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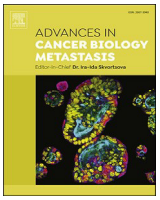

The role of exosome heterogeneity in epithelial ovarian cancer

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ABSTRACT

Ovarian cancer results in more deaths than any other gynecological malignancy, with a 5-year survival of only 30%. It is typically diagnosed after it has spread from the primary site to the secondary site. Exosomes are membrane-bound nanovesicles that play a critical role in tumor biology and metastasis by promoting intercellular communication. Tumor-associated exosome populations are widely acknowledged to be heterogenous, as various cell types and hallmark tumor microenvironment stressors impact exosome synthesis. Ovarian cancer cells metastasize using intraperitoneal fluids that are rich in exosomes, suggesting that these circulating exosomes assist detached cancer cells to maintain invasive phenotypes prior to secondary site invasion. Studies show that tumor-secreted exosomes direct organ-specific colonization by fusing exosome integrins with target cells in a tissue-specific fashion. Exosome signaling molecules (mRNA, miRNA, proteins) are encapsulated by cholesterol-rich membranes, and thus protects biomaterials from enzymatic degradation. Therefore, they represent an ideal system for studying the expression of sensitive proteins and RNA and for future drug delivery vehicles. Proteins and RNA exchanged through exosomes also influence the molecular and mechanical properties of ovarian cancer cells promoting adaptations that contribute to invasive and metastatic cell behavior. Tumor-derived exosomes also interact with stromal cells to alter their molecular profiles, thus promoting the development of a more malignant tumor microenvironment (TME), invasive cell behavior, and cancer progression. This review provides an overview on exosome structure and biogenesis and summarizes recent studies on ovarian cancer exosomes, exosome mediated interactions in the tumor microenvironment, and exosome heterogeneity.

1. Introduction

Exosomes are membrane-bound nanoparticles that play a critical role in tumor biology and metastasis by promoting intercellular communication [1]. Recent studies also show that exosomes can direct organ-specific metastases [2]. In part to their cholesterol-rich membranes, exosomes protect signaling molecules (mRNA, miRNA, proteins) from enzymatic degradation; thus, they represent an ideal system for studying the expression of sensitive proteins and RNA [1].

These nanovesicles shed via endosomal trafficking into extracellular spaces and contain cargo that is important during paracrine and autocrine signaling [1]. Internal cargo can include proteins, DNAs, RNAs, and miRNAs—most of which highly mirrors the parent cell. Interestingly, more current work demonstrates that exosomes can possess content that is unique from parent cell populations [3]. Exosome exchange can lead to the transferring of various biomaterials and recipient cells taking on unique physical and molecular behaviors [4]. This exchange can also lead to the recipient cell exhibiting characteristics similar to the cell that is secreting exosomes.

Tumor-associated cells—i.e. cancer, fibroblasts, immune, stromal—readily secrete exosomes, making the exosome composition in the local and distal TME very heterogeneous [5]. The aberrant properties of malignant tissues (i.e.: acidic and hypoxic environments with leaky vasculature and increased physical pressures from growing tumor and stiffening extracellular matrix) can alter the physical characteristics, secretion rates, and internal cargo of secreted exosomes [6,7]. These tumor microenvironment conditions have also been shown to alter exosome properties in single cell cultures [8]. Therefore, exosome heterogeneity can be explored *in vitro* using single cell or multicellular cultures and *in vivo* using fluid or tissue samples, such as blood, ascites, or tumor tissues.

Numerous studies highlight the importance of exosomes as secreted factors that dictate metastasis and as active contributors in Paget's "seed and soil" theory [9,10]. This review will particularly focus on the role of

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exosomes in epithelial ovarian cancer (EOC) progression. Exosomes play a unique role in EOC metastasis as cancer cells are shed directly into ascites [11]. These fluids are rich in growth factors and exosomes [11]. Ovarian cancer cells (seeds) migrate using ascites-rich fluids for secondary site attachment and invasion (soil). Exosomes and their respective biomaterials play critical roles in priming the secondary site for metastasis (pre-metastatic niche).

Exosome secretion and exchange assist in local, primary tumor growth and distal progression. However, engineering exosomes to serve as natural vehicles to deliver anti-cancer therapies has gained an immense amount of attention [12]. The low immunogenicity combined with efficient drug or miRNA encapsulation make exosomes prime candidates for new drug delivery avenues [13]. Cell-derived exosomes and nanoghosts (formed from exosome membranes) have been proposed as potential vectors for drug and gene delivery to tumor tissues [14].

We will review how exosome heterogeneity regulates EOC progression and how heterogenous exosome content can provide unique miRNA profiles for future novel therapeutic targets.

2. Exosome overview

2.1. *Exosome Biogenesis and Secretion*

Exosomes are 40–160 nm vesicles that cells (prokaryotes and eukaryotes) secrete into extracellular spaces via exocytic processes [15]. Proteins, such as those that constitute the ESCRT complex (–0,-I, –II, –III), work in concert to form invaginations on the endosomal limiting membrane; these inward budding vesicles are known as intraluminal vesicles (ILVs) [15]. Mature endosomes that are loaded with ILVs are known as multivesicular bodies (MVBs) [15]. MVBs then fuse with the plasma membrane and release the ILVs that then become exosomes. MVBs docking to the plasma membrane rely heavily on tethering interactions with cytoskeletal and actin-associated proteins, a cohort of Rab GTPases, and SNARE proteins (Fig. 1) [16].

Sinha et al. show that cortactin modulates exosome secretion by regulating branched actin dynamics that transport MVBs to the plasma membrane [17]. Rab GTPases (i.e.: 27a/b, 5a/b) play pivotal roles in vesicle trafficking along actin and tubulin and MVB fusion [18]. Knockdown and overexpression studies of specific Rabs across various cell lines demonstrate the importance of these proteins in exosome secretion [18]. SNARE complexes form coiled-coil helices that mediate vesicle-membrane fusion [19]. Specifically, inner plasma membrane target SNARE (t-SNARE) binds with vesicular SNARE (v-SNARE) on the MVB [19].

Additional proteins that are critical in exosome biogenesis and secretion include syndecan, syntenin, and ALIX [20]. In fact, the syndecan-syntenin-ALIX pathway plays an important role in this process. Syndecans are single transmembrane protein receptors that bind and interact with adhesion molecules via heparan sulfate chains [21]. Syntenin is a scaffold protein that binds to the syndecan cytosolic tail and with ALIX, a protein that is associated to the ESCRT complex proteins [21]. The direct mechanistic interaction of syntenins, along with syndecans and ALIX, to the ESCRT complexes indicate that this protein cohort play important roles in intraluminal vesicle formation and support exosome biogenesis [21]. Baietti et al. highlight that syndecan-syntenin-ALIX are important regulators of endosomal membrane trafficking and exosome budding formation [20]. Specifically, this study indicates that overexpression of syntenin directly increases accumulation of exosomal syndcan and ALIX and increases exosome production by 2–3 fold [20]. Table 1 further illustrates and highlights various reported proteins that are responsible for exosome biogenesis and secretion.

Although exosome content and composition vary with their respective parent cell populations, there are multiple components that are ubiquitous to all exosomes. These include i) membrane-bound and surface proteins, ii) surface lipids, and iii) coding and non-coding RNA.

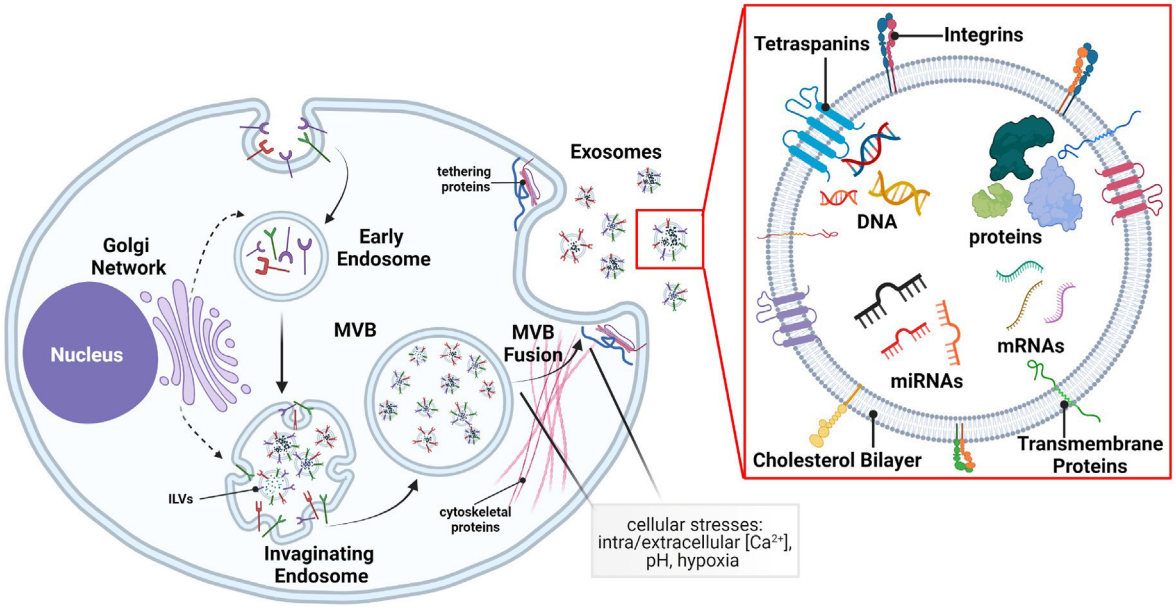

Fig. 1. Exosome Biogenesis and Secretion. Exosomes are generated via an endosomal process through consecutive invaginations of various membranes. Initial membrane invagination begins at the plasma membrane where cell surface proteins are endocytosed and trafficked to form early endosomes (1). Early endosomes can potentially interact or fuse with golgi networks or golgi budding vesicles. Continued surface invaginations lead to the development of intraluminal vesicles (ILVs) (2). An ILV is known to act as the exosome precursor. Further endosome modification gives rise to multivesicular bodies (MVBs) (3). MVBs can either fuse with a lysosome for subsequent degradation (not illustrated) or fuse with the luminal side of the plasma membrane for subsequent exosome release (4). MVBs are shuttled to the plasma membrane via cytoskeletal proteins, such as actin and microtubules, and docked to the membrane with tethering proteins, such as SNAREs. Exosome physical phenotype and molecular content is highly variable. However, exosomes exhibit stable cholesterol-rich membranes decorated with surface protein markers (i.e. integrins, transmembrane proteins, tetraspanins). Many exosomes further contain biomaterials, such as proteins, DNA, RNAs, and miRNAs that are highly indicative of the parent cell and endosomal processing. Tumor microenvironment stresses (gray box) not only heavily influence exosome content, but also change the mechanisms and rate of exosome release. *Created in BioRender.*

Table 1
List of proteins responsible for exosome synthesis—membrane invagination, tethering proteins, secretion proteins.

Protein	Function	References
VAMP7	involved with the SNARE complex that fuses vesicle with plasma membrane	[16]
ESCRTs (–0,I,II,III)	0: recruits ubiquitinated proteins I/II: triggers budding of ILVs III: pinches ILVs that become EXOs	[121,122]
TSG101	subunit of ESCRT-I complex	[123,124]
ALIX	binds to syntenins and syndecans to work with ESCRT complexes for ILV invagination	[125]
VPS4A/B, LIP5, BRO1, BROX, Hrs	Various protein complexes that interact with ESCRTs during ILV formation	[126–128]
RAB isoforms (1A, 1B,2B,4,5A, 7, 9A, 11, 27A, 27B, 35)	GTPases that regulate vesicle budding, trafficking along cytoskeletal proteins, docking to plasma membrane and fission/ secretion	[18]
SNAREs (t-SNARE, v-SNARE)	work with tethering proteins for membrane fusion	[129]
Ceramide	Lipid that promotes endosomal invagination and budding by inducing negative membrane curvature	[130]
Cytoskeletal and motor proteins (actin, cortactin, dyein)	pivotal in transporting MVB to plasma membrane and subsequent exosome secretion	[131]
Tetraspanins (CD9, -63, –81)	transmembrane proteins that play important roles in ILV formation	[132]
LAMP (1,-2)	Surface exosome marker that is expressed in lysosomes; traffic from plasma membrane to lysosomes	[133,134]
Heat Shock Proteins (HSP20, -27, –70) and nSMase	Exosome markers and are involved in ESCRT-independent exosome secretion	[135]
Lipid rafts (caveolin, sphingolipids, cholesterol, phospholipase, diacylglycerol, flotillin)	Contributes to plasma membrane invagination, ILV formation, and ILV shape maintenance	[136]

Hallmark examples include i) integrins, tetraspanins, MHC class I/II, RAB isoforms and ESCRT proteins, ii) sphingomyelin, cholesterol, and ceramides, and iii) mRNA, miRNA, and other non-coding RNAs [22].

2.2. *Exosome function*

Exosomes play critical roles in cell-cell crosstalk, often altering physical/molecular phenotypes and downstream signaling pathways in recipient cells [4]. The cholesterol-rich membrane allows exosomes to serve as stable vehicles during intercellular communication and resist enzymatic degradation after secretion [23]. Many *in vitro* studies show that bulk exosome treatment can modulate cell and nuclear morphologies, cytoskeletal protein organization, and various molecular pathways [4]. Exosome exchange can promote or even suppress aggressive cell phenotypes. Costa-Silva et al. report that Kupffer cells treated with pancreatic cancer exosomes elevate TGF-B secretion and fibronectin production [9]. This promotes the rapid development of a fibrotic microenvironment and increases recruitment of bone marrow-derived macrophages [9]. Conversely, exosomes can attenuate disease progression and act as therapeutic factors. Ji et al. demonstrate that stem cell-derived exosomes combat fibrotic development via YAP-mediated pathways [24]. These studies indicate that exosomes are important factors in cell-cell communication as they can influence recipient cells to take on unique physical and molecular phenotypes.

In addition to inducing distinct physical and molecular cellular phenotypes, exosome exchange can modulate immune cell activity, promote chemoresistant behaviors, and induce site-specific metastasis. Studies report that exosomes can activate immunostimulatory or immunosuppressive cellular behaviors [25]. Specifically, EOC cell exosomes can upregulate immunosuppressive functions in regulatory T cells and myeloid-derived suppressor cells [26]. Both cell types are critical in tumor immune evasion processes and can further facilitate tumor metastasis [26]. Exosome surface membranes are also packed with adhesion proteins and integrins that can be used to target specific recipient cells [2]. Packaged exosome content and integrins play important roles in disease progression [2]. Hoshino et al. revealed that exosome-specific integrins can direct organ-site metastasis by fusing exosomes with target cells to prepare the pre-metastatic niche for site-specific metastasis [2]. This led to the finding that exosomes are potent homing factors that can alter the tropic site of metastatic cancer cells (i.e., exosomes from lung-tropic cancer cells can cause bone-tropic cancer cells to metastasize to the lung) [2]. In conjunction to promoting tumor metastasis, exosomes can also induce chemoresistance [27]. Aberrant exosome miRNAs derived from chemoresistant cancer cells can regulate post-transcriptional modifications that confer chemoresistance to neighboring drug-sensitive cancer cells [28,29]. For example, Li et al. highlighted that exosomal transfer of miRNA-429 to cisplatin-sensitive EOC cells led to increased cell proliferation and the drug-resistance development via the STAT3 pathway [30].

Finally, these nanovesicles can act as useful clinical diagnostic/prognostic biomarkers and more recently as drug delivery vehicles [31,32]. Exosomes are packaged with functional biomolecules, such as protein, RNA and miRNA that are highly representative of parent cells during ILV formation [1]. Studies have shown that cancer cells under hallmark TME conditions secrete elevated amounts of exosomes and that these exosomes encapsulate unique miRNA profiles [32]. Differentially regulated exosome miRNA profiles are stabilized and protected from enzymatic degradation by exosome membranes [1]. This allows circulating exosomes to be harvested from biofluids, such as peritoneum lavage fluid, and subsequently analyzed for disease-related miRNA biomarkers [33]. Tokuhisa et al. collected exosomes from peritoneal fluids and identified several miRNAs that can be used as predictive metastatic markers [33]. This allows aberrant exosome production and differential exosome cargo expression profiles to act as useful diagnostic and prognostic biomarkers [32]. Exosomes can also serve as site-specific delivery systems, which can be loaded with chemotherapeutics, to tissues of interest. Zhuang et al. used exosomes to encapsulate curcumin to combat brain tumors and brain inflammatory diseases [34]. It was reported that using exosomes as drug delivery vehicles effectively delayed brain tumor growth and induced apoptosis [34].

3. Exosomes in serous epithelial ovarian cancer (EOC)

Numerous studies show the importance of exosomes in mediating cancer progression [35]. Currently, all examined cells readily and naturally secrete exosomes and play pivotal roles in tumorigenesis. For this review, we will specifically examine the role of exosomes in EOC.

3.1. *EOC overview*

Ovarian cancer is the most lethal gynecological malignancy and accounts for approximately 2.3% of cancer-related deaths [36]. There are three types of ovarian cancers: epithelial, germ cell, and stromal cell [37]. EOC is the most predominant form, accounting for 90% of new cases, and often originates from the fallopian tubes [38]. High-grade serous epithelial ovarian cancer constitutes 75% of ovarian cancers [39]. This disease is unfortunately often diagnosed at late stage after the cancer has metastasized [36].

Many metastatic processes involve extravasation into the vasculature and subsequent intravasation into secondary sites [40]. However, EOC metastasis is uniquely characterized by the shedding of cells (single-cell or spheroid aggregates) into local peritoneal fluid, followed by cells

implanting onto the omentum—a fat pad lined with mesothelial cells (Fig. 2) [41]. However, clinical biopsies have shown that mesothelial cells are not present under tumor masses that are implanted onto the omentum or surrounding peritoneal cavity walls [42–44]. Iwanicki et al. highlight that EOC cell spheroids use integrin-dependent activation of myosin and matrix traction force to clear mesothelial cells that line target organs [45]. This allows EOC cells to potentially invade more rapidly and gain access to sub-mesothelial tissues.

Aberrant peritoneal fluid (ascites) build-up is common in late stage EOC [46]. This ascites is an abundant source of cell-secreted exosomes that contain and guide proteins, transcription factors, and miRNAs through potent tumor environments that impact cell behavior at metastatic sites [46].

Integrative proteomic analyses of EOC patient-harvested ascites have identified thousands of novel targets that can potentially serve as biomarkers when diagnosing women [47]. However, the heterogenous nature of ascites composition between patients makes it difficult to establish additional diagnostic markers [48]. This challenge further underscores the importance of examining exosome content.

Further omics analysis of circulating exosomes isolated from ascites showed overlapping proteins that are linked to late stage EOC [49]. Several of these include: mucin16 (MUC16 or CA125), epithelial cell adhesion molecule (EpCAM), and tubulin B (TUBB). MUC16 is a transmembrane glycoprotein and remains as the most important EOC serum biomarker [49]. It is also used to measure and monitor chemotherapy response [50]. Growing evidence shows that EpCAM is overexpressed in epithelial tumors like ovarian cancer [51]. Its role is not only limited to cell-cell adhesion, but also is involved in Wnt signaling pathways [52].

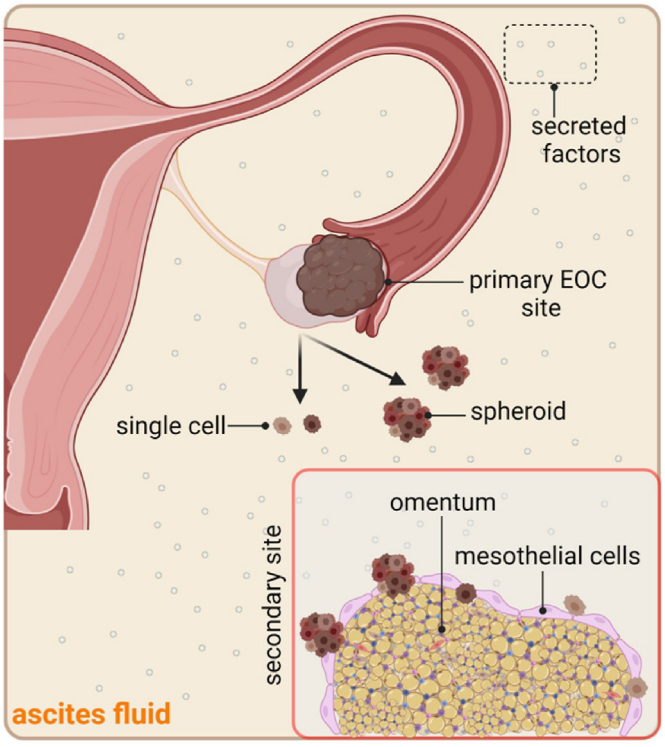

Fig. 2. EOC Metastatic Process. EOC cells are shed from the primary tumor site as single cells or spheroid aggregates. These cells and clusters secrete exosomes into ascites, where there is already an abundance of secreted factors, such as growth factors, cytokines, and other exosomes. The accumulation of ascites is a common side effect of late stage EOC. Secreted factors found in these fluids maintain and stabilize the EOC cells and aggregates for secondary site attachment and further metastasis across the mesothelium, which lines tissues and organs in the peritoneal cavity. Common EOC secondary sites include the omentum—a fat pad that is rich in adipocytes, immune cells, and fibroblasts and lined with mesothelial cells. *Created in BioRender.*

Yet, clear understanding of EpCAMs role in mediating EOC progression via epithelial-to-mesenchymal transition (EMT) remains unclear, largely in part to tumor heterogeneity and differences in EOC cell populations [51].

3.2. *Mixed EMT signatures in EOC*

EOC metastasis exhibits mixed EMT characteristics [53]. EOC cells, like other metastatic processes, naturally take on mesenchymal-like behaviors [53]. This is characterized by elongated cancer cells that exhibit increased migration, invasion, and development of drug resistance [53]. However, studies reveal that surface invaginations on the ovarian surface epithelium elevate levels of E-Cadherin (E-Cad) and that upregulation of this transcription factor serves as an early indicator of EOC formation [54]. E-Cad can activate pathways, such as PI3K/AKT and MEK/ERK, that are responsible for cell proliferation and survival in EOC [55].

EOC cells that shed from the primary site as densely packed multicellular clusters further retain epithelial phenotypes. Spheroids are held together and migrate collectively via adherens junctions and cadherins [56,57]. These epithelial characteristics are maintained when spheroids and single cells anchor to mesothelial cells on the omentum [58]. Cells packed in spheroids present mesenchymal characteristics when invading into the mesothelium layer [59]. Studies show that exosomes are responsible for inducing EMT/MET during cell-cell communication in tumor metastasis [60]. Therefore, exosomes and surface integrins not only guide location-specific metastasis, but can also regulate EMT/MET phenotypes during EOC metastasis.

4. Exosome heterogeneity in EOC tumor microenvironment (TME)

Various factors and characteristics (molecular and physical) contribute to exosome heterogeneity in the EOC TME. Hallmark TME conditions—low pH, increased hypoxic conditions, mechanical and physical stresses, and bystander irradiation effects—significantly alter exosome secretion [8,61]. Different parent cells naturally secrete exosomes with unique content [62,63]. Varying ILV biogenesis processes also leads to heterogenous vesicle diameters and content [16]. Many studies examine exosome heterogeneity derived from heterogenous parent cell sources; however, even a single EOC cell line can secrete different exosome populations under different culture conditions, which can contribute to single cell exosome heterogeneity [64] (Fig. 3).

4.1. *Multicellular exosome heterogeneity*

The TME includes multiple cell types that are readily secreting exosomes and contributing to multicellular exosome heterogeneity. Cancer cells that vary in metastatic potential, cancer-associated fibroblasts, immune cells, adipocytes, mesothelial cells, and stromal cells are several types of cells found in the TME [65]. These exosomes are crucial in heterogeneous cell-cell communication as they can serve as paracrine and autocrine signaling molecules that help reprogram recipient cells in the TME to recruit additional cells or alter their behavior to promote or suppress the malignancy [66]. Table 2 summarizes heterogenous exosome exchange and reported downstream changes.

Cancer cells. Tumor sites comprise of a diverse collection of cancer cells that vary in molecular and physical signatures that differentially alter primary and distal site metastasis [67]. Numerous studies underscore that exosomes mediate pre-metastatic niche formation and support organotropism [2,9]. For example, Zhang et al. report that gastric cancer cell exosomes promote cancer cell metastasis to the liver [68]. Gastric cancer cell exosomes encapsulate EGFR that is delivered to liver stromal cells [68]. EGFR translocation directly activates hepatocyte growth factor and supports the attachment and proliferation of metastatic cancer cells [68].

Further, exosome regulated cell-cell communication has pro-

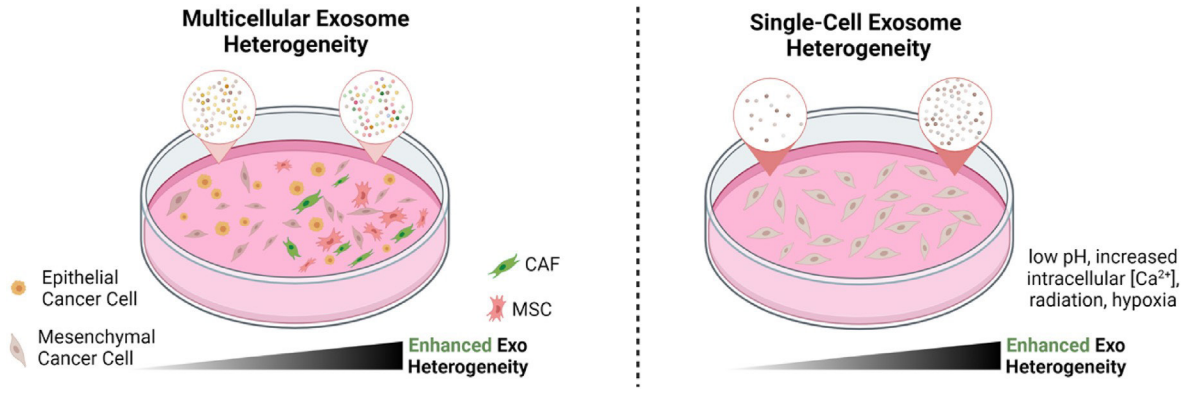

Fig. 3. Multicellular vs. Single-cell Exosome Heterogeneity. Exosome heterogeneity can be examined two-fold: multicellular (left) and single-cell (right). EOC tumor microenvironments are composed of heterogenous cell populations, including cancer cells exhibiting varied levels of EMT phenotypes, fibroblasts (normal and activated), stromal cells, and immune cells. All examined cell types have been reported to readily secrete exosomes. Heterogeneous cells secrete unique exosome populations that vary in size, content, and function. Tumor microenvironment stresses, such as changes in pH, fluid pressure or other physical stresses, radiation or chemotherapy, tumor hypoxia, and tumor induced hypercalcemia, can drive cancer cells to secrete exosomes with different content, leading to heterogeneity in single cell exosome populations. These exosome populations can differ in their physical properties (size and shape) and exosome content. *Created in BioRender.*

Table 2
This table summarizes heterogenous exosome treatment and downstream impact/response for cells that are critical in the EOC TME.

Cell-Secreted Exosome Population	Exosome Recipient-Cell Population	Downstream Impact and Response	References
Cancer cells (mesenchymal)	Cancer cells (epithelial)	Transfer of "metastatic potential"; activation of EMT	[69]
EOC cells	Fibroblasts	Induce myofibroblast phenotypes; altered morphologies, cytoskeletal architecture, migration, adhesion, and spread profiles	[4]
Cancer-associated fibroblasts	EOC cells	Increased EOC cell migration and invasion; dysregulated SMAD activity	[4,72]
Macrophages	EOC cells	Increased EOC cell cisplatin-resistance	[76]
EOC cells	Mesothelial Cells	Increased CD44 expression; mesothelial cells secreted MMP9	[78]
Ovarian cancer spheroids	Mesenchymal stem cells	Increased MSC migration; MSCs secreted factors that induced endothelial angiogenesis	[81]
Adipocytes	EOC cells	Suppressed apoptosis and elevated chemoresistant behaviors	[85]

tumorigenic effects in the primary TME. More invasive, mesenchymal cancer cells secreting exosomes can transfer "metastatic potential" to less invasive, epithelial-like cells [69]. Tumor-derived exosomes can initiate EMT hallmarks in recipient, neighboring tumor cells that lead to invasive cancer progression phenotypes, such as inflammation, proliferation, and migration [69].

Cancer-associated Fibroblasts (CAFs). Fibroblasts play important roles in the primary TME. These cells can secrete matrix proteins and enzymes that alter matrix composition [70]. This allows tumor cells to rapidly disseminate from the primary site or invade subsequent secondary sites [70]. CAFs found in the EOC TME secrete abnormal amounts of matrix metalloproteinases (MMPs), such as MMP9 and MM2 that can break down basement membranes [70]. Further, CAFs secrete and deposit increased amounts of matrix proteins, such as collagen and fibronectin, that subsequently increase tumor tissue stiffness [71]. The remodeling of local tissue from increased stiffness and MMP production allow cancer cells to proliferate and readily disseminate from primary sites [71].

Studies indicate that exosome exchange between cancer cells and fibroblasts can induce fibroblast differentiation to form myofibroblast-like phenotypes [4]. For example, we report that EOC cell-secreted exosomes induce more aggressive phenotypes in EOC patient-derived fibroblasts [4]. Exosome-treated fibroblasts alter physical phenotypes, such as fibroblast morphologies, filamentous actin and focal adhesion protein structures, fibroblast adhesion strength and kinetics, and increase directional and random migration [4]. Conversely, studies highlight that EOC cells treated with CAF exosomes increase EOC cell invasion and collective migration via dysregulated SMAD expressions [72].

Macrophages. Macrophages are responsible for triggering immunostimulatory processes. However, exosome exchange can induce macrophages to exhibit more immunosuppressive features [73,74]. Tumor-associated macrophages (TAMs) are one of the most important immune cells and play important roles in subsequent cancer cell migration and proliferation [75]. Zhu et al. demonstrate that exosomes isolated from hypoxic macrophages were enriched with miRNA-223 [76]. Functional analysis highlights that macrophage secreted exosome miRNA-223 increase cisplatin-resistance in EOC cells. These results indicate that hypoxic macrophage-cancer cell exosome exchange, an environment that mirrors *in vivo* EOC conditions, promotes drug-resistant phenotypes [76].

Mesothelial Cells. EOC cells can directly contact mesothelial cells during initial stages of metastasis [77]. Studies show that EOC cell exosome treatment elevate CD44 expression in peritoneal mesothelial cells [78]. This, in turn, leads to mesothelial cells secreting increased levels of MMP9. MMP9 clears mesothelial linings and allows for rapid EOC cell invasion into secondary sites [78].

Mesenchymal Stem Cells (MSCs). MSCs are multipotent stromal cells and are recruited to primary TME sites [79,80]. MSCs can differentiate into various lineages, such as adipocytes, and take on activated, pro-tumorigenic phenotypes [79]. These activated MSCs can subsequently increase metastatic potential in neighboring cells. Vera et al. show that exosomes harvested from cisplatin-treated ovarian cancer spheroids elevate MSC migration, MMP expression, and inflammatory cytokine secretion [81]. Further, exosome treated MSCs induce endothelial cell-driven angiogenesis and increase migration in ovarian cancer cells [81].

MSCs accumulated in tumor tissues can become senescent and release a senescence associated secretory phenotype (SASP) that is critical in cancer development [82]. SASP is characterized by the increased accumulation of inflammatory cytokines, growth factors, and more recently exosomes [63,83]. Studies show that senescence elevates exosome

secretion and that these exosomes can activate pathways involved in inflammation, extracellular matrix remodeling, and metastasis [63].

Adipocytes. EOC cells largely metastasize to adipocyte-rich omental tissues because adipocytes secrete homing factors [84]. Studies highlight that the cell-cell communication between adipocyte-EOC cells is critical because adipocytes can directly transfer lipids and proteins that promote rapid tumor growth [84]. In fact, this study suggests that these adipocyte-secreted factors serve as an energy source for subsequent EOC metastasis [84]. These factors, along with specific miRNA content, can be packaged into exosomes. Yeung et al. report that adipocyte-secreted exosomes have high levels of miRNA-21 [85]. Exosome exchange between adipocytes and EOC cells suppresses apoptosis and leads to chemoresistant EOC cell behaviors [85].

4.2. Single-Cell Exosome Heterogeneity

Aberrant mechanical and chemical stresses that are exerted on primary tumor-associated cells trigger increased secretion of exosomes that have unique physical and molecular content [4]. These stresses elicit the release of heterogenous exosome populations from the same parent cell line [4]. The established EOC exosome literature has primarily focused on heterogeneity in bulk, multicellular populations; however, exosome heterogeneity derived from single cell lines induced by hallmark TME conditions remains largely unknown.

pH. Studies indicate that tumor tissues are more acidic (6.4–7.0) compared to healthy tissues (7.2–7.5) [86]. Intracellular pH plays an important role in exosome biogenesis and secretion [7,87]. A dynamic pH shift occurs when the acidic late endosomal MVB fuses with the neutral plasma membrane [88]. Ban et al. reveal that acidic conditions lead to the increased release of exosome protein, RNA, and surface markers [89]. Specifically, exosomes harvested from acidic conditions (pH 4) exhibit a five-fold increase in exosome protein than those collected under neutral pH conditions [89]. Dynamic pH conditions can also alter physical characteristics of exosomes. Exosomes harvested from cells cultured in acidic environments display increased membrane rigidity [87]. Consequently, these exosomes fuse with tumor cells both in acidic and neutral conditions more effectively [87]. These findings highlight that acidic pH increases exosome secretion [87,89].

Hypoxia. The primary TME vasculature is highly dysfunctional—with leaky and compressed blood vessels—which results in aberrant microcirculation and reduced oxygen concentrations [32]. Increased hypoxic conditions that recapitulate physiological TME can increase exosome release [6]. In addition, there is evidence that hypoxic-induced exosomes enhance EMT in cells [90]. As Ramteke et al. report, hypoxic-induced exosomes remodel adherens junctions in epithelial cells that promote EMT [90].

Hypoxia alters exosome miRNA content. EOC cell exosomes that were cultured and harvested under hypoxic conditions show increased expression of miRNA-940 than normoxia-cultured exosomes [61]. These hypoxic-derived exosomes induce more potent M2-like macrophage phenotypes [61]. M2-like macrophages highly resemble TAMs—whereby these cells lead to stroma remodeling, angiogenesis, immune suppression, and cancer metastasis [91]. M2 macrophages readily secrete proinflammatory chemokines, such as IL-10, that assist in EMT and tumor cell invasiveness [91]. This study further shows that miR-940-induced M2 macrophages and elevates EOC cell migration and proliferation [92].

Mechanical Stress. Mechanical cues play a critical role in activating cells in the TME to establish a tumor-promoting environment. This includes mechanical cues from local EOC tissues, rapidly proliferating tumor cells, and stiffening matrix [93].

Studies report that inducible mechanical stresses result in distinct exosome release profiles [94]. Exosomes collected from fibroblasts that had pull forces applied exhibit increased levels of TGF-B compared to static fibroblasts [94]. These pulled fibroblasts also secrete significantly more exosomes than control groups and contain unique miRNA profiles that induce tumor angiogenesis [94].

Chemotherapy and Radiation-based Treatment. Common treatment strategies for EOC often include aggressive administration of platinum-based chemotherapy [95]. The use of radiation therapy to combat local tumor growth is limited, but is often used after metastasis on surrounding or distal organs. Both treatment strategies alter exosome content and function [96] [–] [98].

Cisplatin is a common platinum-based drug used to treat EOC [99]. Exosomes released in response to cisplatin treatment induce potent bystander effects in neighboring ovarian cancer cells by activating cell invasiveness via JNK signaling and increasing drug resistance [97]. This suggests that cisplatin-stress released exosomes induce aggressive phenotypes to local EOC cells, potentially reducing the therapeutic effects of cisplatin.

Exosomes harvested from γ-irradiated cells promote cell migration and activate Akt pathways [100]. Proteomic analysis of exosome content show differential profiles that supported the observed pro-migratory profile [100].

Intracellular and Extracellular Calcium Concentrations. Intracellular calcium regulation is pivotal in cell regulation, but also in exosome secretion [101]. Studies show that calcium is necessary in exosome synthesis and increased intracellular calcium concentration positively enrich exosome release profiles [101]. Dynamic changes in intracellular calcium concentrations can alter exosome physical characteristics, such as exosome diameter [101].

Changes in extracellular calcium concentrations can also lead to the release of unique exosome populations [4]. Extracellular calcium chelation— *in vivo* conditions that often mirror gynecological malignancies, such as preeclampsia—trigger the secretion of a unique population of ovarian cancer cell exosomes (Fig. 4) [4]. These exosomes possess unique miRNA content and induce differential physical changes during exosome exchange [4]. Specifically, 1,019 miRNAs are differentially regulated between naturally secreting exosomes and

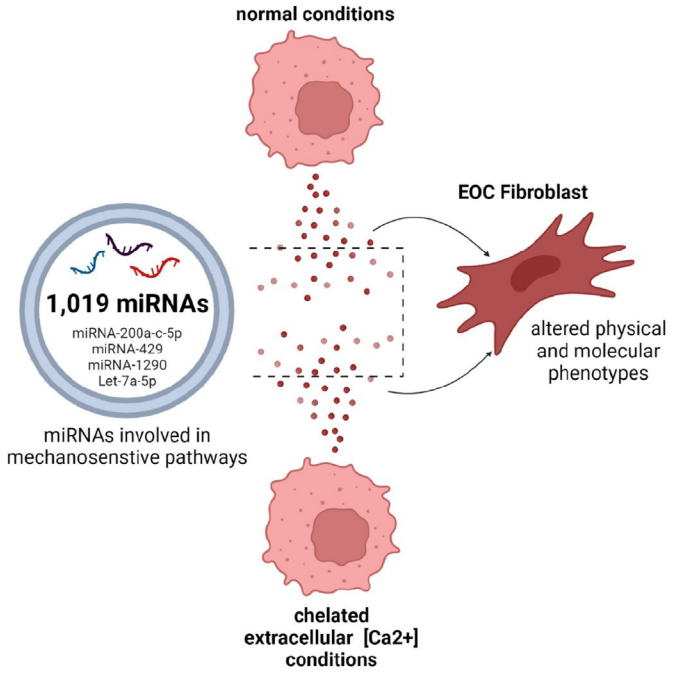

Fig. 4. TME Conditions (Extracellular Calcium Concentrations) Lead to Single-Cell Exosome Heterogeneity. Extracellular calcium chelation led to EOC cells secreting heterogenous single-cell exosome populations. These exosomes contained 1,019 differentially expressed miRNAs compared to EOC cells cultured in normal extracellular calcium concentrations. Many of these exosome miRNAs targeted mechanosensitive pathways (i.e.: focal adhesions and cytoskeletal organization) and altered physical and molecular EOC fibroblast phenotypes. *Created in BioRender.*

chelation-induced exosomes [4]. Many of the miRNAs target mechanotransduction-related proteins [4]. Chelation-induced exosome treatment induces dynamic filamentous actin organization and vinculin protein localization, migration, and adhesion profiles in patient-derived ovarian cancer fibroblasts [4].

Unique exosome populations derived from single-cell populations provide novel miRNA profiles that can be used in various therapeutic targeting strategies. These exosome miRNAs can also serve as biomarkers for aggressive cancers or TME conditions.

5. Exosome miRNAs as a clinical EOC diagnostic marker

miRNAs are a class of short, conserved non-coding RNAs (19–25 nucleotides) that modulate post-translational gene expression [102]. Up- and down-regulated miRNA expression levels regulate various metastatic processes important in metastatic initiation, progression, and dissemination [32]. miRNAs cohorts found in tissues, ascites, and exosomes can serve as differential biomarkers between various types of ovarian cancers (serous epithelial, mucinous, clear cell) and a patient's disease stage [103]. Previous research relies on uncharacteristic circulating or tissue miRNA expression profiles for early ovarian cancer diagnosis [103]. The heterogenous EOC nature and the rapid degradation of miRNAs, however, make it difficult to rely on miRNAs as a detection tool. Therefore, more current work has shifted to examining exosome miRNA content. Studies show that exosome miRNA profiles more accurately represent tumor cell molecular phenotypes compared to other circulating signaling molecules [104].

There are numerous circulating exosome miRNAs that are critical in EOC metastasis, diagnosis, and prognosis [105]. Commonly enriched ovarian cancer exosome miRNAs include positive upregulation of miRNAs-141 and -200a-c; downregulated miRNAs include −125b, −140, and-199a [105]. These miRNAs are consistently used as clinical diagnostic markers [105].

The miRNA-200 cohort is largely responsible for maintaining epithelial-like features in EOC and is known as a the “universal indicator” of epithelial phenotypes in cancer cells [106]. It suppresses EMT through inhibition of mesenchymal transcription factors, such as ZEB1, and TGF-B. miRNAs-200a-c are crucial in EOC metastasis because it is well known that this family support EOC cell growth and metastasis [107]. Exosomes isolated from fluids of malignant EOC patients display elevated levels of −200a-c cohort in comparison to benign patients [106]. However, EOC cells that retain more epithelial-like phenotypes secrete exosomes that express higher levels of miRNAs-200a-c compared to invasive EOC cells [108]. Differential miRNAs-200a-c expression levels are not only evident across heterogeneous EOC cell lines, but also variable in single-cell exosome populations. We previously show that miRNAs-200a-c are uniquely enriched between exosome populations that are secreted under different extracellular calcium concentrations [64].

miRNA-141 is closely related to miR-200a-c and often targets the same genes. It also shares the same seed sequence—the first 2–8 nucleotide sequence on the 5’ end of a mature miRNA that binds to target transcripts—as miRNA-200c [108,109]. miRNAs-141 and -200a target p38 [109]. This modulates oxidative stress responses and increases cisplatin-sensitivity and resistance in EOC cells [109]. miRNA-141 also promotes anoikis resistance by targeting KLF12 [110]. This downstream enriched Sp1-survivin expression inhibits apoptotic pathways and promotes EOC cell survival [110]. The increased miR-141 expression is transferred to secreted exosomes. miRNA-141 was also shown to be elevated in serum exosomes from women diagnosed with late stage EOC [110].

Downregulation of miRNA-199a has been correlated with cisplatin-resistance in EOC patients [111]. Alterations in miRNA-199a expression is critical in EOC because this miRNA targets multiple proteins and pathways that regulate hallmark EOC cell behaviors, including cell cycle progression, proliferation, and apoptosis [111].

miRNA-199a targets and regulates CD44, an important transmembrane protein that is responsible for tumor metastasis and chemoresistance development [112]. Cheng et al. report that overexpression of this miRNA via transfection inhibits CD44 activity in ovarian cancer-initiating cells [112]. Cancer-initiating cells are stem cells that rapidly proliferate and exhibit drug-resistant properties [113]. This resulted in chemosensitive cells that reduces proliferation, migration, and invasion. miRNA-199a further arrests cell cycle progression (G2/M) in transfected cells, which inhibits cell growth [112].

Similarly, miRNA-199a targets Yes-associated protein (YAP1) [114]. Yap1 is a potent oncogene that is elevated in EOC patients and that positively modulates proliferation, invasion, and viability [114]. Studies report that overexpression of miRNA-199a in EOC cells directly target Yap1 and decrease cell viability and increase apoptotic rates [114]. Therefore, enhancing miRNA-199a expression could act as a therapeutic target for EOC patients. Inhibiting or overexpressing miRNAs that are associated with poor EOC prognosis can differentially target tumorigenic genes during post-translational modification; this process can mitigate or promote TME cell behaviors.

6. Exosomes as drug delivery vehicles in cancer therapy

Exosomes are prime candidates for drug delivery vehicles due to their a) intrinsic endogenous nature to the body, leading to non-immunogenic and non-toxic characteristics and b) site-specific attachment due to specific surface integrin profiles [115]. Exosomes can evade macrophage-driven phagocytosis and the stable cholesterol-rich lipid bilayers increase the systemic circulation time [116]. These nanovesicles can therefore act as natural drug, miRNA, and siRNA carriers that prolong the half-life of encapsulated anti-cancer therapies.

Tian et al. loaded immature dendritic cell exosomes at 20% encapsulation efficiency with doxorubicin to combat tumor growth [117]. Exosome surfaces were modified with targeting ligands (i.e.: Lamp2b, a ubiquitous exosome membrane protein) and were able to specifically adhere to tumor cell and tissues (i.e.: αv-integrin positive tumor cells), and thus inhibited tumor growth [117].

Studies have also engineered a combinatorial therapeutic approach by loading exosomes with miRNAs and anti-cancer drugs. Liang et al. used exosomes to deliver 5-FU, a drug used to treat colorectal cancer, and a miRNA-21 inhibitor oligonucleotide [118]. Tumor cells were able to efficiently uptake engineered exosomes [118]. Results indicate that the engineering exosomes could deliver this miRNA inhibitor to the cytoplasm of recipient cells and silence the function of miRNA-21 and its corresponding targets [118]. miRNA-21 targets include PTEN and $hMSH_2$. The codelivery system recovered both regulatory gene expressions and subsequently reversed drug resistance, cell proliferation and induced apoptosis [118].

Triptolide, a drug that possesses anti-inflammatory properties and is an active ingredient in several anticancer treatments, can serve as a promising adjunct to chemotherapy for ovarian cancer; however, low aqueous solubility and hepatotoxic profiles limit its bioavailability and use as clinical therapeutic, respectively [119,120]. To address this limitation, Liu et al. engineered triptolide-encapsulated exosomes that yielded high drug encapsulation efficiency [119]. The study revealed that triptolide-encapsulated exosomes inhibited EOC cell growth and tumor proliferation over four weeks [119]. Yet, histopathology results showed that prolonged exosome treatment led to liver and spleen dysfunction. Although these engineered exosomes can lead to organ toxicity after multiple applications, Liu et al. showed that exosomes can serve as tissue-specific delivery systems for encapsulated drugs that present complicated absorption characteristics [119]. These studies demonstrate the potential usefulness of exosome delivery systems for targeted cancer therapy of drugs with low bioavailability, which should be balanced with toxicity research to limit the organ toxicity issues.

7. Concluding remarks

Exosomes are TME signaling vesicles that contribute to the complexities of tumor heterogeneity. These nanovesicles are of particular importance to the EOC TME because tumor cells and tumor-associated cells rely on the exosome-rich ascites when metastasizing from primary to distal sites. They play important roles in paracrine and autocrine communication, such as reprogramming recipient cells to take on unique, heterogenous phenotypes and priming secondary sites as a metastatic niche. Heterogenous cellular responses in EOC (driven via exosome exchange) lead to dual metastatic responses—retain epithelial traits vs. induce mesenchymal phenotypes (i.e.: mixed EMT). Because exosome exchange and exosome content influence tumor heterogeneity, we believe that it is critical to examine the various factors that alter exosome biogenesis and secretion.

The importance of exosome heterogeneity derived from various cell types is widely established and acknowledged. However, it is critical to examine how hallmark TME conditions, such as hypoxia, mechanical stress, and low pH conditions, alter proteins and pathways that are necessary for exosome synthesis. These dynamic conditions can alter miRNA content contributing to variation in exosome populations secreted by individual cells—a type of exosome heterogeneity that is not as widely studied. Single-cell exosome heterogeneity can ultimately reveal unique miRNAs and targets that regulate EOC metastasis under tumor microenvironment stress. These can serve as biomarkers or be used as a metric to diagnose stages of EOC. More importantly, these miRNAs can lead way to new engineered exosomes that serve as cancer drug delivery vehicles. Combining the need to examine exosome heterogeneity more in-depth with new ways to use exosomes as drug delivery vehicles can provide new ways to combat deadly ovarian cancers.

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Declaration of competing interest

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