding stromal cells. EVs have been identified as important mediators of these intercellular communications, acting over distinct "Hallmarks of Cancer" [14,81].

The role of tumor-derived EVs in tumor colonization and progression has been increasingly reported, with these EVs promoting tumor proliferation, angiogenesis, invasion, metastasis and immunosuppression (Figure 2) [33,82]. Literature has extensively described the involvement of EVs on the horizontal transfer of oncogenic cargo content from cancer cells to the surrounding tumor and stromal cells, thus facilitating tumor development and invasion [33,82]. Moreover, tumor-derived EVs are also able to transfer their cargo to a distant site, supporting the pre-metastatic niche formation and tumor

metastasis (Figure 2) [82]. Interestingly, it has been demonstrated that cancer cells release more EVs than normal cells and that the cargos of EVs derived from cancer cells also differ from those released by non-neoplastic cells [14,25,83-85].

Cell Proliferation

A phenomenon observed in many cancer cell types, such as bladder and gastric cancers, is the activation mediated by EVs of tyrosine kinase receptors and their downstream signaling pathways, such as MAP/ERK and PI3K/AKT, conferring tumor cells the ability to proliferate and avoid apoptosis [86,87].

The active transfer of onco-miRNAs also contributes to tumor cell proliferation [88,89]. For instance, in breast cancer cells, EVs transfer miR-1246 to other cancer cells, suppressing cyclin-G2 levels, thus affecting cancer cell growth [88,89]. In human nasopharynx cancer, EVs contain different miRNAs, such as miRNA-106a-5p and miRNA-891a, promoting cell proliferation through downregulation of the MARK1 signaling pathway [88,89].

Similarly, long non-coding RNAs (lncRNA) present in the cargo of tumor-derived EVs may also favor cancer cell proliferation [90,91]. For instance, the lncRNA ZFAS1 in EVs shed by gastric cancer cells and lncRNA PVT1 in EVs released by colon cancer cells promote the progression of gastric and colorectal cancers, respectively [90,91].

Cell Death

Apoptosis, a mechanism of programmed cell death, is a tightly regulated process that acts to selectively eliminate damaged or abnormal cells [92]. The deregulation of this homeostatic mechanism is one of the most remarkable features of cancer and tumor-derived EVs have been demonstrated to play an important role in it [14,93].

For instance, inhibitors of apoptosis (IAPs), including survivin, were found in the cargo of cancer-derived EVs, promoting both tumor- and tumor microenvironment (TME)-cellular resistance to apoptosis. For example, cervical cancer HeLa cell secretion of

survivin supported recipient cancer cell proliferation and evasion from apoptosis [94]. The detected extracellular survivin was further demonstrated to be exosome-encapsulated [95]. Similarly, an upregulation of survivin levels in the cargo of MDA-MB-231 cell-released EVs was reported following treatment with paclitaxel [96]. When treated with these cancer-derived EVs, serum-deprived paclitaxel-treated fibroblasts and SKBR3 breast cancer cells demonstrated a significant apoptosis reduction, which was proven to be strongly related to survivin through siRNA-guided gene specific silencing. Interestingly, it was recently demonstrated that pancreatic ductal adenocarcinoma (PDAC) patient-derived exosomes express higher levels of survivin, than those of non-PDAC patients [97]. Accordingly, the same study also demonstrated that exosomes released by *KRAS* mutated fibroblasts and PDAC cell lines were equally enriched in survivin and capable of increasing tumor cell survival in a survivin-dependent manner [97].

Furthermore, EVs shed by human bladder cancer cells inhibited cancer cell apoptosis by increasing the levels of anti-apoptotic proteins, such as Bcl-2 and Cyclin D1, and decreasing the levels of pro-apoptotic proteins, such as Bax and caspase-3 [98]. This effect was later shown to be linked to the activation of the ERK/Akt signaling pathway. In agreement, another study demonstrated that EVs released by colorectal cancer cells significantly reduced tumor cell apoptosis through activation of the ERK pathway [99].

Moreover, tumor-derived EVs were also reported to carry onco-miRNAs impacting tumor cell evasion to apoptosis. Indeed, miRNAs with the ability to inhibit apoptotic-related genes (*e.g.* miR-21-5p, miR-143-3p and miR-148-3p) were found in the cargo of EVs released by human osteosarcoma cell lines [100]. Also, EVs shed by colorectal cancer cells suppressed colorectal cancer recipient cells' apoptosis through the active transfer of miRNA-361-3p [101].

Remarkably, some studies demonstrated the ability of tumor-derived EVs to simultaneously suppress tumor apoptosis and increase the autophagic process [102-104]. Indeed, EVs released by PC-9 non-small cell lung cancer (NSCLC) cells following

treatment with gefitinib decreased the antitumor effect of the drug treatment, reduced apoptosis and induced autophagy [102]. Similarly, PD-L1-containing EVs released by stem cells increased glioblastoma cell resistance to temozolomide, activating AMPK/ULK1-mediated autophagy and reducing apoptosis [103]. Furthermore, EVs shed by human breast cancer cell lines (MCF-7 and MDA-MB-231) transferred miR-1910-3p to MCF-10A epithelial recipient cells, causing a decrease in apoptosis and activating the autophagic process [104].

Angiogenesis

Emerging evidence suggests that tumor-derived EVs also participate in tumor angiogenesis, a process that forms new blood vessels from preexistent capillaries, stimulated under hypoxia conditions [105]. Through angiogenesis, the tumor is provided with oxygen and other essential nutrients [82,85]. Therefore, angiogenesis is crucial for tumor expansion and metastasis, as tumor cells resulting from a primary tumor are transported to a distant locus via blood vessels to implant and grow into a secondary tumor [82,85].

Thus, tumor-derived EVs can carry pro-angiogenic factors, such as miR-135b, miR-210, miR-494, lncRNA H19, lncRNA CCAT2 and lncRNA POU3F3, which are transferred to vascular endothelial cells (ECs), promoting their proliferation and migration, thus facilitating angiogenesis [14,105]. Indeed, this intercellular transfer of EV's cargo, between tumor cells and ECs, leads to an upregulation of the vascular endothelial growth factor (VEGF) on recipient ECs [85,105]. For example, in classical Hodgkin's lymphoma and oral squamous cell carcinoma, the level of miR-494 was elevated and the angiogenic potential was encouraged via induction of the phosphatase and tensin homolog (PTEN) – protein kinase B (AKT) – endothelial nitric oxide synthase (eNOS) axis [85,105]. Likewise, tumor-derived EVs can deliver lncRNA H19 to ECs, leading to enhanced expression of the fibroblast growth factor 2 (FGF-2) and VEGF via nuclear factor kappa B (NF-κB) upregulation in hepatocellular carcinoma cells [85,105]. Moreover, it was reported that chronic lymphocytic leukemia cells released exosomal miR-21 and miR-146a, which target MSCs and ECs,

contributing to the production of pro-angiogenic molecules [85,105]. Similarly, prostate cancer cells released EVs that induce alterations in the ECM and promote angiogenic activity [85].

Invasion and Metastasis

As major players of intercellular communication, tumor-derived EVs and their associated cargo have a significant role in cancer invasion and metastasis, by stimulating tumor cell motility and migration, and interfering with the TME [106].

Indeed, tumor-derived EVs promoted tumor metastasis through the active intercellular transfer of different molecules involved in tumor cell motility and migration, such as lysyl oxidase-like 4 (LOXL4) [107], ErbB2/CRK [108], S100 calcium-binding protein A4 (S100A4) [109], Caveolin-1 [110] or SMAD family member 3 (SMAD3) [111]. In particular, EVs shed by human gastric cancer cells delivered EGFR to liver stromal cells and suppressed miR-26a and miR-26b to activate liver hepatocyte growth factor, when using *in vitro* co-culture models [89]. Using a mouse *in vivo* model, it was demonstrated that the upregulation of liver hepatocyte growth factor promoted hepatotropic metastasis. Likewise, EGFR-enriched EVs derived from highly metastatic nasopharyngeal cells upregulated EGFR and downregulated ROS in low metastatic cells through the PI3K/Akt signaling pathway. Furthermore, these EVs increased low metastatic tumor cell migration and motility, namely through promotion of the epithelial-to-mesenchymal transition (EMT) process [112].

Interestingly, tumor-derived EVs and their associated cargo have also promoted tumor metastization by inducing macrophage M2 polarization [113-115]. For instance, colorectal cancer cell-derived EVs carrying high levels of miR-934 promoted THP-1 macrophage M2 polarization. The M2 macrophages further stimulated colorectal cancer liver colonization *in vivo* [113]. Interestingly, EVs secreted by pancreatic cancer cells under hypoxic conditions expressed higher levels of miR-301a-3p, being able to induce macrophage M2 polarization, which in turn promoted migration, motility and, therefore, invasion of tumor

cells, by inducing a switch from an epithelial to a mesenchymal phenotype (EMT) [116].

The role of miRNAs, found in the cargo of tumor-derived EVs, on tumor metastasis has been extensively reported. For instance, exosomal miR-1260b and miR-660-5p were found upregulated in NSCLC patients' plasma, and pre-clinical models were used to demonstrate the ability of these exosomal miRNAs to promote a NSCLC metastatic phenotype through inhibition of HIPK2 and KLF9, respectively [117,118]. Similarly, higher levels of miR-423-5p and miR-222 were found in the cargo of EVs derived from gastric and breast cancer patients with lymph node metastasis, correspondingly [119,120].

Moreover, EVs shed by cancer cells have been identified as important mediators of intercellular transfer of miRNAs between highly metastatic donor cells and low metastatic recipient cells, increasing the latter's metastatic potential [121,122]. Indeed, exosomal miR-196a-1 derived from highly metastatic gastric cancer cells promoted low metastatic cells invasion and metastasis *in vitro* and *in vivo*, through inhibition of the tumor suppressor SFRP1 [121]. Accordingly, metastatic hepatocellular carcinoma cell-derived EVs transferred miR-92a-3p to low metastatic hepatocellular carcinoma cells, promoting their motility and invasion through the PTEN/Akt pathway [122].

Additionally, tumor-derived EVs also carry miRNAs that interfered with the TME remodeling and formation of a pre-metastatic niche, potentially promoting metastasis. A recent study demonstrated that miR-181a-5p-rich EVs, derived from highly-metastatic colorectal cancer cells, persistently activated hepatic stellate cells (HSCs), which were shown to secrete the chemokine CCL20. The latter activated the CCL20/CCR6/ERK1/2/Elk-1/miR-181a-5p positive feedback loop, contributing to colorectal cancer cell migration and invasion *in vitro* and to the formation of liver pre-metastatic niches *in vivo* [123]. Furthermore, highly metastatic ovarian cancer cell-derived EVs transferred miR-630 to normal fibroblasts, promoting their differentiation into CAFs via KLF6 inhibition and NF-kB signaling pathway activation, thus increasing low metastatic ovarian cancer cells ability to spread

through the EMT process [124]. Similarly, breast cancer cell-derived EVs transferred miR-146a to normal fibroblasts promoting their differentiation into CAFs through activation of the Wnt signaling pathway. This activation increased breast cancer cell invasion and metastasis [125]. Interestingly, metastatic osteosarcoma cell-derived EVs carrying miR-675 promoted fibroblast migration and invasion through downregulation of CALN1, which was strongly associated with osteosarcoma patient's metastatic phenotype [126].

Metabolic Reprogramming

Another remarkable feature of cancer is the reprogramming of cellular energy metabolism. It has been shown that tumor-derived EVs also play an important role in this hallmark of cancer [79,127].

Research demonstrated that pyruvate kinase M2 (PKM2) was highly present in the cargo of EVs derived from both prostate cancer cell lines and prostate cancer patients serum [128]. Importantly, a correlation between the elevated levels of PKM2 in prostate cancer patient-derived EVs and metastasis was found. Furthermore, using both *in vitro* and *in vivo* models, the same study revealed that EVs mediated the horizontal transfer of PKM2 from prostate cancer cells to bone marrow stromal cells, promoting an increase in CXCL3 production by bone marrow stromal cells which translated into increased seeding and growth of prostate cancer in the bone marrow. Moreover, a recent study showed that the co-culture of lung cancer cell lines with EVs derived from irradiated lung cancer cells increased lung cancer cellular growth and motility *in vitro* [129]. These effects were further related to an increase in the glycolytic activity of recipient cells, resulting from an elevated presence of the metabolic enzymes ALDOA and ALDH3A1 in the EVs.

Importantly, tumor-derived EVs were consistently shown to prompt tumor progression through the metabolic reprogramming of the tumor microenvironment, namely by promoting the formation of the so-called pre-metastatic niches [130]. Indeed, a recent study demonstrated that EVs derived from six melanoma cell lines contributed to extracellular acidification – critical for

pre-metastatic niche formation – through stimulation of a significant increase in aerobic glycolysis and reduction in oxidative phosphorylation (OXPHOS) in human adult dermal fibroblasts [131]. This metabolic reprogramming was further related to the presence of miR-155 and miR-210 in the cargo of the melanoma-derived EVs. Interestingly, a study from 2015 revealed that breast cancer cells suppressed glucose uptake by non-tumor cells in the pre-metastatic niche, through secretion of miR-122-enriched EVs [132]. The observed suppression of glucose uptake by non-tumor cells resulted in increased nutrient availability in the pre-metastatic niche and promoted breast cancer metastasis *in vivo*.

Immune Response and Inflammation

The stress of the TME, such as microenvironmental acidosis, interferes with the tumor immune escape and progression [28]. Tumor-derived EVs repressed the antitumor immune response by recruiting and inducing the differentiation of immunosuppressive cells, including regulatory B cells, regulatory T cells, myeloid-derived suppressor cells (MDSCs), M2-like tumor-associated macrophages (TAMs) and neutrophils [28,82]. The presence of the heat shock protein 72 (HSP72) on the surface of tumor-derived EVs activated the signal transducer and activator of transcription 3 (STAT3), through the HSP72-toll-like receptor 2 axis-mediated signaling and the autocrine production of interleukin-6 (Il-6), which triggered the T cell-dependent immunosuppressive function of MDSCs [28,133]. The activation of molecular signals via EVs can result in the reprogramming of tumor-infiltrating immune cells, like natural killer (NK) cells and dendritic cells (DCs) [28,85]. For instance, tumor-derived EVs expressing HSP70 in their surface stimulated the migratory and cytolytic activity of NK cells [28,85]. Moreover, EVs shed by hypoxic cancer cells delivered miRNA-23a and cytokine TGF-β, suppressing NK cells' function [28,85].

Importantly, DCs are considered to be the main antigen-presenting cells involved in the regulation of the tumor immune response, and tumor-derived EVs were also found to carry and transfer tumor antigens to DCs, leading to immune tolerance or encouraging immunity [28,85]. Moreover, it was also described

that tumor-derived EVs present Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), and programmed death-ligand 1 (PD-L1) on their surface, which inhibits $CD8^+$ T cell proliferation and response through the induction of apoptosis following receptor/ligand interactions [28,133]. In addition, tumor-derived EVs can deliver cargo content to facilitate the transition to M2-like profile on macrophages [82]. For instance, epithelial ovarian cancer-derived EVs transferred miR-222-3p to macrophages, affecting the suppressor of cytokine signaling 3 (SOCS3)/STAT3 signaling pathway, which induces polarization of the M2 phenotype [134]. Tumor-associated macrophages are prone to induce angiogenesis through secretion of various angiogenic growth factors, such as VEGF, Il-6, granulocyte-colony stimulating factor, and tumor necrosis factor α (TNF-α) [82].

Moreover, upon fusion with recipient cancer cells, tumor-derived EVs can also perpetuate inflammation within the TME, by inducing the expression of several inflammatory factors, such as Il-8, Il-6, TGF-β, and TNF-α [14,82]. Interestingly, this inflammatory microenvironment contributes to the establishment of the premetastatic niche and the promotion of angiogenesis [14,82].

Non-Mutational Epigenetic Reprogramming

In addition to the genomic instability and mutagenesis that characterize cancer, growing evidence attests the key role of epigenetic reprogramming in regulating tumor progression [80,135]. Epigenetics consists in the study of functional, heritable and reversible alterations in gene expression, that occur independently of the underlying DNA sequence, and comprises different mechanisms, including DNA methylation/demethylation, histone modification and regulatory non-coding RNAs [135,136]. Tumor-derived EVs have been demonstrated to interfere with these mechanisms, given their ability to dynamically carry and transfer bioactive cargo from donor to recipient cells [136].

Indeed, recent evidence suggests that tumor-derived EVs may interfere with the expression levels of onco- and tumor

suppressor genes, by affecting the methylation pattern of their regulatory CpG regions. In particular, EVs derived from chronic myeloid leukemia K562 cells were demonstrated to induce a leukemia-like malignant phenotype in normal mononuclear cells through the horizontal transfer of BCR-ABL1 mRNA and protein [137]. Interestingly, an increase in the global DNA methylation levels as well as promoter hypermethylation of the tumor suppressor genes *P53* and *RIZ1* were observed when normal mononuclear cells were incubated with the aforementioned EVs. These effects were further correlated with an upregulation of DNA methyltransferases, DNMT3a and DNMT3b, which was severely hampered when the leukemia-derived EVs were subjected to RNase treatment. Importantly, another study from the same group revealed that the observed DNMT3 upregulation was linked to the presence of miR-106a/b and large intergenic non-coding (linc) RNA lincPOU3F3 in the K562-derived EVs, which were involved in the transcriptional regulation of *DNMT3a* and *DNMT3b* genes [138]. Moreover, a recent study found that DNMT1 mRNA is upregulated in the cargo of EVs derived from pediatric acute lymphoblastic leukemia patients' serum, when compared with EVs derived from healthy donors' serum. Remarkably, using a co-culture system of normal immortalized B cells (JM1, Sup-B15, and NALM-6) with EVs derived from pediatric acute lymphoblastic leukemia patients serum, the authors demonstrated the delivery of exosomal DNMT1 mRNA transcripts into normal immortalized B cells and raised the possibility that exosomal DNMT1 mRNA may modulate the epigenetic landscape of healthy cells, promoting their transition towards a leukemic-like phenotype [139].

Another important mechanism of epigenetic reprogramming is histone modification. Indeed, histone modifications (*e.g.* histone acetylation, phosphorylation or methylation) affect chromatin organization and accessibility to transcription factors, thus exerting a strong impact on gene expression [140]. The role of tumor-derived EVs in histone modification is beginning to be explored. Indeed, a study demonstrated the synthesis and subsequent release via EVs of elevated levels of histone H1º mRNA and protein by G26/24 oligodendroglioma cells; however, this was not observed with normal astrocytes under the

same conditions [141]. Recently, research from the same authors demonstrated similar results in A375 melanoma cells [142]. The authors suggested that cancer cells could be selectively packaging this protein into EVs, as a way to escape differentiation and its associated reduction on stemness and self-renewal capacity.

In addition to DNA methylation and histone modifications, another important mechanism of epigenetic reprogramming is associated with regulatory non-coding RNAs (ncRNAs) [136]. Indeed, miRNA, lncRNA and circular RNA (circRNA) are the most abundant ncRNA species found in the EVs cargo, playing a significant role in post-transcriptional regulation [143]. Their dysregulation plays a significant role in tumor progression [144]. In this context, the epigenetic role of ncRNAs present in the cargo of tumor-derived EVs has been extensively studied and their involvement in the acquisition of distinct hallmarks of cancer has been demonstrated. Indeed, it was demonstrated that miR-1290 levels were elevated in the cargo of EVs derived from gastric cancer cell lines (SGC7901, AGS, and BGC823) and patient's serum [145]. Importantly, these authors observed an increase in tumor cell proliferation, migration and invasiveness when gastric cancer cell lines were incubated with miR-1290-containing EVs. Using both *in vitro* and *in vivo* models, these effects were further linked to the direct inhibition of naked cuticle homolog 1 by miR-1290. Moreover, another study found increased levels of the lncRNA HOTAIR in the cargo of EVs isolated from lung cancer blood samples and cell lines (A549 and H1299) [146]. Importantly, the incubation of several lung cancer cell lines with EVs shed by lung cancer A549 cells resulted in an increased cellular expression of lncRNA HOTAIR accompanied by an increase in cancer cell proliferation, migration, and invasion resulting from HOTAIR mediated miR-203 sponging. Likewise, research revealed that EVs derived from hepatocellular carcinoma cell lines overexpressing circRNA Cdr1 promoted tumor cell proliferation and migration *in vitro* [147].

Regarding angiogenesis, research revealed that miR-135b contained in the cargo of EVs shed by SGC7901 gastric cancer cells inhibited forkhead box O1 (FOXO1) expression in HUVEC

endothelial cells, promoting angiogenesis both *in vitro* and *in vivo* [148]. Interestingly, another study demonstrated that miR-155 contained in the cargo of EVs shed by the same gastric cancer cell line produced a similar effect through inhibition of FOXO3a [149]. Furthermore, a study from 2018 found elevated levels of miR-210 in the cargo of EVs isolated from the serum of hepatocellular carcinoma patients, which was further positively correlated with increased microvessel density in human hepatocellular carcinoma tissues [150]. Moreover, these authors also revealed that miR-210 contained in the cargo of EVs shed by hepatocellular carcinoma cells promoted tumor angiogenesis both *in vitro* and *in vivo*, and *in vitro* studies further demonstrated that this pro-angiogenic effect resulted from the inhibition of the expression levels of SMAD4 and STAT6 induced by EV-derived miR-210. Likewise, lncRNAs and circRNAs contained in the cargo of tumor-derived EVs interfered with the process of new vessels formation. In fact, lncRNA RAMP2-AS1 was found in the cargo of chondrosarcoma cell-derived EVs and its internalization into HUVEC endothelial cells was demonstrated [151]. Upon internalization, RAMP2-AS1 promoted HUVEC endothelial cells angiogenic capacity through the RAMP2-AS1/miR-2355-5p/VEGFR2 axis. Moreover, a recent study showed that circRNA-100338 was highly expressed in both hepatocellular carcinoma cell lines and derived EVs and, importantly, circRNA-100338 contained in the cargo of hepatocellular carcinoma-derived EVs stimulated HUVEC endothelial cells' pro-angiogenic capacity both *in vitro* and *in vivo* [152].

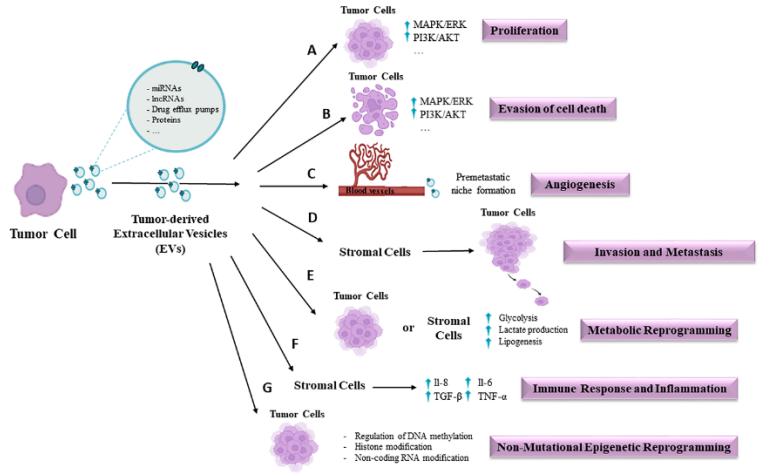

Figure 2: Roles of Extracellular Vesicles (EVs) released by tumor cells on the hallmarks of cancer. EVs released by tumor cells promote tumor proliferation **(A)** and evasion of cell death **(B)**, e.g. through the activation of tyrosine kinase receptors and their downstream signaling pathways (as MAP/ERK and PI3K/AKT). In addition, EVs released by tumor cells promote angiogenesis and support the premetastatic niche formation, facilitating cancer metastasis **(C)**. EVs released by tumor cells can also promote the epithelial-mesenchymal transition (EMT) phenotype, which enhances cancer cells' ability to invade adjacent structures and metastasize **(D)**. Indeed, EVs carry various molecules to support these phenomenon, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), drug efflux pumps, transforming growth factor-β (TGF-β), and programmed death-ligand 1 (PD-L1). Moreover, EVs released by tumor cells can mediate the process of metabolic reprogramming by inducing glycolysis, lactate production and lipogenesis, which further supports tumor proliferation, angiogenesis, metastasis and immunosuppression **(E)**. Importantly, EVs can modulate the immune and inflammatory response, e.g. by suppressing T cell activation or inducing the expression of several inflammatory factors (such as Il-8, Il-6, TGF-β, and TNF-α), respectively **(F)**. Finally, EVs also play a role in DNA methylation, histone modification and transmission of non-coding RNA in the cancer microenvironment, thus participating in non-mutational epigenetic reprogramming **(G)**.

The Impact of Tumor-derived EVs on Cancer Drug Resistance

The relevance of the intercellular transfer of a drug-resistant phenotype by EVs derived from drug-resistant cancer cells to recipient drug-sensitive cancer cells has been described in several studies, supporting the notion that EVs contribute to the

dissemination of the drug resistance phenotype and to challenges regarding the clinical management of cancer patients (Figure 2) [32,82,153,154].

Interestingly, it was recently demonstrated that multidrug-resistant (MDR) cells secrete more EVs than their sensitive counterparts, and drug-sensitive cells capture more EVs than their MDR counterparts [155]. Furthermore, it is known that MDR tumour cells tend to secrete larger EVs than their sensitive counterparts [156]. These studies confirm the potential impact of EVs on drug resistance and their potential as resistance biomarkers.

ATP-binding Cassette (ABC) transporters, commonly known as drug efflux pumps, such as P-glycoprotein (P-gp), breast cancer resistance protein, and multidrug resistance-associated protein 1 (MRP1), are transferred from donor MDR cells to sensitive cells through EVs [32,36,153]. The overexpression of Ubiquitin C-terminal hydrolase L1, a deubiquitinating enzyme, in circulating EVs was reported to upregulate P-gp protein expression levels through the MAPK/ERK signaling pathway, thereby enhancing MDR in breast cancer cells [157].

Moreover, EVs can elicit anti-apoptotic signals in tumor cells by targeting apoptosis regulators, which also contributes to cancer drug resistance [14,158]. For example, EVs from drug-resistant chronic myeloid leukemia cells transferred to recipient breast and lung tumor drug-sensitive cells inhibitors of apoptosis-related proteins, such as XIAP, IAP, and survivin, thereby promoting the induction of drug resistance [159].

Furthermore, there are other molecules in EVs' cargo that are implicated in the transfer of drug resistance, including miRNAs and lncRNAs [32,157]. For instance, there is evidence that miRNAs can post-transcriptionally regulate the expression of drug efflux pumps, including the ones mentioned above, through direct targeting of drug efflux mRNAs, with an inverse correlation in terms of expression levels [154]. For example, the overexpression of miR-138, that directly targets the multidrug resistance protein 1 (MDR1) gene, sensitizes leukemic cells to numerous drugs, by downregulating MDR1 gene expression and decreasing P-gp expression [154]. Moreover, some miRNAs may

regulate drug resistance of hematological malignancies by indirect targeting drug efflux, as shown by the overexpression of miR-631 that targets UbcH10, thus inhibiting MDR1 ubiquitination and consequently supporting its expression [154]. The downregulation of tumor-suppressive miRNAs, such as miR31-5p, miR-155, and miR-1238, present in EVs cargo, was shown to enhance drug resistance in diverse types of cancer, including renal cell carcinoma, breast cancer, lung cancer and glioblastoma [158].

Similarly, specific lncRNAs are recognized as regulators of drug resistance, even though their contribution remains poorly understood [32,160]. For example, a study conducted in hepatocellular carcinoma cells, that were sensitive to sorafenib, identified EV lincRNA ROR as a mediator of chemoresistance in response to TGF-β [161]. Furthermore, vesicular lncRNA ARSR and HOTTIP were detected in the serum of patients with renal cell carcinoma and with gastric cancer who exhibited tolerance to sunitinib and cisplatin, respectively, thus suggesting their clinical relevance [158].

Lastly, lipids may also mediate drug resistance, as supported by the enrichment in the lipid ceramide in EVs mediated MDR, possibly via ABC transporters [14,162,163].

Interestingly, EVs shed by drug resistant cells typically have a distinct cargo from the ones shed by their drug-sensitive counterparts [153,154]. A recent study identified a distinct transcriptomic profile between EVs released by MDR cells and their drug-sensitive counterparts. This work showed the presence of pseudogenes, including RNA 5.8S, and miRNAs as potential MDR biomarkers in both MDR NSCLC and acute myeloid leukemia cell lines [164].

Additionally, according to several studies, metabolic alterations can induce modifications in the EVs cargo and release. Such alterations include increased lactate production, inhibition of glutamine metabolism, and the presence of various enzymes involved in glucose and glutamine metabolism [163,165,166]. It has been reported that EVs shed by MDR cells can stimulate a metabolic switch towards a MDR phenotype in recipient drug-sensitive cells, inducing metabolic reprogramming to glycolysis

and enhancing the levels of detoxifying enzymes such as Glutathione S-transferase P [14,153].

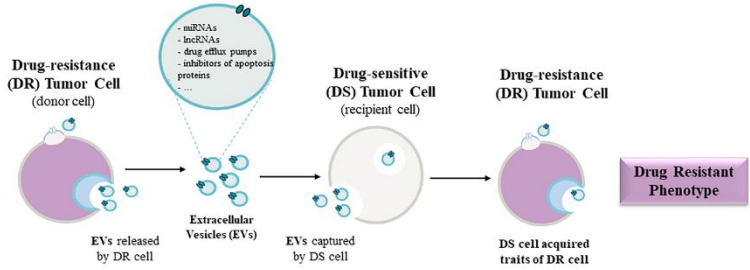

Figure 3: Schematic representation of the dissemination of the drug resistance phenotype via EVs. Drug-sensitive tumor cells may temporarily present a more drug-resistant phenotype following the uptake of EVs shed by drug-resistant (donor) tumor cells. This is due to the transfer of EVs cargo from donor to recipient cells, including miRNAs, lncRNAs, drug-efflux pumps and inhibitors of apoptosis proteins (e.g., XIAP and survivin).

Conclusion

Over the last decade, several studies have demonstrated that EV-mediated intercellular communication, acting at close proximity and even at distant sites, has an important impact on cancer progression and therapy resistance. Tumor derived EVs interfere with the various "Hallmarks of Cancer", and are important mediators of horizontal transfer of drug resistance traits.

Despite multiple challenges, including standardization and characterization analysis, the field of EVs has gained importance in clinical research due to the EVs unique features, such as their abundance in biological fluids, and ability to protect their diverse and multifunctional cargo from external enzymatic degradation. These biological properties explain the interest in their potential use as drug delivery vehicles, as well as in their role as biomarkers in liquid biopsies. In fact, EVs show promise in the areas of early cancer diagnosis, detection of measurable residual disease and of therapy resistance.

EVs may come to provide us with new tools for precision medicine, with relevant clinical applications, namely in the field of oncology. Further elucidation of their role as disease-mediators should also be thoroughly explored.

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