



Review

Extracellular vesicles and anti-cancer drug resistance

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ABSTRACT

Extracellular vesicles (EVs) including exosomes, microvesicles, oncosomes, and microparticles have been associated with communicating anti-cancer drug-resistance. The *in vitro*, pre-clinical *in vivo* and patients' data linking EVs to drug-resistance (and the specific drugs involved) in breast cancer, prostate cancer, lung cancer, ovarian cancer, haematological malignancies, colorectal cancer, gastric cancer, pancreatic cancer, glioblastoma, neuroblastoma, melanoma, kidney cancer and osteosarcoma. Details of the mechanisms by which the resistance seems to be occurring (e.g. EVs transferring drug-efflux pumps from drug-resistant cancer cells, EVs binding monoclonal antibodies in the peripheral circulation and so reducing their bioavailability, EVs from tumour microenvironment cells, etc.) are outlined, as are efforts to try to block such resistance. Research to date strongly supports EVs as playing a key role in drug-resistance. Further studies including tailored clinical studies are now warranted to determine how best to prevent this occurring, in the interest of patients and also for economic benefit. Furthermore, efforts to exploit safe (non-cancer origin) EVs as anti-cancer drug delivery vehicles that may achieve efficacy with more limited side-effects than free drug, deserve further investigation.

1. Introduction

Approximately 30 years ago, exosomes were described as involved in reticulocyte maturation by transporting transferrin receptor out of the cell [35]. Building on this knowledge, over recent years increasing evidence indicates that substantial cargos of information are released from cells *via* lipid bilayer-enclosed vesicles typically termed exosomes and microvesicles. These vesicles are proposed to be tailor-made specialised mini-maps of their cell of origin; are transported in the bloodstream and other body fluids; and much evidence indicates them to be involved in cell-to-cell communication. Exosomes and microvesicles, collectively termed extracellular vesicles (EVs), are often defined and sub-grouped based on size and cellular origin (exosomes ~30 nm–120 nm, endosomal origin; microvesicles/ectosomes > 120–1000 nm, from the cell membrane). It should be noted that some reports use additional or alternative terms including, but not limited to, ectosomes, microparticles, oncosomes and prostasomes; all of which are EVs, as, indeed, are apoptotic bodies. However, once outside the cell and released into the environment (for example, the bloodstream) we cannot be certain if the EVs originated from the cells' endosomal region or directly from the cell membrane. Furthermore, EV size distinctions are not absolute *i.e.* there is no known reason why vesicles budding from the cell membrane cannot be < 120 nm. In diseases such as cancer, regardless of the size and origin of EVs released, arguably the

problems that EVs contribute to when released are of much importance to understand. Evidence from pre-clinical and clinical specimens' studies, by ourselves and others, strongly associate EVs with transmitting anti-cancer drug-resistance from cell-to-cell in multiple cancer types.

This phenomenon, known as multi-drug resistance or multiple-drug resistance (MDR) and initially described > 30 years ago [8], is manifest as cancer cells being resistant to anti-cancer drugs that are structurally and mechanistically unrelated. MDR may present as innate/primary or it may be acquired. Innate MDR means that, from the outset, the cancer cells are already equipped to be resistant to the anti-cancer drug being used. Acquired resistance occurs when cancer cells initially respond to treatment, but develop resistance mechanisms overtime. Advancing on early work where the *mdr1* gene (which encodes the ABC transporter, ABCB1, also known as P-glycoprotein (P-gp) and now well established as causally involved in MDR) was cloned [72], many research studies and reviews (exemplified by [17,26,28,40,43,45,56–58,73,79,84]; but too many to detail here) have been published on this topic. MDR is a substantial concern in cancer management.

Our understanding is that the first study showing transmission of drug-resistance by EVs, was a study performed by our group in prostate cancer [18]. Numerous important studies by other research groups have been reported. A good understanding of this undesirable communication of drug-resistance by EVs may help pave the way to its circumvention or, indeed, prevention, and so have therapeutic benefit for

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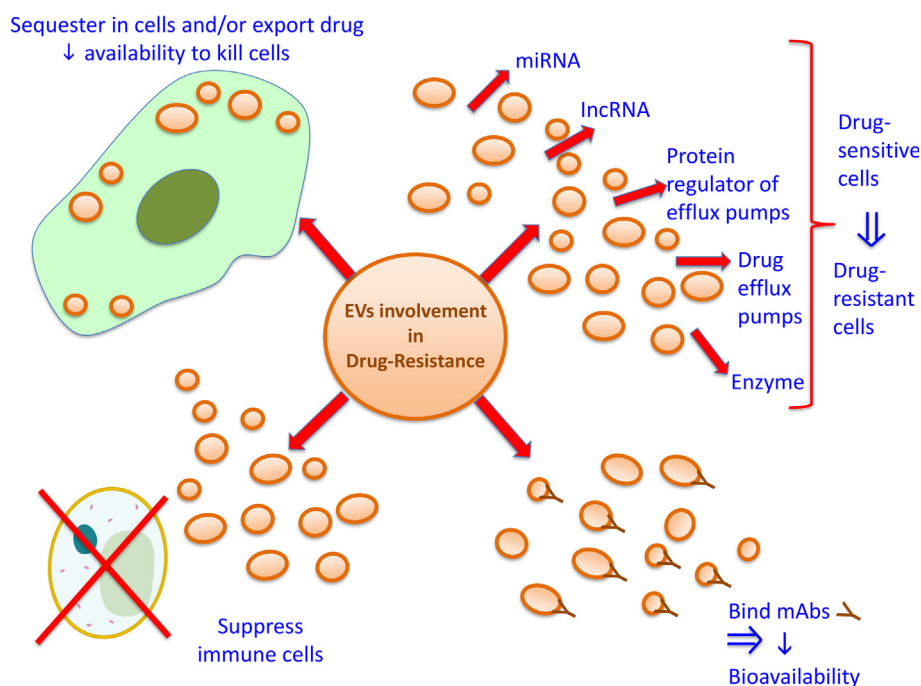


Fig. 1. Multiple mechanisms whereby EVs released from drug-resistant cancer cells have been associated with transmitting resistance to other cells.

cancer patients (Fig. 1 summarises example mechanisms associated with this resistance). Thus, here we review the emerging data in this field across multiple cancer types. Of note, throughout the document we have used the term EV for all vesicles studies but in Table 1 - where we have summarised studies in chronological order- we have included the specific vesicle type, as suggested by the researchers reporting on each study. Furthermore, although EV isolation methods involving ultracentrifugation are still the most commonly used [25] an increasing number of methods and variations of methods have been used. This information is also indicated in Table 1.

2. Breast cancer

One of the earliest investigations into drug-resistance associated with EV structures was in breast cancer. Notably, however, these vesicles were studied when they remained intracellular, so were not extracellular vesicles *per se*. This study reported that a variant of MCF-7 cell line with 20-fold resistance to mitoxantrone (thus termed MCF-7/MR cells) had, confined to its cell-cell attachment zones, an increase in EV-like structures containing the ATP-binding cassette (ABC) transporter protein ABCG2, which is also termed breast cancer resistance protein (BCRP). These vesicles, which the authors termed EVs (although they were not analysed extracellular *per se*), sequestered mitoxantrone and so promoted drug-resistance. Specifically, by removing drug out of the cytoplasm and into EVs, MCF-7/MR cells were drug-resistant compared to MCF-7 cells. This mitoxantrone-resistance was inhibited using the ABCG2/BCRP inhibitor ko143. It was concluded that these EVs serve as drug disposal chambers shared by multiple neighbour cells [32].

In breast cancer, Chen et al. [13] reported a role for EVs in mediating drug-resistance. Here, EVs from adriamycin- and docetaxel-resistant cell lines, MCF-7/Adr and MCF-7/Doc respectively, transferred resistance to previously drug-sensitive MCF-7 cells, with the uptake of fluorescently (PKH26)-labelled EVs by the MCF-7 cells recorded by confocal microscopy and flow cytometry. A decrease of apoptosis in response to docetaxel exposure resulted when the MCF-7 cells were incubated with resistant cell-derived EVs. Investigating possible mechanism involved, miR-100, miR-222, miR-30a and miR-17 levels were found to be significantly increased in previously drug-sensitive MCF-7

cells following transfer of EVs from the docetaxel-resistant variants. In three follow-up reports involving these cells, the same group reported other EV-carried miRNAs from the resistant cells could be transferred to the previously drug-sensitive MCF-7 cells and may be causally involved in the EV-transmitted drug-resistance [14,49,96]. More recently the same group showed the phase II metabolising enzyme glutathione-S-transferase P1 (GSTP1) which detoxifies anti-cancer drugs by conjugating them with glutathione, to be at significantly higher levels in the MCF-7/Adr cells and their EVs respective to the MCF-7 cells and their EVs. The GSTP1 - evaluated as GSTP1 mRNA in the recipient cells- could be transferred by EVs in a dose-dependent manner from the resistant cells, resulting in acquired resistance to adriamycin. This resistance, evaluated as reduced apoptosis, was dose-dependent on the quantities of EVs added prior to adriamycin exposure. Then considering patients specimens, the researchers reported significantly higher quantities of EVs (reported in μg , based on protein analysis as a surrogate for EV quantities) carrying higher amounts of GSTP1 mRNA in serum from patients who did not respond ($n = 14$) to neoadjuvant anthracycline/taxane-based chemotherapy compared to those who did respond ($n = 16$) [94].

As evident from above, some of the EV-carried molecules implicated in drug-resistance are RNAs, including miRNAs. Similarly, EVs from MCF-7's tamoxifen-resistant variant MCF-7^{TamR} transferring miR-221/222 have been reported as a mechanism of tamoxifen-resistance [88]. Furthermore, our group found the aggressive triple negative breast cancer (TNBC) cell line variant Hs578Ts(i)₈ to contain reduced miR-134 levels compared to its less aggressive parental cell line Hs578T. Interestingly, in turn, Hs578Ts(i)₈-derived EVs showed similar low levels of miR-134 to their cell of origin. Subsequent delivery of miR-134 to Hs578Ts(i)₈ cells (*via* miR-134-enriched EVs or by direct transfection) increased the cells' sensitivity to cisplatin and to anti-Hsp90 compounds 17-AAG and PU-H71 [55].

Another class of non-coding RNA, long non-coding RNA (lncRNA), can also be transferred *via* EVs transmitting drug-resistance. Previously thought to be bystanders, lncRNAs are now recognised as having a role in gene regulation causing adverse effects when they become dysregulated [29]. Xu et al. [92] analysed the tamoxifen-resistant variant of MCF-7, termed LCC2, and found the tamoxifen-resistance to be associated with increased levels of the lncRNA urothelial cancer associated

Table 1
Summary of many studies, in chronological order, that support EVs being involved in communicating anti-cancer drug-resistance.

Cancer type/subtype	EV Type (as reported by authors)	Isolation Method	Anti-cancer drug(s) in question	Cell lines/Specimens	Reference
Breast	Extracellular vesicles (termed EVs but only analysed intracellularly)	No isolation-immunohistochemical localisation and TEM	Mitoxantrone	MCF-7 and resistant variants MCF-7/MR, MCF-7/FLV1000	[32]
Ovarian	Exosomes	Centrifugation, Ultracentrifugation	Cisplatin (DDP)	Ovarian carcinoma 2008 cells and 2008/CI3*5.25 (resistant)	[76]
Acute lymphoblastic leukaemia (ALL)	Microparticles	Differential centrifugation	Daunorubicin	CCRF-CEM, VLB ₁₀₀	[7]
B-cell lymphoma	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Rituximab	B-cell lymphoma cell lines Su-DHL-4, Balm3, OCI-Ly1	[6]
Breast	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Trastuzumab Lapatinib	Lymphoma patients blood specimens (n = 6) SKBR3, BT474, MDA-MB-231 Murine NIH3T3 <i>neu</i> , NIH3T3 WT (Fibroblast cells)	[16]
Prostate	Exosome	Differential centrifugation, Filtration, Ultracentrifugation Isolated from human sera by ExoQuick-TC exosome precipitation solution	Docetaxel	Sera of healthy volunteers and HER2-overexpressing cancer patients (n = 11 early stage and n = 11 advanced stage) DUI45, DUI45RD, 22Rv1, 22Rv1RD, LNCap Serum of prostate cancer patients (n = 6) and healthy volunteers (n = 6). Further sera from prostate cancer patients (n = 8) before and during docetaxel therapy	[18]
Ovarian	Exosomes	Centrifugation, 30% sucrose-deuterium oxide-cushion ultracentrifugation	Platinum-based chemotherapy (e.g. DDP, paclitaxel, carboplatin, cyclophosphamide)	Drug-sensitive epithelial ovarian cancer cell line SKOV3 and A2780. Resistant derivatives SKOV3/Cis and A2780/Cis Ovarian cancer patients sera (n = 50) Normal female donor sera (n = 30)	[95]
Prostate	Extracellular vesicles	Centrifugation, ultracentrifugation	Camptothecin (CPT)	DUI45, RC1 (resistant) non-malignant human prostate epithelial cells (PnEC), Prostate cancer patients tissue(n = 5)	[61]
Breast	Exosomes	Centrifugation, Filtration, Ultracentrifugation	Docetaxel	MCF-7, MCF-7/DOC (resistant)	[13]
Breast	Exosomes	Centrifugation, Filtration, Ultracentrifugation	Docetaxel Adriamycin	MCF-7, MCF-7/DOC, MCF-7/ADR	[14]
Breast	Microvesicles	Centrifugation, Ultracentrifugation	Adriamycin	MCF-7, MCF-7/ADM, Human microvessel endothelial cells (HMEGs), BALB/c AnNCr-nu/nu mice	[21]
Breast	Extracellular vesicles	Differential centrifugation, Ultracentrifugation	Paclitaxel Adriamycin	MCF-7, MCF-7/ADM Nude female mice (n = 7)	[47]
Breast	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Tamoxifen	Breast cancer tissue (n = 26) and peripheral blood specimens (n = 33) MCF-7 MCF-7 ^{tamk}	[88]
Lung	Extracellular vesicle	Serial centrifugation, sucrose cushion ultracentrifugation, Optiprep density gradient ultracentrifugation	Gefitinib	PC9, PC9R	[15]
Lung	Exosomes	ExoQuick-TC exosome Precipitation Solution, Ultracentrifugation	DDP	A549	[91]
Ovarian	Microvesicles	Differential centrifugation, Ultracentrifugation	Paclitaxel Adriamycin	A2780, A2780/PTX	[100]
Colon	Microvesicles	Centrifugation, Ultracentrifugation, Filtration	Fluorouracil (5-FU)	DLD-1, DLD-1/5FU	[2]
Colon	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Oxaliplatin	Human colon cancer cells (HCT116, SW480-ADH, SW1417) Fibroblast cells BJ-5ta, Human brain vascular pericytes (HBVP), HUVECs	[77]
Hepatocellular carcinoma (HCC)	Extracellular vesicles	Differential centrifugation, Filtration, Ultracentrifugation	GPT Doxorubicin Sorafenib	Colon cancer tumour and blood specimens (n = 69), Athymic nude-Fox1nu mice HepG2, PLC-PRF5 Non-malignant human hepatocytes	[78]

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Table 1 (continued)

Cancer type/subtype	EV Type [as reported by authors]	Isolation Method	Anti-cancer drug(s) in question	Cell lines/Specimens	Reference
Multiple myeloma (MM)	Exosomes	Concentrated (Centriprep centrifugal filter 3 K device), Filtration, ExoQuick-TC exosome precipitation solution, centrifugation	Doxorubicin Bortezomib JNJ-26481585	Murine MM cell lines 5T33MMvt and 5T33MMvv Bone marrow stromal cells (BMSCs) derived from healthy donors, MM patients, naïve mice and 5T33MM mice Mice (C57BL/KaLwRij and 5T33MM) Human MM cell line RPMI8226	[85]
Breast	Exosomes	Differential centrifugation, Ultracentrifugation, Sucrose density gradient centrifugation	Z-guggulsterone Bexarotene Doxorubicin	MDA-MB-231	[38]
Breast	Extracellular vesicles	Filtration and Ultracentrifugation	DDP Anti-Hsp90 compounds 17-AAG and PU-H71	Hs578T and Hs578T <i>(i)</i> s	[55]
Ovarian	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	DDP	A2780, CP70, OVCAR5, OVCAR8 and IGROV1	[64]
Colon	Exosomes	Total exosome isolation kit Differential centrifugation and Ultracentrifugation	5-FU Oxaliplatin	Human colon cancer cells SW260 Fibroblasts derived from normal colon tissues 18Co Cancer-associated fibroblasts isolated from primary colorectal cancer tissue	[30]
Neuroblastoma (NBL)	Exosomes	Centrifugation, Precipitation using ExoQuick-TC exosome precipitation solution, centrifugation	DDP	Female NOD/SCID (<i>n</i> = 4) and BALB/c- <i>nu</i> mice (<i>n</i> = 5) NBL primary tissues (<i>n</i> = 20) Female <i>nu/nu</i> mice (<i>n</i> = 12) Human NBL cell lines SK-N-BE(2), CHLA-255, IMR-32, LA-N-1, and KNCR	[11]
Gastric	Exosomes	Density gradient centrifugation	5-FU DDP	Human monocytes isolated from healthy male donor PBMCs Mesenchymal stem cells isolated from human umbilical cord, Human foetal lung fibroblast (HFL1), Gastric cancer cell lines (HGC-27, MGC-803, and SGC-7901), Male BALB/c <i>nu/nu</i> mice (<i>n</i> = 24) MCF-7, MCF-7/Adr	[34]
Breast	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Adriamycin	MCF-7, LLC2 (tamoxifen-resistant)	[49]
Breast	Exosomes	Differential centrifugation, Ultracentrifugation	Tamoxifen	MCF-7, MCF-7/Adr	[92]
Breast	Exosomes	Differential centrifugation, Ultracentrifugation	Adriamycin	DUI45 and PC3 cell line and resistant variants DUI45-TXR and PC3-TXR	[96]
Prostate	Exosomes	Differential centrifugation, Ultracentrifugation		PC9 (NSCLC) and A549	[41]
Lung	Exosomes	ExoQuick-TC exosome precipitation solution Differential centrifugation	Gefitinib DDP	Primary CAFs and CAAs (cancer associated fibroblasts and adipocytes) from ovarian cancer patients	[42]
Ovarian	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Paclitaxel	Primary fibroblasts and adipocytes derived from normal ovaries and omental tissue respectively Human ovarian cancer cell lines A2780,ALST, OVCA432, OYCA433, OVCAR5, HeyA8, HeyA8-MDR, SKOV3ip, SKOV3-TR, SKOV3, PEA1 and PEA2, Female BALB/c euthymic nude mice Human HCC cell lines SMMC-7721, MHCC-97H, MHCC-97L and LO2	[5]
HCC	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Sorafenib	Male BALB/c <i>nu/nu</i> mice (<i>n</i> = 25)	[68]
Renal cell carcinoma (RCC)	Exosomes	Filtration, Differential centrifugation, Ultracentrifugation	Sunitinib	RCC cell lines 786-O and ACHN Sunitinib-resistant cell lines 7Su3rd, ASCu3rd and 771R, HK2 cell line (epithelial cells from human kidney), HUVECs Male athymic BALB/c nude mice RCC patients tumour tissue specimens (<i>n</i> = 84) RCC patient blood specimens (<i>n</i> = 71)	[67]
Osteosarcoma	Exosomes	Differential centrifugation, Ultracentrifugation	Doxorubicin	Human osteosarcoma cell line MG-63, MG-63DXR30 (resistant variant)	[81]

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Table 1 (continued)

Cancer type/subtype	EV Type [as reported by authors]	Isolation Method	Anti-cancer drug(s) in question	Cell lines/Specimens	Reference
Breast	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Adriamycin	MCF-7, MCF-7/ADM	[54]
Breast	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation (MCF-7 cells)	Adriamycin	MCF-7, MCF-7/ADR	[94]
Prostate	Extracellular vesicles	Total exosome isolation reagent (patient serum specimens)	Enzalutamide	Peripheral blood specimens from breast cancer patients (n = 30), breast cancer tissue (n = 42) before and after chemotherapy	[22]
Lung (NSCLC)	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	Gemcitabine	VCap, 22Rv1 and 22Rv1/CR1 (mesenchymal-like cell line derived from 22Rv1)	[44]
Lung	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	Parental human bronchial epithelial cells (HBECs)30KT	[65]
Lung	Exosomes	Differential centrifugation, Filtration, Ultracentrifugation	DDP	HBECs with p53 knockdown, KRAS ^{V12} overexpression and LKB1 knockdown 30KTP ^{p53/KRAS/LKB1}	[66]
Lung	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	A549, A549/DDP (resistant)	[87]
Lung	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	A549, A549/DDP	[90]
Lung	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	Male BALB/c athymic nude mice	[97]
Lung	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	NSCLC patients (n = 50), Female SCID mice (n = 16), A549, A549/GR	[19]
Ovarian	Exosomes	Differential centrifugation, Ultracentrifugation	Carboplatin	NSCLC patients (n = 50), Female SCID mice (n = 16), A549, A549/GR	[69]
HCC Cervical cancer	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	BEAS-2B, A549, PC9 (NSCLC) and H1299 (NSCLC)	[101]
Gastric	Exosomes	Differential centrifugation, Ultracentrifugation	DDP	Sera from lung cancer patients (n = 56) and normal patients (n = 19)	[97]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	A549, A549/DDP	[51]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Sera from advanced NSCLC patients (n = 100)	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Human ovarian cancer cells A2780, C30, CP70, C200, OVCAR5, A1847, and OVCAR10	[83]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	HCC cell line HepG2	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Human cervical cancer cell line Hela	[101]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Gastric cancer cell lines MFC and MGC-803	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Murine bone marrow-derived macrophages (BMDM) isolated from C57BL/6 male mice,	[101]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Male athymicC57 nude mice (n = 5)	[51]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	PDAC cell lines Panc1, MiaPaCa2, PSN1	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Gemcitabine-resistant cell lines established from Panc1 (Panc1-GR1, GR3 and GR4)	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	PDAC patient specimens (n = 45, with plasma specimens from 23 patients)	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Female non-obese mice with diabetes/SCID (n = 5)	[71]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Patient-derived fibroblasts,	[71]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	AsPC-1, BxPC-3, PANC-1 and L3.6 cells,	[71]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	NOD/SCID mice	[71]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	MiaPaCa and Colo-357	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	HL60, HL60/AR (resistant variant)	[9]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Human melanoma cell line LM-MEL-64 established from resected melanoma metastases	[83]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	LM-MEL-64R3 (resistant)	[83]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	A431	[83]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Human GBM cell line U87, A172, U118 and LN18	[99]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	Primary human N3 and K3 GBM cells	[99]
Pancreatic (PDAC)	Exosomes	Differential centrifugation, Ultracentrifugation	Gemcitabine	HBMVECs and Normal human astrocytes (NHAs)	[99]

1 (UCA1). Pre-treatment with EVs from these tamoxifen-resistant LCC2 cells, which like their cells of origin carried increased amounts of UCA1, protected MCF-7 viability upon subsequent tamoxifen treatment. This tamoxifen-resistance was associated with decreased expression of cleaved caspase-3 and inhibition of apoptosis. The subsequent knock-down of UCA1 in LCC2 cells indicated that the lncRNA plays a mechanistic role in tamoxifen-resistance, as EVs from these UCA1-knock-down cells had a reduced effect on MCF-7 cell survival when the cells were exposed to tamoxifen.

EV-carried proteins have also been implicated in promoting drug-resistance in breast cancer. The Ca^{2+} -permeable transient receptor potential channel 5 (TrpC5) is reported to regulate P-gp [46]. Advancing on this knowledge and using MCF-7 models, EVs from adriamycin-resistant MCF-7 cells (MCF-7/Adr) were found to transfer TrpC5 (and P-gp) to recipient human microvessel endothelial cells (HMECs) and further induce *de novo* expression of P-gp. This P-gp induction was diminished if the EVs were pre-treated with T5E3, a TrpC5-specific blocking antibody [21]. The subsequent study from this group reported that EVs from peripheral blood plasma of mice bearing MCF-Adr xenografts carried TrpC5, *mdr1*, as well as MUC1 and flotillin2 mRNAs. In keeping with this and considering the potential clinical relevance, immunohistochemistry performed on breast cancer tissues demonstrated a correlation between TrpC5 and treatment-resistance, with non-responders' tumours showing increased amounts of TrpC5 [47]. Then progressing to EVs from plasma specimens of breast cancer patients, the 4 mRNAs (*i.e.* TrpC5, *mdr1*, as well as MUC1 and flotillin 2) were amplified from EVs from patients being treated with chemotherapy ($n = 17$), but not those without chemotherapy ($n = 12$). The researchers thus suggested that the TrpC5-containing EVs circulating in the bloodstream may transfer drug-resistance to non-resistant cells.

Of note, in keeping with the association between drug efflux pumps and drug-resistance in breast cancer, using MDA-MB-231 cell line model, the combination of guggulsterone (a farnesoid X receptor antagonist and bexarotene (retinoid X receptor agonist)) was found to reduce cellular BCRP levels by approximately 80% by inducing its association and secretion with EV. This resulted in increased doxorubicin retention and enhanced cell death [38].

Survivin is an anti-apoptotic protein [59] that has been substantially analysed and found at the protein level [36], but not at the mRNA level [60], to be significantly associated with survival outcomes from breast cancer. Treating the TNBC cell line, MDA-MB-231, for 8 h with drugs that affect the microtubules (paclitaxel and nocodazole), but not other chemotherapeutic drugs, induced the release of EVs carrying survivin protein. These EVs, isolated using filtration and centrifugation of the conditioned medium (CM), were able to protect serum-starved or paclitaxel-treated HER2-overexpressing cells (SKBR3) and fibroblasts from death, except when survivin was knocked-down in the EV-generating MDA-MB-231 cells. This strongly implicated EV-carried survivin protein as causally involved in the cells' protection [39]. This would seem to suggest, however, that either paclitaxel and/or nocodazole drugs were not carried out of the MDA-MB-231 cells in EVs or that the drug(s) carried did not act on the receiving cells. In contrast, in studies including the same cell line MDA-MB-231, Wang et al. [86] also reported induced EV release in response to paclitaxel but, in that study, the released EVs carried paclitaxel and inhibited migration of recipient cells. Differences here may be, at least partly, due to the former study involving treatment with 50 nM paclitaxel for 8 h, while the latter study used 500 nM paclitaxel for 24 h. The two studies also used different recipient cells for EV analysis.

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that plays an important role in protein degradation through recycling free ubiquitin by cleaving ubiquitylated peptides. UCH-L1 mRNA levels are significantly greater in breast tumour ($n = 100$) compared to normal breast tissue ($n = 24$) with higher UCH-L1 mRNA levels in breast tumours associated with ER-/PR- and poor prognosis [52]. UCH-L1 over-expression has been reported to enhance MDR in

breast cancer by up-regulating P-gp protein expression levels via the MAPK/ERK signalling pathway. In a study of MCF-7 and its adriamycin-resistant variant (in this study termed MCF-7/ADM cells), transmission electron microscopy (TEM) analysis showed enhanced EV generation on the surface of the resistant cells. Reflecting the UCH-L1 and P-gp status of their cells of origin, EVs released by the MCF-7/ADM cells carried increased amounts of UCH-L1 and P-gp. When sensitive MCF-7 cells were incubated with EVs from MCF-7/ADM cells there was a shift towards increased UCH-L1, p-ERK and P-gp protein levels in the recipient cells associated with a substantial induction of adriamycin-resistance. Analysing the EVs from serum of patients with breast cancer ($n = 93$) for the presence of UCH-L1 showed an association between EV-UCH-L1 (evaluated by flow cytometry) and outcome, with above average levels of UCH-L1 in EVs associated with poorer response to adjuvant anthracycline/taxane-based chemotherapy compared to those patients whose EVs had below average levels of UCH-L1 [54].

Resistance associated with EVs is not restricted to small molecules, but also biologics such as monoclonal antibodies. Initial studies with EVs from HER2-positive cell lines SKBR3 and BT474 showed the EVs bound to the anti-HER2 monoclonal trastuzumab and so reduced its bioavailability, as expected this was not found with EVs from the HER2-negative cell line MDA-MB-231. The results were supported by studies of serum specimens from breast cancer patients that showed that the majority of (8 of 11) EV preparations from advanced-staged patients had HER2-positive EVs that bound to trastuzumab, compared to only 2 of 11 early stage patients [16].

Our group has shown that HER2-positive cells that are resistant to HER2-targeting drugs, as well as their released EVs, have increased amounts of the immunosuppressive cytokine $\text{TGF}\beta_1$, when compared to the drug-sensitive HER2-positive parent cells and their corresponding EVs. Furthermore, EVs from the drug-resistant cells were able to increase levels of $\text{TGF}\beta_1$ in drug-sensitive cells. In our neo-adjuvant clinical trial including trastuzumab and lapatinib, $\text{TGF}\beta_1$ levels were significantly higher in EVs isolated from the serum of patients with HER2-overexpressing breast cancers who went on to not respond to HER2-targeted drug treatment ($n = 4$), compared to those who experienced complete or partial response ($n = 26$). While the numbers of patients' specimens available were too few to make any substantial claims, the EV levels of $\text{TGF}\beta_1$ correlating with patients' response *versus* resistance to HER2-targeted drugs suggests a potential use of EV- $\text{TGF}\beta_1$ as a minimally-invasive companion diagnostic for such treatment in breast cancer [50].

3. Prostate cancer

To the best of our knowledge, the first studies associating EVs with drug-resistance in prostate cancer were performed by our research group. These studies centred around docetaxel and included analysis of 22Rv1 and DU145 cell lines and their respective docetaxel-resistant variants 22Rv1RD and DU145RD. The research showed EVs from the docetaxel-resistant cells transmitted docetaxel-resistance to previous drug-sensitive parent cell lines. The resistant cells and their released EVs carried substantial amounts of P-gp indicating that they may, at least partly, be responsible for the acquired-resistance. Considering clinical specimens, EVs from serum of prostate cancer patients compared to those from healthy controls ($n = 6$ each) significantly increased invasion and proliferation of recipient DU145 and 22Rv1 cells. Subsequently, EVs isolated from sera of a small cohort of prostate cancer patients classified as non-responders to docetaxel treatment (2 out of a total of 8 patients were non-responders *i.e.* their PSA levels increased during the course of treatment) protected both 22Rv1 and DU145 cells from the effects of docetaxel, while EVs from the 6 responders seemed to enhance the effects of docetaxel on these cells line [18]. Investigating miRNAs that may be causally involved in transferring this resistance to docetaxel, we subsequently performed global profiling of miRNAs and found a strong correlation between the

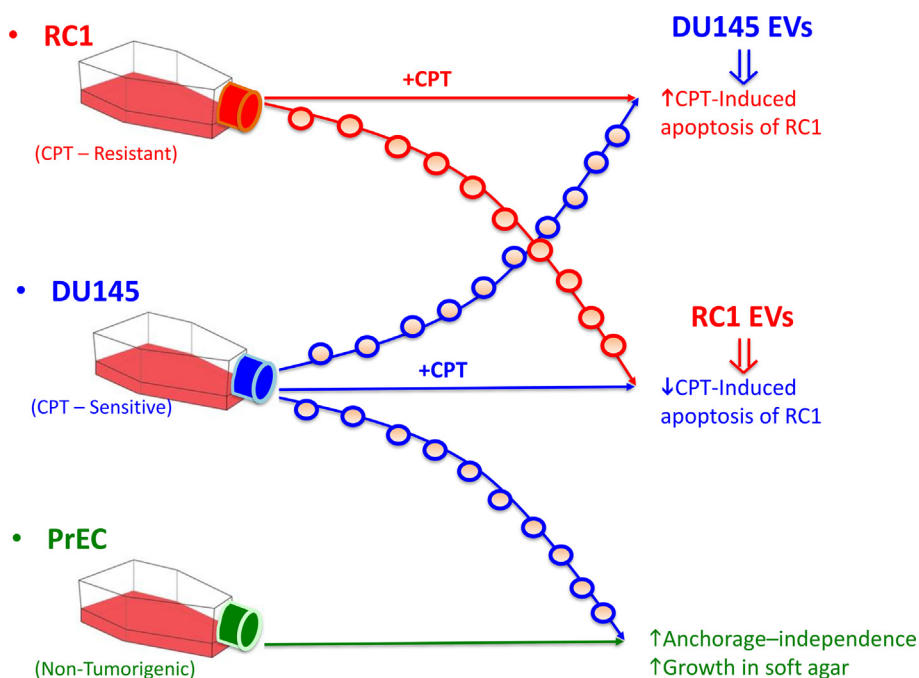


Fig. 2. Studies of drug-resistant and drug-sensitive prostate cancer cells, as well as non-tumorigenic prostate cells, show that the EVs from the drug-resistant (the drug being camptothecin (CPT)) cells transmit resistance to the previously drug-sensitive cells. Conversely, EVs from the drug-sensitive cells sensitised previously resistant cells to drug. Furthermore, EVs from the cancer cells induced anchorage-independence and growth in soft agar to the non-tumorigenic cells.

detection of miRNAs in EVs and the corresponding drug-resistant and drug-sensitive cell lines; supporting EVs being “mini-maps” of their cells of origin. Of the miRNAs chosen for further validation and clinical assessment, decreased miR-34a levels showed substantial clinical relevance and so was chosen for further functional analysis. Manipulating miR-34a in the prostate cancer cells confirmed that this miRNA regulates BCL-2 (a target of docetaxel) and thus may, in part, regulate response to docetaxel.

As summarised in Fig. 2, a subsequent study included DU145 cells, their camptothecin (CPT)-resistant variant RC1, and non-tumorigenic immortalised prostate epithelial cells (PrEC). Firstly, drug-sensitive DU145 cells co-cultured with RC1-EVs were found not to undergo apoptosis in response to CPT while, conversely, the drug-resistant RC1 cells were sensitised to CPT by co-culturing with DU145-EVs and so underwent substantially increased levels of apoptosis. Furthermore, co-culturing PrECs with EVs from DU145 cells induced their anchorage independence and ability to form colonies in soft agar. Evaluation of the conditioned medium remaining post-EV isolation indicated that EVs were required to produce this event (it must be noted that all EVs used in this study were isolated using 24,000 g centrifugation, rather than 100,000–110,000 g that is typically used). Extending the study to EVs from 2 prostate cancer patients ($n = 2$) with high grade tumours, these induced significantly growth in soft agar of the non-tumorigenic PrEC cells, which was associated with increased 14-3-3 zeta, pRKIP and prohibitin in these cells. Remarkably also, co-culturing DU145 cells with EVs from PrECs cells prevented DU145's colony formation in soft agar, indicating that anchorage-independent growth was significantly suppressed [61].

Further support of the potential relevance of EVs in conferring resistance to a broad range of anti-cancer drugs (not just classical chemotherapy) was recently reported where the androgen-dependent prostate cancer cell line VCaP acquired mesenchymal traits (specifically, enhanced migration and invasion) and decreased sensitivity to the anti-androgen drug enzalutamide in the presence of the EVs derived from a mesenchymal-like prostate cancer cell line 22Rv1/CR-1, also known as Mes-PCa. Of note and as summarised in Table 1, the EVs here were isolated by low-speed spins and ultra-centrifugal filter 100 kDa units. The Mes-PCa-EVs were found to induce TGF β activity and to inhibit androgen receptor expression in the VCaP cells, with 3 specific miRNAs -proposed to be AR-associated- dysregulated in the

cells by the Mes-PCa-EVs [22]. Interestingly, a study of EVs from DU145, PC3 and their respective paclitaxel-resistant variants (DU145-TXR and PC3-TXR) also identified a hub of miRNAs that they proposed to be regulating AR, PTEN and T-cell factors/lymphoid enhancer-binding factors 4 (TCF4) in prostate cancer, although there was no overall commonality in the miRNAs identified in these two studies [41].

4. Lung cancer

One of the first studies associating EVs with drug-resistance in lung cancer included the non-small cell lung cancer (NSCLC) cell line PC9 and its gefitinib-resistant subline PC9R that carries an EGFR T790M mutation. Here, EVs shed from the PC9R cells were found to stimulate proliferation, invasion and drug-resistance to gefitinib-induced apoptosis in parental PC9 cells. Key proteins of the AKT/mTOR pathway were detected in the EVs and by treating PC9R cells with BEZ235 - a dual inhibitor of AKT and mTOR- a role for this pathway in gefitinib-resistance was substantiated [15].

Around the same time, Xiao et al. [91] demonstrated that the A549 NSCLC cells release a greater amount of EVs when treated with cisplatin. It is noteworthy that this quantification was based on surrogate protein analysis. (Of note, many EV researchers argue that EV quantification should be based on EV numbers only rather than protein analysis. This is because the proteins analysed could be proteins of the EVs, proteins that were spun down with the EVs, or a combination of both -and so may or may not accurately reflect EV quantities. However, based on choice and/or lack of equipment and expertise to actually quantify EVs, protein analysis is sometimes performed and considered as a surrogate for EV amounts *i.e.* it does not inform of EV numbers. Culturing A549 parent cells with the EVs released in response to cisplatin treatment, in turn induced cisplatin-resistance. In a subsequent study this group established a cisplatin-resistant variant of A549, termed A549/DDP, and showed that their released EVs conferred cisplatin-resistance/reduced apoptotic rate of recipient A549 parent cells. As in breast and prostate cancers, miRNA transported by EVs have been linked drug-resistance in lung cancer. Here, miRNAs profiling identified miR-100-5p - which targeted mTOR- as substantially down in A549/DDP and their EVs, compared to their drug-sensitive counterparts. *In vitro* and *in vivo* A549 xenograft studies (where tumours were grown and then mice administered cisplatin +/- EVs) indicated A549/DDP

transmittance of cisplatin-resistance is via an EV miR-100-5p-dependent way [65,66].

A study investigating resistance that occurred when using a combination of gefitinib and cisplatin showed EVs from PC9 gefitinib-treated (for 24 h) cells reduced anti-tumour effects of cisplatin and resulted in significantly less apoptosis and higher autophagic activity (measured by LC3-II conversion and p62 degradation) when compared to cisplatin treatment alone. However, EVs from cisplatin treated PC9 cells did not substantially affect gefitinib's anti-cancer effects [42]. Efforts to inhibit EV secretion with GW4869 resulted in only modest beneficial effects from cisplatin and gefitinib. However, it must be noted that the EVs used through this study were isolated using ExoQuick which has been described as one of the precipitation solution-based techniques that does not succeed in extracting all exosomal particles but co-precipitates non-exosomal impurities and thus is not considered to be EV-specific [82].

In 2017, a plethora of studies on lung cancer drug-resistance mediated by EV were reported. Using a similar approach to that of [22] in prostate cancer, one such study set out to determine if mesenchymal NSCLC cells, which are more resistant to therapy, could transfer their resistance phenotype to epithelial NSCLC cells. Using a human bronchial epithelial cell line 30KT and inserting common NSCLC mutations such as p53 knockdown, KRAS^{V12} over-expression, and LKB1 knockdown, mesenchymal cell line variant termed 30KT^{p53/KRAS/LKB1} was developed. This variant which includes a substantial increase in its stem-like (CD24^{low}/CD44^{high}) population was shown to be resistant to both cisplatin and gemcitabine. While the quantity of EVs it released was not different to that of the parent 30KT cells, the 30KT^{p53/KRAS/LKB1}-EVs conferred resistance to gemcitabine and gemcitabine-cisplatin combination (but not cisplatin alone) to 30KT parent cells and induced their epithelial-mesenchymal transition (EMT) as shown by induced transcription of ZEB1, a master EMT transcription factor [44].

As in breast cancer, miR-222 family members have been implicated in EV-associated drug-resistance in lung cancer. Specifically, miR-222-3p was detected in EVs released from gemcitabine-resistant A549 (A549-GR) cells and shown to induce migration, invasion, anoikis-resistance and gemcitabine-resistance in recipient parent A549 cells. Analysis of sera from NSCLC patients ($n = 50$) indicated a correlation between EV-miR-222-3p levels and gemcitabine response, with higher levels of EV-miR-222-3p associated with limited response to gemcitabine [87].

Reflecting the increased levels of miR-96 detected in lung cancer ($n = 56$) compared to normal lung tissue ($n = 19$), using ExoQuick for EV isolation, substantially higher levels of EV-miR-96 were found in serum from the cancer patients ($n = 56$) compared to the controls ($n = 19$). miR-96 was found to target and inhibit expression of the tumour suppressor gene LMO7. Conversely, overexpression of LMO7 in A549 cells reduced the drug-resistance, thus restored drug-sensitivity, supporting a miR-96/LMO7 axis in lung cancer facilitated by EV transfer [90].

In a study focused on serum procured from patients with advanced NSCLC after cisplatin-based chemotherapy, again using ExoQuick for EV isolation, low versus high EV-miR-146a-5p levels were associated with shorter progression-free survival (PFS). Of note, this study reported that serum specimens were collected from $n = 100$ patients but for assessment of response after 2 courses of treatment, $n = 6$ patients were defined as cisplatin-resistant and another $n = 6$ as cisplatin-sensitive. In turn, decreasing levels of miR-146a-5p were observed in cisplatin-resistant A549 variants A549/DDP (which is approx. 5-fold resistant to cisplatin) and A549/DDP-500, A549/DDP-1000 and A549/DDP-2000 (strongest resistance, cultured in final concentration of 2000 ng/mL cisplatin) cells. Supporting functional relevance of EV-miR-146a-5p in lung cancer, when this miRNA, proposed to target Atg12 to inhibit autophagy, was transfected back into the resistant A549/DDP cells, cisplatin-sensitivity increased [97].

5. Ovarian cancer

One of the earliest studies on EVs involvement in drug-resistance was performed in ovarian cancer with the ovarian carcinoma cell line 2008 and its cisplatin-resistant 2008/C13*5.25 variant. Specifically, Safaei et al. [76] examined the secretory pathway that led to cisplatin exportation from cells. The cisplatin-resistant cells were observed to have a reduced sized lysosomal compartment and to export 2.6-fold more cisplatin by EVs than their drug-sensitive counterparts. The proteins released in EVs by 2008/C13*5.25 cells included the drug efflux proteins MRP2, ATP7A, ATP7B and lysosome-associated protein 1 (LAMP1).

Annexin A3 was also reported to be highly expressed by cisplatin-resistant ovarian cancer cell lines A2780/cis and SKOV3/cis, compared with their respective sensitive parent cells A2780 and SKOV3. Using TEM, the resistant cells were found to have relatively more vesicles in their cytoplasm, at least some of which carried annexin A3 protein. Subsequent studies of ovarian cancer patients' sera ($n = 50$) showed elevated levels of annexin A3 for cisplatin-resistant patients compared to cisplatin-sensitive patients and sera from healthy controls. As EVs were not isolated from the serum to check if the annexin A3 was EV-associated, it would be beneficial for future studies to address that [95].

As we reported in prostate cancer [18], evidence also suggest that in ovarian cancer EVs carrying P-gp may be involved in transmitting resistance. Here A2780 and its paclitaxel-resistant sub-clone A2780/PTX (which is also adriamycin-resistant) were studied. The resistant cells were observed to prevent adriamycin entering their nucleus by apparently capturing it in vesicular structures on the cell membrane periphery, whereas adriamycin accumulated in the nuclei of A2780. Furthermore, A2780/PTX cells were found to secrete larger amounts of EVs compared to A2780 cells. EVs that budded from A2780/PTX carried P-gp, while P-gp was barely detectable in A2780 cells. Incubation of A2780/PTX EVs with A2780 cells rendered the latter 5-fold resistant to both adriamycin and paclitaxel. This was proposed to be due to EV transfer of P-gp into A2780 cells, enabling adriamycin to only accumulate in the peripheral part of the cell leading to its exportation via P-gp-containing EVs [100].

As in other cancer types, EV-carried miRNAs have been implicated in drug-resistance in ovarian cancer. Pink et al. [64] analysed A2780 and its cisplatin-resistant variant, here termed CP70, for dysregulated miRNAs and found miR-21-3p -which targets neuron navigator 3 (NAV3) - to be 50-fold higher level in the resistant cells. Its transfection into drug-sensitive A2780 cells induced cisplatin-resistance. EVs from cisplatin-resistant CP70 cells induced significant resistance in A2780. Although this EV-induced resistance was accompanied by an increase in miR-21-3p in the recipient cells, the increase in miR-21-3p was not significant, suggesting that the EVs may be inducing resistance via a miR-21-3p-independent manner.

More recently Crow et al. [19] used a number of ovarian cancer cell lines with different levels of carboplatin sensitivity/resistance to investigate the ability of EVs to confer resistance. A2780 cells pre-treated with EVs released from its drug-resistant C30 and CP70 variants developed decreased drug-sensitivity. A similar result occurred when A2780 cells were treated with EVs released from the innately carboplatin-resistant OVCAR10 cells. DNA sequencing of the ovarian cancer cell lines revealed that somatic mutations in SMAD4 were present in the platinum-resistant cell lines (C30, CP70, and OVCAR10). Further investigating showed that engineered SMAD4 mutations in A2780 cells had up-regulated EMT markers, were carboplatin-resistant, and released EVs that induced carboplatin-resistance on previously sensitive A2780 cells.

It is very well established that the tumour microenvironment, including stromal cells such as fibroblasts, adipocytes, lymphocyte infiltrates, endothelial cells and macrophages, can contribute to cancer progression. Analysing primary cancer-associated fibroblasts (CAFs) and cancer-associated adipocytes (CAAs) isolated from ovarian cancer

tissue, Au Yeung et al. [5] demonstrated that miR-21 was transferred *via* EVs into recipient ovarian cancer cell line SKOV3ip. Two other ovarian cancer cell lines OVCA432 and SKOV3 treated with EVs isolated from mouse embryonic fibroblasts that express miR-21 (^{miR21+ / miR21+}MEFs), compared to cells incubated with EVs from ^{miR21- / miR21-}MEFs, had higher cell numbers remaining after paclitaxel treatment, establishing a role for EV-carried miR-21 in drug-resistance. OVCA432 and SKOV3 cells expressed decreased levels of apoptotic protease-activating factor 1 (APAF1) protein when transfected with miR-21 and, conversely, were re-sensitised to paclitaxel when transfected with APAF1; indicating a potential miR-21/APAF1 axis involved in paclitaxel-resistance. Overall, this study suggested that EV-miRNA from neighbouring stromal cells may contribute to the malignant phenotype and paclitaxel-resistance in ovarian cancer.

6. Haematological malignancies

EVs have been implicated in numerous blood malignancies. Studies of serum EVs in multiple haematological malignancies showed elevated levels of EVs reported in many cancer types ($n = 102$ patients) compared to those in serum of health controls (28 healthy controls). Specifically EV levels were substantially elevated, as assessed by a flow cytometric method, in acute myeloid leukaemia (AML), multiple myeloma (MM), myeloproliferative neoplasms (MPNs), Hodgkin's lymphoma (HL), Waldenstrom's macroglobulinemia (WM) and to a lesser extent in chronic lymphocytic leukaemia (CLL) and non-Hodgkin's lymphoma (NHL) compared to healthy controls [10].

Bebawy et al. [7] reported that EVs (of note, here relatively large microparticles of 0.1–1 μm isolated with max. spin speed of 24,000 g) released by the resistant acute lymphoblastic leukaemia (ALL) cell line, VLB₁₀₀ (that overexpresses the MDR1/P-gp gene), conferred resistance to the sensitive ALL cell line, CCRF-CEM, by the transfer of P-gp protein. Here resistance was considered as a reduced accumulation of P-gp substrates rhodamine 123 or doxorubicin.

Multidrug resistance-associated protein 1 (MRP1, also known as ABCC1) is another ABC transporter protein associated with MDR [26,40]. Using a promyelocytic leukaemia cell line HL60 and its daunorubicin-resistant variant HL60/AR which overexpresses MRP1, an increase in daunorubicin-resistance was observed when previously drug-sensitive HL60 cells were treated with EVs from the HL60/AR cells. This was associated with a decrease in reactive oxygen species (ROS) generation. MiR-196 and miR-20a were at higher levels in EVs from the drug-resistant cells compared to sensitive cell-derived EVs, although they were more highly expressed by the drug-sensitive cells themselves compared to the resistant cells. It was proposed that this selective expulsion of specific miRNAs out of the cell may be a function of MRP1 to maintain drug-resistance [9].

Rituximab is a monoclonal antibody that targets the surface protein CD20 on tumour cells and causes cell death *via* multiple mechanisms including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [48]. Rituximab has therapeutic benefit in B-cell lymphoma. EVs from aggressive B-cell lymphoma cell lines Balm3, Su-DHL-4 and OCI-Ly1 were found to bind rituximab *via* CD20 and so reducing drug bioavailability. Isolation of EVs from patient plasma specimens ($n = 6$) confirmed that, 3 h post-administration, half of all plasma rituximab was bound to EVs suggesting that much of the rituximab administered was actually unavailable for therapeutic benefit. The ABC transporter, ABCA3, previous shown to confer MDR in leukaemia cells [12] was found here to be critical for the amounts of EVs released from each of the three cell lines, suggesting an ABCA3-dependent pathway of EV release [6]. In a subsequent study, this group showed that disrupting ABCA3 expression, using the cyclooxygenase inhibitor indomethacin, decreased EVs release previously observed upon doxorubicin treatment and increased efficacy of doxorubicin and pixantrone suggesting that nuclear trapping through inhibition of EV export, by indomethacin, increased anti-cancer drug efficacy.

In multiple myeloma (MM) and bone marrow stromal cells (BMSCs) interaction studies, where EVs were isolated from murine and human cell lines using ExoQuick, it was found that MM and BMSCs could mutually exchange EVs carrying certain cytokines and that EVs induced MM cell resistance to the proteasome inhibitor bortezomib. EVs isolated from both MM patients' and healthy donors BMSCs ($n = 3$ each) prevented loss (by approx. 25%) of cultured RPMI-8226 MM cells when treated with bortezomib [85].

7. Gastrointestinal (GI) cancers

7.1. Colon cancer

In colon cancer, a study of the drug-sensitive DLD-1 cell line, its fluorouracil-(5-FU)-resistant variant (DLD-1/5-FU) and their corresponding EVs pre- and post-5-FU treatment showed that the levels of miR-34a and miR-145, established anti-oncomirs in colon cancer, were not substantially different between drug-sensitive and -resistance cells. Upon treatment with 5-FU, miR-145 and miR-34a cellular levels increased in the sensitive DLD-1 cells, but not in the resistant variant. For the sensitive cells, treatment with 5-FU resulted in reduced levels of miR-145 in its released EVs. For the resistant cells, the level of miR-34a were down-regulated in the cells and, instead, increased in their EVs. This supports the notion of extracellular disposal of tumour-suppressor miRNAs as an undesirable protective mechanism in drug-resistance [2].

ΔNp73 , a TP73 gene-derived isoform, has been reported to inhibit the tumour suppressor function of TP53 or and induce a set of genes involved in tumorigenesis [98]. The colon cancer cell line HCT116 was transfected to over-express ΔNp73 and this resulted in substantial ΔNp73 mRNA in the released EVs, compared to those released by mock transfected HCT116 cells. Pre-treatment of drug-sensitive HCT116 cells with EVs from ΔNp73 over-expressing cells conferred oxaliplatin resistance, reflecting that of the ΔNp73 -overexpressing HCT116 cells from which these EVs were derived. Furthermore, EV- ΔNp73 conferred oncogenic potential on xenograft tumours [77]. Specifically, in *in vivo* studies, administering (by tail vein) EVs from ΔNp73 -overexpressing HCT116 or EVs from HCT116-mock cells immediately after HCT116 mock cell inoculation and then at 3-day intervals resulted in significantly larger tumours with significantly higher levels of ΔNp73 in the xenograft tumour. In a clinical study including blood specimens procured from patients ($n = 69$) prior to oxaliplatin-based therapy, those with low levels of EV- ΔNp73 had a 5-year disease-free survival advantage (57% vs. 49%) over those with high levels of EV- ΔNp73 .

There is increasing evidence that undifferentiated CD133⁺ cancer stem cells (CSCs) exist in colorectal tumours and that they are inherently resistant to chemotherapy. CAFs have been shown to interact and maintain the CSC pool, through the release of soluble factors. Thus, targeting CAFs in colorectal tumours is a proposed strategy to prevent drug-resistance observed in many patients [70]. In 2015, Hu et al. [30] investigated the role of CAFs in transmitting drug-resistance through the priming of CSCs in colorectal cancer. EVs from 18Co (*i.e.* fibroblasts from normal colon) and from CAFs from colorectal cancer tissue (from $n = 1$ patient) were found to promote sphere-formation and tumorigenic capabilities of CSCs purified by FACS from SW620 cell line. In pre-clinical xenograft model, CAF-EVs significantly inhibited the anti-tumour activity of oxaliplatin; evident by tumour volume not being so substantially reduced by the drug. This study proposed that the EVs primed CSCs through the Wnt signalling pathway.

7.2. Liver cancer

Hepatocellular carcinomas (HCC) are notoriously resistant to chemotherapy. Culturing HepG2 cells with increasing concentrations of their own EVs conferred a level of resistance to the tyrosine kinase inhibitor sorafenib, as well as the classical chemotherapeutic drugs camptothecin and doxorubicin. As TGF β is associated with acquired

drug-resistance and direct treatment with TGF β was found to also reduce the sensitivity of the cells to these drugs, the authors profiled lincRNAs in HepG2 cells and their derived EVs following TGF β treatment and identified a substantial increase in lincRNA-ROR in cells and their EVs. Treatment with sorafenib increased linc-ROR in HepG2 cells and EVs, with the transfer of lincRNA into recipient HepG2 cells transferring drug-resistance. siRNA silencing of linc-ROR increased apoptosis in HepG2 cells incubated with sorafenib, camptothecin and doxorubicin. This suggests that EV-lincRNA is a mediator of drug-resistance and that targeting linc-ROR may help restore drug-sensitivity [78].

Sorafenib-resistance in HCC was also investigated by Qu et al. [68]. *In vitro* analysis demonstrated that EVs from invasive HCC cells, MHCC-97 L and MHCC-97H, induce sorafenib resistance and lowered the apoptotic rate in SMMC-7721 HCC cells; with most effect seen with EVs from the more invasive MHCC-97H cells. Data indicated that this was through the delivery of the hepatocyte growth factor (HGF) cytokine activating the HGF/c-MET/AKT pathway and sorafenib resistance in recipient cells was demonstrated by an increase in the levels of phosphorylated Met, Akt and VEGFR2. In a sub-cutaneous xenograft mouse model, the mice treated with EVs in addition to sorafenib had much larger tumours than those treated with sorafenib alone, indicating that EVs from invasive cells inhibited the therapeutic effect of sorafenib. Thus, the EVs from the more invasive cell line conferred most resistance to sorafenib *in vivo* as *in vitro*.

7.3. Gastric cancer

Mesenchymal stem cells (MSCs) are implicated in the potentiation of drug-resistance in gastric cancer. Firstly, a subcutaneously xenograft model using the gastric cancer cell line HGC-27 was established. MSCs (isolated from human umbilical cord) and human foetal lung fibroblast (HFL1) cells were included in this study. MSC-EVs or HFL1-EV were then co-injected with 5-FU into tumour-bearing mice. MSC-EVs substantially inhibited 5-FU effects and increased tumour size and weight resulted, while HFL1-EVs had minimal effect. *Ex vivo* analysis of the tumours showed that MSC-EVs induced drug-resistance in association with elevated mRNA and protein levels of MDR-associated MDR, MRP, and lung resistance protein (LRP), and a reduction in their apoptotic rate. Further analysis showed that MSC-EVs may induce drug-resistance by activating the CaM-Ks/Raf/MEK/ERK signalling pathway [34].

Zheng et al. [101] developed M2-like macrophages mimicking tumour-associated macrophages (TAMs) to investigate the effects of EVs released from TAMs on gastric cancer cells. Gastric cancer cell lines, MFC and MGC-803, cultured with the TAM-like macrophages exhibited a reduced level of apoptosis in response to cisplatin compared to cells incubated with unactivated macrophages or normal control cells. MFC cells treated with cisplatin had reduced cell death when co-incubated with TAM-like macrophage derived EVs. This development of drug-resistance was supported by *in vivo* studies where a subcutaneous model was developed with MFC cells which had been pre-treated with or without EVs derived from TAM-like macrophages, followed by administration of cisplatin ten days later. The presence of the EVs alone had minimal effect on tumour growth, however they substantially inhibited the anti-cancer effects of cisplatin. miRNA microarray analysis showed a significant increase of miR-21 in TAM-like macrophage and qPCR demonstrated miR-21 amounts to be increased in MFC cells after incubation with TAM-like cells' EVs. Subsequent transfection of MFC cells with miR-21 resulted in decreased PTEN mRNA and protein and increased AKT phosphorylation, suggesting that miR-21 delivered by the TAM-like macrophage-derived EVs modulates drug-resistance by up-regulating the PTEN/PI3K/AKT pathway in recipient cells.

7.4. Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer (90% of all cases) and gemcitabine (GEM) is the

standard chemotherapeutic agent used to treat locally advanced and metastatic pancreatic cancers [3]. A study exploring the ability of EVs, isolated using ExoQuick, to transfer GEM-resistance in PDAC reported EVs from GEM-resistant Panc 1 (Panc1-GR) cells transmit GEM resistance to previously GEM-sensitive Panc1 parent cells. A higher amount of miR-155 was found in Panc1-GR compared to GEM-sensitive Panc1 cells, which contributed to increased EV secretion and increased miR-155 content in those EVs. Overexpressing miR-155 in Panc1 cells also caused an increase in quantities of EVs released. EVs from miR-155-overexpressing Panc1 and MiaPaCa2 cells induced GEM-resistance and inhibition in apoptosis in Panc1 and MiaPaCa2 cells, respectively. Considering the clinical relevance of miR-155 in pancreatic cancer, tissue samples resected from PDAC cancer patients treated with subsequent GEM chemotherapy ($n = 45$) showed that a high expression of miR-155 in tumour tissue correlated with a poorer prognosis compared to patients with a lower level of miR-155 [51].

A similar result was observed when drug-sensitive pancreatic cancer cells MiaPaCa and Colo-357 were treated with CM from GEM-treated MiaPaCa and Colo-357 cells. Specifically, these cells were treated with GEM for 8 h, or vehicle as control, cultured in fresh medium for 48 h, and this CM was used to pre-treat parent cells for 12 h prior to GEM treatment. The GEM-CM substantially reduced the GEM toxicity on these cells. Subsequent analysis of the EV (isolated using ultracentrifugation) and soluble fraction showed that the EVs component of the CM was responsible for decreased GEM toxicity. Here, the previously drug-sensitive cells became resistant, with higher miR-155 expression levels and decreased apoptotic rates. Mechanistically, the increased miR-155 was found to target the GEM-metabolising enzyme, deoxycytidine kinase (DCK), down-regulating its cellular levels and inducing GEM-resistance [63].

Also in pancreatic cancer, Richards et al. [71] demonstrated that CAFs increase their EV secretion, assessing EVs isolated using ExoQuick, when under stress from GEM and nab-paclitaxel. Panc1, L3.6, AsPC-1 PDAC cell lines treated with CAF-derived EVs had increased survival rates and GEM-resistance. Further analysis revealed that GEM increased miR-146a and SNAIL in CAF EVs, apparently contributing to the drug-resistance.

8. Glioblastoma

Temozolomide is the standard-of-care pharmacological treatment for glioblastoma multiforme (GBM), but the median survival still remains at approximately 15 months [93]. EVs have been implicated as a cause for temozolomide-resistance, resulting in this short survival time. RNA sequencing identified a recurrent receptor-type tyrosine-protein phosphatase zeta (PTPRZ1)–MET fusion, known as ZM fusion, present in $n = 1$ of 13 grade III astrocytomas and $n = 3$ of 20 secondary GBM specimens and associated with poor survival. EVs from U87 cells containing the ZM fusion (U87/ZM) delivered MET and p-MET into U87 cells that did not contain the ZM fusion, resulting in an increase of MET and p-MET biological activity, EMT, cell migration and invasion and temozolomide-resistance. Using a sub-cutaneous U87 cell model in mice, pre-incubation of the U87 cells with EVs from U87/ZM cells compared to EVs from U87 cells resulted in much larger tumours. The relevance of the ZM fusion was investigated by analysis of tumour tissue specimens from GBM patients. The study described that patients treated with temozolomide ($n = 53$) whose tumours did not have the ZM had a longer overall survival compared to patients that did not receive temozolomide ($n = 9$). Conversely, those whose tumours had the ZM fusion and that were treated with temozolomide ($n = 7$) gained no overall survival benefit compared with patients that did not receive treatment temozolomide ($n = 4$); indicating that the patients whose tumours carried ZM fusion were temozolomide-resistance [99]. ZM fusion in corresponding EVs from patients was not investigated. However, it is noteworthy that this study did not use current criteria to evaluate GBM status of patients, so it is not clear if all would have been truly GBMs by

current standards. Secondly, it is not clear that all patients were undergoing surgery for primary tumours as opposed to recurrence, although the implication is that these were primary tumours. Finally, it was not clear why some patients did not receive TMZ.

9. Neuroblastoma

Neuroblastoma (NBL) is a form of cancer that develops in the sympathetic nervous system in immature nerve cells found in the neural crest in the developing foetus or in early infancy and is the most common tumour diagnosed during infancy [20]. Like many other cancers, resistance to chemotherapy can arise and TAMs in the tumour microenvironment have been implicated in this drug-resistance through the secretion of EVs. Studies including NBL SK-N-BE(2), CHLA-255 and IMR-32 neuroblastoma cell lines and monocytes supported EVs to be causally involved in cross-talk between neuroblastoma and microenvironment cells. Treating monocytes with EVs from NBL cells resulted in a 12-fold increase in their miR-21 levels. Additionally, an increase in miR-155 occurred in TAMs which were co-cultured with NBL cell lines SK-N-BE(2) and CHLA-255. Apparently miR-155 targets TERF1, a telomerase inhibitor in the NBL cells, causing an increased telomerase activity thereby modulating drug-resistance [11].

10. Melanoma

Approximately 40–60% of melanomas carry oncogenic BRAF mutations (although the percentages seem to differ from population to population), with the majority being a V600E mutation. For this reason, RAF inhibitors (BRAFi) have been approved for V600E-mutant melanoma [89]. Recently Vella et al. [83] investigated EV contribution to BRAFi resistance. Using a BRAF V600E-mutant cell line LM-MEL-64, a resistant variant termed LM-MEL-64R3 was developed by exposing cells to the BRAF kinase inhibitor PLX4720 for 10 weeks. Receptor tyrosine kinase phospho-antibody array analysis of whole cell lysates identified PDGFR β to be the resistance driver. Furthermore, EVs released by PLX4720-resistant cells were enriched in PDGFR β . It was concluded from *in vitro* studies that these EVs could deliver PDGFR β to LM-MEL-64 cells and induce PLX4720 resistance *via* the activation of the PI3K/AKT signalling pathway.

Interestingly, in a study including Me30966 and Me501 metastatic melanoma cells' EVs, low pH mimicking microenvironmental acidity was found to markedly impair cisplatin uptake by the cells. Cisplatin quantities found in EVs released from these cells correlated with the pH of the CM in which the cells were cultured. Both *in vitro* and pre-clinical *in vivo* studies indicated that proton pump inhibitors (PPIs, drugs commonly used for the treatment of acid reflux, indigestion, and peptic ulcers and ranked among the top 10 prescribed classes of drugs) helped to increase cisplatin uptake by cells and to reduce the release of EVs carrying cisplatin. This two-pronged effect could thus help maintain cisplatin within the cancer cells, supporting the potential of PPIs to prevent this mechanism of drug-resistance [24].

11. Renal cell carcinoma

Sunitinib is a tyrosine kinase inhibitor that blocks VEGF, PDGFR, and stem cell growth factor receptor. While a pooled study of data from 6 trials including $n = 1059$ patients showed approximately 38% patients with of renal cell carcinoma (RCC) achieved objective response from sunitinib, the majority did not [53]. Studies performed by Qu et al. [67] identified that EVs from the resistant RCC xenograft cell lines 7Su3rd and ACSu3rd conferred resistance to previously sunitinib-sensitive RCC 786-0 cells, by means including the transfer of a lncRNA which they termed lncRNA-ARSR (*i.e.* active in RCC sunitinib-resistance). lncRNA-ARSR was found to competitively bind miR-34/miR-449, resulting in the upregulation of AXL/c-MET, leading to the activation of STAT3, ERK and AKT signalling. It was thus proposed that this

AXL/c-MET/ERK/AKT signalling axis may offer a new target in the treatment of RCC in efforts to overcome sunitinib-resistance.

12. Osteosarcoma

Treatment of osteosarcoma, cancer of the bone, often fails due to drug-resistance. Torreggiani et al. [81] used the human osteosarcoma cell line MG-63 and its doxorubicin-resistant variant MG-63DXR30 to indicate that short-term pre-treatment (for 4 h) with EVs from MG-63DXR30 cells apparently transmitted doxorubicin-resistance to previously drug-sensitive MG-63 cells *via* the transportation P-gp.

13. Resistance transmission from one cancer type to another

Considering drug-resistance transfer from one cancer type to another, as example study EVs from cisplatin-resistant liver (HepG2) cells were taken up by ovarian (HeLa) cells and, in turn, decreased their sensitivity to cisplatin. The EVs from the drug-resistant HepG2 cells, compared to those from drug-sensitive HepG2 cells, were found to have reduced levels of miR-106a and miR-106b. Sirtuin 1 (SIRT1), an enzyme that deacetylates regulatory proteins, has been identified as a target of miR-106a/b. Protein and mRNA levels of SIRT1 were increased in HeLa cells treated with EVs derived from cisplatin-resistant HepG2 cells. This study demonstrated that EV secreted by one drug-resistant cancer type can confer drug-resistance on another cancer type [69].

14. EVs as drug delivery vehicles

On the positive side, there is growing evidence that EVs have potential to be exploited as naturally drug delivery vehicles. Some examples of efforts in the regard are summarised here. Based on concerns that cells release relative low quantities of EVs and so a more pro-active approach may be needed to generate EVs as drug delivery vehicles, Jang et al. [33] aimed to generate “EV mimics” carrying anti-cancer drugs. To achieve this, whole monocyte or macrophage cells were mixed with drug and the cells were then broken down by serial passing through filters that had diminishing pore sizes (10, 5, and 1 μm). The effects of the resulting EV mimics were compared to both EVs released from cells that were drug-loaded by incubating with doxorubicin for 2 h and with doxorubicin-loaded liposomes. The EV mimics were reported to have many similar characteristics but 100-fold production yield, when compared to EVs released from the drug-loaded cells. In pre-clinical studies, following *i.v.* injection the EV mimics reduced tumour growth to the same extent as 20-fold higher doses of free drug and without systemic side-effects. Similarly to the EV mimics, the naturally released EVs had counter-receptors (*e.g.* LFA-1 for endothelial CAMs) and so were trafficked to the tumour. Conversely, the drug-loaded liposomes that did not carry the targeting proteins were inefficient in reducing tumour growth. Overall, beneficial effects were observed with EV mimics and naturally-released EVs when compared to free drug, but the ability to produce EV mimics in much higher quantities than naturally-released EVs supports the further investigation of this approach.

Advancing on the naturally released drug-loaded EV approach, Pascucci et al. [62] loaded murine SR4987 mesenchymal stromal cells with paclitaxel by incubating with high dose of drug for 24 h before feeding with fresh medium and subsequently collecting the released EVs using ultracentrifugation. The resulting paclitaxel-loaded EVs substantially reduced the proliferation of pancreatic adenocarcinoma cells, CFPAC-1. Another example involved using a different approach where EVs from prostate cancer (LNCap- and PC-3) cells were loaded with paclitaxel by directly incubating the EVs suspension with drug for 1 h and subsequently re-collecting the washed EVs again using ultracentrifugation. These EVs were found to help take drug into recipient cells through an endocytic pathway, increasing the cytotoxic effect of

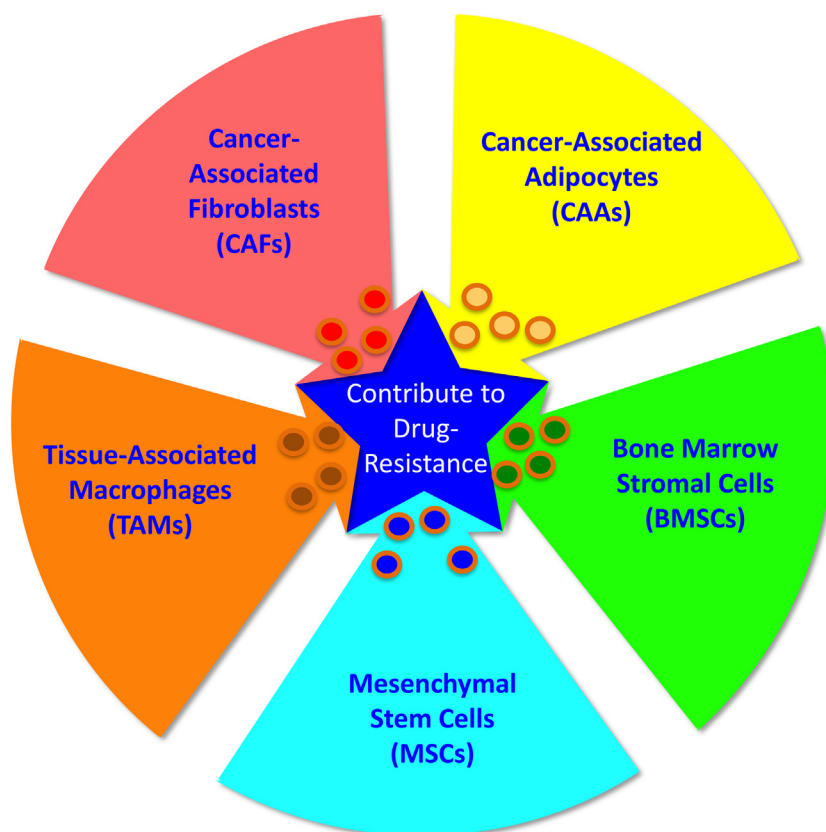


Fig. 3. EVs from many other cell types, in addition to cancer cells, have been implicated in contributing to drug-resistance in cancer.

the drug [74]. Of note, fluorescence lifetime imaging microscopy (FLIM) has recently been reported as a novel method to investigate such EV-mediated cellular uptake pathways of anti-cancer drugs and, as a tool, may add substantially to our understanding of these mechanisms [75].

In a preliminary investigation and subsequent more extensive study, Rizzolio's group [27,80] reported that EVs isolated, using ExoQuick, from CM of cancer cell lines (MDA-MB-231 and HCT-116) and loaded with doxorubicin by electroporation had 40% reduced accumulation in the hearts of mouse models of breast and ovarian cancer and no cardiotoxicity. This indicated that loading of the drug into EVs reduced cardiac toxicity when compared to free drug. Again these studies show, in principle, safety benefits of EV-loaded drug compared to free drug; granted because of what other molecules they may be carrying, of course cancer cells would not be a suitable source of EVs to be used as delivery vehicles in humans. Using EVs released from macrophages derived from the blood of healthy donors, rather than cancer cell lines, Iessi et al. [31] reported that EVs increased the delivery and cytotoxicity of the tumoricidal dye acridine orange into Me30966 melanoma cells *in vitro*. Modifications of this approach have also shown success in pre-clinical studies of pulmonary metastasis using macrophage-derived EVs which were isolated using ExoQuick and loaded with paclitaxel [37].

Considering safe sources of EVs that could be obtained at large-scale, ourselves and others are working on using milk-derived EVs as delivery vehicles. For example, milk EVs have been loaded with paclitaxel and delivered orally. This study reported these EVs to achieve the same therapeutic efficacy as free paclitaxel delivered *i.p.* in mice bearing ovarian [4] and lung [1] tumour xenografts. While the application of EVs as drug delivery vehicles requires much more research, studies to date give hope that such EVs will contribute substantial to the future of cancer management and, indeed, nanomedicine in other disease settings [23].

15. Conclusion

It is evident from multiple studies by multiple research groups across both solid and non-solid cancer types that EVs from drug-resistant cancer cells and/or tumour microenvironment cells (Fig. 3) are causally involved in transmitting resistant to anti-cancer drugs thus contributing to challenges experienced with anti-cancer treatments. Through *in vitro*, pre-clinical *in vivo* and/or *ex vivo* studies on patients' specimens it is evident that the EVs involvement is *via* numerous mechanisms. We propose that larger multi-institutional studies including more pre-clinical and clinical analyses, using consistent and best methods for EV isolation and evaluation, and samples sharing for independent validation are now warranted to move this field forward, in a timely way, for the benefit of patients. Furthermore, while still in its infancy as a research area, studies to date investigating the utility of EVs as naturally delivery vehicles for anti-cancer molecules -in order to achieve efficacy at lower drug doses and so with reduced side-effects-suggest that this approach holds much promise.

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