

Title

Intercellular communication through extracellular vesicles in cancer and evolutionary biology

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Abstract

Extracellular vesicles (EVs) are nano-sized membrane enclosed vesicles that are released by cells. While initially thought to be cellular detritus or particles involved in eliminating waste from cells, EVs have been recognised as important mediators of intercellular communication by transferring their bioactive cargoes. Notably, over the last two decades, a substantial research effort has been undertaken to understand the role of EVs in cancer. It is now understood that tumour derived EVs can transfer their contents to influence metastatic behaviour, as well as establish favourable microenvironments and pre-metastatic niches that support cancer development and progression. EV-mediated intercellular communication in cancer will be of importance to understanding the emerging paradigm which views cancer as the establishment of a new species within the host organism. Here, we provide a concise overview of EVs and the current understanding of their role and application in cancer. In addition, we explore the potential wider role of EVs in the transfer of inherited characteristics and evolutionary biology.

Introduction

For decades, cancer has been defined as the accumulation of genetic mutations in a cell that results in uncontrolled proliferation (1). However, in recent years a new perspective to this somatic mutation theory of cancer has emerged. Cancer has begun to be regarded as cellular niches where evolution occurs on a cellular level and cancer grows and develops interactively within tissues; a sort of “cellular Darwinism” (2,3). Just as life on earth has emerged from unicellular organisms that adopted multicellularity for its various advantages, cancer emerges from a single cell within a multicellular environment with mutations which facilitate its evolution from a healthy state to a cancerous state. Evolutionary processes such as mutation and selection of cells playing a role at various stages of cancer (4).

Additionally, as Denis Noble presents within his article in this Special Issue of Cancer and Evolution, the harnessing of stochasticity may play a key role in cancer development, and

these mutations may be secondary to new attractor cellular states that preside over the genome(3). Becoming cancerous allows these cells to buck the limitations that are posed on healthy multicellular systems and utilize the tenets of cancer (1).

As we start viewing cancer as an ecosystem in which tumour and host cells interact, the importance of cellular communication comes to the forefront. In a healthy state, cells and tissues which compose multi-cellular organisms communicate successfully with one another to facilitate healthy homeostasis (5). Furthermore, cells must communicate with one another to gain effective control over stochasticity and push cells towards a particular phenotype (3). Cancer therefore represents a failure of homeostasis and communication between cells that results in cells evolving into a harmful unregulated state. More specifically, over the last few decades, the role that cell derived, membranous nanoparticles called extracellular vesicles (EVs) play in cancer growth and development has been rigorously scrutinized.

By delivering protein, nucleic acid and small molecule cargoes to recipient cells, EVs have been shown to be involved in numerous processes of tumour progression and cancer metastasis (6). As a result EVs and their associated biogenetic machinery and cargoes have become interesting targets for the development of cancer diagnostics and anticancer therapeutic strategies.

Here we provide the reader with a concise overview of EVs and their roles in cancer. In addition, we explore their potential contribution to quasi-evolutionary processes that underlie cancer biology, as well as their potential role in influencing wider genetic inheritance and evolution in a manner that resonates with some themes of Darwin's theory of Pangenesis.

The history of extracellular vesicles

Cells release membrane-bound nanoparticles called extracellular vesicles (EVs) that contain proteins, lipids and nucleic acids. EVs play a role in intercellular communication by delivering these cargoes to recipient cells (7–9). They are released as part of normal physiological processes and in response to various stimuli, including variations in pH and oxygen availability, injury, cellular stress and complement activation (10–13). EVs have been identified in many biological materials and fluids, including blood, urine, faeces, synovial fluid and ascites fluid (14).

The discovery and classification of EVs and their various subtypes has so far taken place over the best part of a century. EVs are widely regarded to have been first alluded to in the 1940s in a study by Chargaff and West (1946) that investigated the presence of procoagulants in the blood. They reported the presence of procoagulant platelet-derived particles in plasma that could be sedimented at speeds of 31,000 g (15). These particles were later described as “platelet dust” by Wolf in 1967 (16).

During the 1970s and 80s various studies documented the presence of membranous vesicles in various biological fluids and in *in vitro* studies. Furthermore, in 1983, EVs were first documented to be released by multivesicular bodies (MVBs) (17). However, throughout this period EVs were simply thought to be a mechanism for eliminating waste from cells(18). It was not until 1996 when Raposo *et al.* reported that EVs derived from B cells were able to induce antigen-specific responses in recipient T cells, in what is widely regarded to be the first study that demonstrated biological function of EVs (19). From here onwards, numerous studies have highlighted the importance of EVs in intercellular communication through the transfer of their bioactive cargoes to recipient cells (20–23).

Despite the great promise of EVs, methodological challenges associated with EV based research and challenges arising from the inherent complexity of EVs remain to be addressed. Findings by Whittaker et al. have for instance shown that factors contaminating isolated EVs can lead to an artefactual increase in the observed bioactivity of EVs (24). Furthermore increasing evidence indicates that EVs represent a heterogeneous population of nanoparticles with the possibility of EV subpopulations carrying out unique functions (25). Technological advances and a better understanding of the aforementioned challenges are required to advance both fundamental and translational EV research (26).

In recent years the number of researchers entering this exciting area of research has grown rapidly and the field founded the International Society for Extracellular Vesicles (ISEV) in 2011 to unify the international EV research community and establish guidelines for the research and publication of EV based research (27,28). To date the society boasts around 1000 international members and publishes its own peer-reviewed open-access scientific journal called The Journal of Extracellular Vesicles.

Cells release populations of extracellular vesicles

Extracellular vesicle is a collective term that describes a cell-derived lipid-bilayer delimited nanoparticle. Cells release a heterogeneous population of EVs that can be classified according to underlying biogenetic and biophysical properties. The precise classification is an ongoing topic of research, but it is generally accepted that cells release two populations that derive from unique biogenesis pathways, termed exosomes and microvesicles (MVs) (29).

Exosomes range in size from roughly 30 nm to 150 nm in diameter and originate from the endolysosomal pathway within cells (30–32). The first step in this process is the formation of membranous vacuoles by the inward budding of the cell membrane. Subsequent invaginations of the endosomal membrane fill endosomes with membrane-bound nanoparticles termed intraluminal vesicles (ILVs). Once filled, endosomes are referred to as multi-vesicular bodies (MVBs) (33). As the endolysosomal pathway also plays a function in autophagy, MVBs displaying specific surface proteins are trafficked to lysosomes to degrade ILV contents, however, MVBs required for exosomal formation are targeted to the cell membrane (34,35). MVBs fuse with the cell membrane, releasing their ILV contents, which thenceforth are termed exosomes (33). Exosome formation within endosomes occurs either dependently or independently of a group of proteins known as endosomal sorting complexes required for transport (ESCRT), often referred to more simply as ESCRT machinery (36,37). ESCRT-independent formation involves various other proteins, including sphingomyelinases (SMases) (38), ADP ribosylation factor 6 (ARF6), phospholipase D2 (PLD2) and tetraspanin family members; CD9, CD63 and CD81, which themselves have been shown to be highly enriched within exosomal membranes (39–43).

In contrast, MVs occupy a much larger size range from roughly 50 nm to 1300 nm in diameter, and MV formation occurs via budding of vesicles directly from the cell membrane into the extracellular environment. Various proteins involved in the formation of exosomes are also involved in these processes, including ARF6 which together with RHO family GTPases rearrange the actin cytoskeleton to help form vesicles (44,45), ESCRT-I associated protein tumour susceptibility gene 101 protein (TSG101) which interacts with accessory proteins ALG-2-interacting protein X (ALIX) in the cell membrane to aid cargo sorting and MV release (46), and SMases which modulate MV release (38,47).

In addition to exosomes and MVs, cancer cells have been shown to release a unique extracellular vesicle population termed large oncosomes with an atypical size that ranges from 1-10 μm in diameter (48,49).

Despite their distinctive underlying biogenesis, EV populations share common biophysical properties including overlapping size range. Conventional EV isolation techniques rely on biophysical properties and as such, one challenge that faces the field at present is the purification of different EV subtypes to investigate potential distinct functions. Findings support that the assumed homogenous exosome population contains subpopulations with distinct biological functions (50,51).

Consequently, in their 2018 position paper, ISEV has recommended that in non-selective studies where specific markers of subcellular origin cannot be reliably identified, heterogenous EV populations should simply be classified according to size; small EVs (S-EVs) for particles smaller than 200nm, and large EVs (L-EVs) for particles larger than 200nm (27). Figure 1 gives a schematic overview of different EV populations, their formation, and size range.

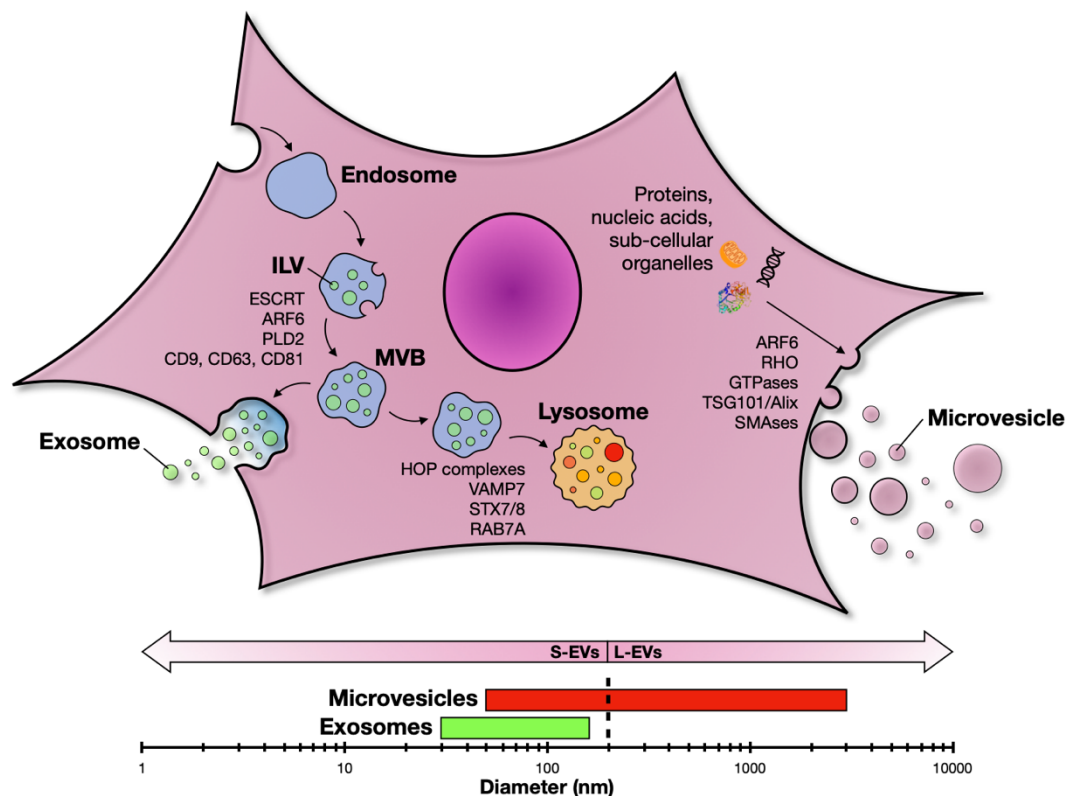


Figure 1: Extracellular vesicles are heterogeneous cell derived nanoparticles with varying biophysical properties and intracellular origins. The two main populations of EVs formed by cells are exosomes (green) and microvesicles (pink). Exosomes are EVs of endosomal origin formed within the endolysosomal system. Multiple inward budding events fill endosomes with intraluminal vesicles (ILVs). Once filled with ILVs these endosomes are termed multivesicular bodies (MVBs). MVBs can be trafficked to the cell membrane where they fuse and release exosomes. Exosome formation occurs either dependent of or independent of ESCRTs. ESCRT independent formation, cargo sorting and release is dependent on various other proteins including ADP-ribosylation factor 6 (ARF6), phospholipase D2 (PLD2) and tetraspanins CD9/63/81. Alternatively, MVBs displaying specific surface

proteins including HSP70/90 organising (HOP) complexes, vesicle associated membrane proteins 7 (VAMP7), syntaxin (STX) 7/8 and RAB7A are trafficked to lysosomes for degradation (34,35). In contrast to exosomes, microvesicles bud directly from the cell membrane. ARF6, Ras homology (RHO) gene family members, GTPases, tumour susceptibility gene 101 (TSG101) protein, ALG-2- interacting protein X (Alix) and sphingomyelinases (SMAases) and involved in microvesicle biogenesis and cargo sorting. Due to the significant overlap in size and the difficulty of selectively purifying EV subtypes, EVs less than 200nm are termed small EVs (S-EVs), while EVs larger than 200nm are termed large EVs (L-EVs).

The role of extracellular vesicles in cancer

A large part of fundamental research investigating biological functions of EVs has focused on cancer derived EVs. Consequently, numerous comprehensive reviews outlining the evidence on the role of EVs in cancer are available (6,52). Here we highlight some of the key findings on their roles.

Tumour derived EVs have been shown to influence various processes underlying tumour progression and metastasis through communication with recipient cancer cells as well as cells in the tumour microenvironment (Figure 2) (25). The biological functions of cancer derived EVs align well with Stephen Paget's 'Seed and Soil' hypothesis, which outlines the need of a receptive microenvironment (i.e. soil) for disseminated cancer cells (i.e. seeds) to establish as metastases (53). For example, Hood et al. reported on the effects of melanoma derived EVs on sentinel lymph nodes, showing that EVs are involved in the recruitment of disseminated melanoma cells and stimulation of metastatic factors and vascular proliferation in the lymph nodes (54). Induction of angiogenesis is one of the hallmarks of cancer and the resulting increase in exchange of nutrients and metabolic waste supports tumours to grow beyond microscopic size. Tumour derived EVs have been shown to transfer oncogenic epidermal growth factor receptor (EGFR) to endothelial cells and induce pro angiogenic processes in surrounding vasculature of the tumour (55,56).

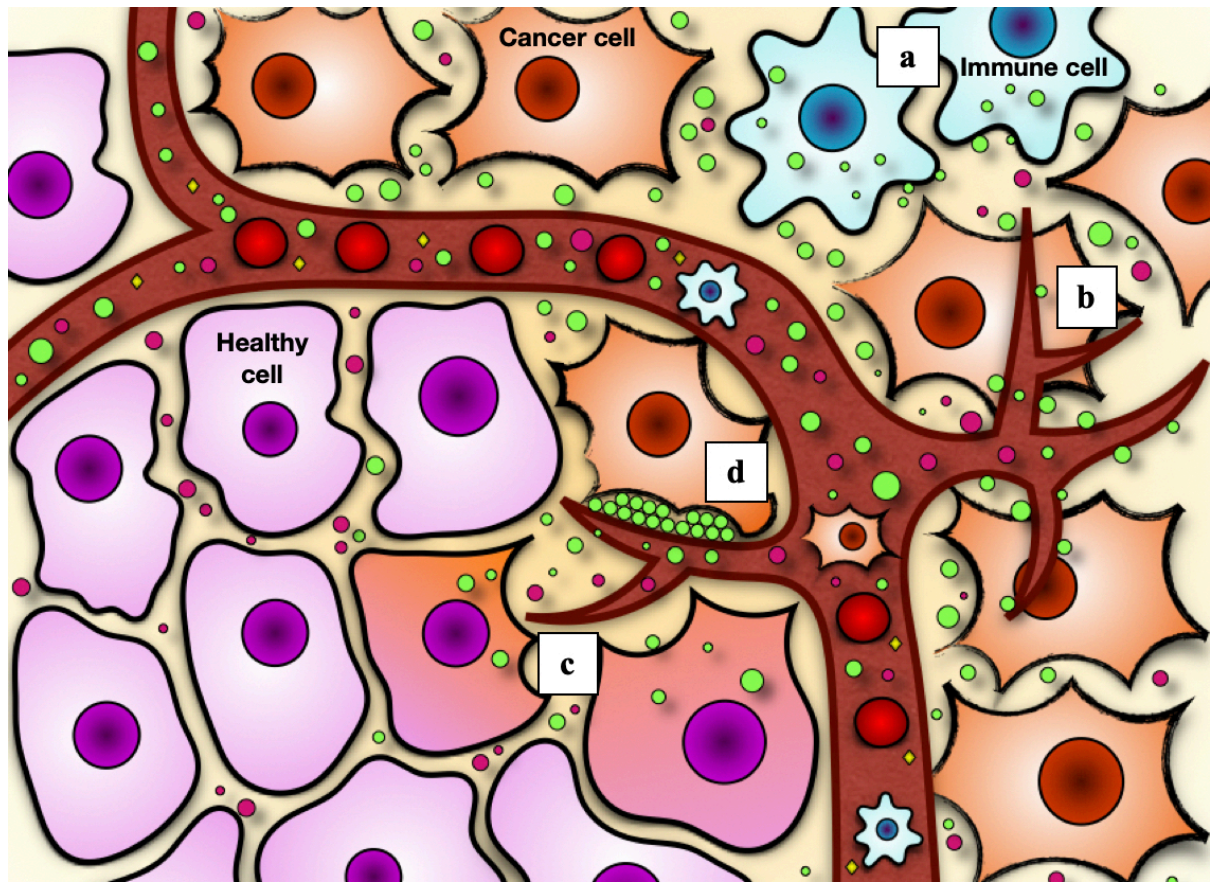


Figure 2: The involvement of EVs in the primary tumour microenvironment and microenvironments at distant sites. **(a)** Tumour derived EVs are involved in immunomodulation, including but not limited to regulation of macrophage activity by transfer of miRNA cargoes (57,58), inhibition of signalling and proliferation of activated CD8⁺ T cells and expansion of suppressor CD4⁺ Treg cells (59), and suppression of CD8 T cell activity by PD-L1 expressing EVs (60). **(b)** EVs have also been shown to induce pro-angiogenic processes in vasculature surrounding tumours, allowing direct delivery of nutrients for proliferative growth (61). **(c)** EVs have been documented to transfer of oncogenic cargoes to recipient cells which can lead to the expression of an oncogenic phenotype in healthy cells, or a metastatic phenotype in tumour cells, enhancing tumourgenesis (62). **(d)** Finally, EVs can initiate pre metastatic niche formation at distant sites by performing the function mentioned above, in addition to initiation to cancer associated fibroblast transition and extracellular matrix remodelling. This creates a favourable microenvironment for tumour cells to travel to and establish metastatic growths (63). EVs also seem to play a prominent role in the adhesion of metastatic tumour cells at new sites, possibly forming scaffold like structures that anchor cells prior to proliferation (64–66).

Along similar lines, Peinado et al. identified that melanoma derived EVs can increase the metastatic tumour burden through effects of EV associated oncoprotein MET receptor tyrosine kinase on bone marrow-derived cells (BMDCs) (67). Importantly, the authors demonstrate that the effects of EVs on the BMDCs are durable. Indicating that genetic or epigenetic changes could be involved in this phenomenon since the cells retain their phenotype following engraftment into a new host.

Another example of the role of EVs the spread of cancer was recently reviewed in the context of ovarian cancer, where EVs are proposed to travel to the abdominal cavity to support the establishment of a pre-metastatic niche. Disseminated ovarian cancer cells can reach the abdominal cavity via the peritoneal fluid and adhere to EV scaffolds, establishing metastatic tumour formation (68). Koumangoye et al. reported that detachment of breast cancer cells is accompanied by the release of EVs which subsequently support cellular adhesion and spreading (65). They postulate that this support could allow cells to gain a foothold and subsequent growth advantage.

De la Fuente et al. have utilised this proposed function of EVs as intermediaries of tumour cell attachment for the development of a therapeutic device that was found to redirect metastasis of ovarian cancer (69). This pre metastatic niche mimetic will be discussed in more detail in the section on EVs as cancer diagnostics and therapeutics.

Interestingly, integrins expressed on EVs seem to play a pivotal role in metastasis by adhering to target tissues, setting up a foundation on which to form new metastatic niches from disseminated tumour cells, and aiding transition of oncogenic molecules to recipient cells(70). In this way, EVs have been shown not only to act by delivering their intraluminal cargoes but through interacting with cell membranes. Furthermore, integrin expression profiles of EVs have been found to correlate with tissue organotropism, were integrins $\alpha 6\beta 4$ and $\alpha 6\beta 1$ were associated with lung metastasis, while integrin $\alpha v\beta 5$ was linked to liver metastasis (71).

In addition to their reported effects in the generation of receptive microenvironments findings show that EVs can alter recipient cancer cells. Melanoma cell derived EVs can induce phenotype switching in recipient primary melanocytes and in colon cancer the transfer of mutant KRAS proto-oncogene has been shown to promote proliferation of wild-type recipient colon cancer cells (72,73)

Furthermore, EVs have been shown to be notable contributors to cancer chemotherapeutic resistance through various mechanisms. Firstly EVs have been shown to be involved in cancer chemotherapeutic expulsion through recruitment of ATP-binding cassettes (ABCs) that directly sequester chemotherapeutics and localize to the limiting membrane of EVs (74,75). ABCG2 mediated mitoxantrone expulsion in breast cancer and ABCA3 mediated daunorubicin expulsion in leukemia are notable examples of this (76,77). Comparably, EVs have also been shown to be involved in the horizontal transfer of drug efflux pumps to spread chemotherapeutic resistance to drug sensitive cancer cells. The most notable example of this is the transfer of the highly active P-gp drug transporter to chemotherapeutic sensitive cancer cells (78). Its transfer has been observed in models of breast cancer where it is involved docetaxel and doxorubicin efflux (79), and in prostate and ovarian cancer where it is involved in taxane efflux (80). Moreover, EVs have also been shown to mediate chemotherapeutic resistance by removal of drugs from extracellular spaces. CD20 expressing B-cell lymphoma derived EVs have been shown to sequester rituximab thus protecting target lymphoma cells from attack (81). Similarly, human epidermal growth factor

receptor-2 (HER2) expressed in EV membrane has been shown to sequester Herceptin in an *in vitro* model of breast cancer (82). There are numerous other examples of EV mediated cancer chemotherapeutic resistance in addition to drug sequestration and efflux, including the transfer of pro-survival proteins and RNAs and immune modulation, however this has been extensively reviewed elsewhere (83–85).

The aforementioned findings highlight the role of EVs in the crosstalk occurring between cancer cells and host environment. Improvements of techniques to study nano-sized EVs and novel models to test hypotheses will undoubtedly contribute to furthering our understanding. Interestingly, transmissible cancers may provide valuable insights into evolutionary processes of cancer (86). Tasmanian devil facial tumour disease (DFTD) and canine transmissible venereal tumours (CTVT) are transmissible cancers that can spread from one individual to another through direct physical transfer of cancer cells (87,88). Studying transmissible cancers in more detail might allow us to study processes occurring upon transfer of cancer cells and their subsequent establishment into cancer within the new host. Interestingly, a recent study by Espejo *et al.* revealed enrichment of proteins associated with epithelial mesenchymal transition (EMT) processes and focal adhesion signalling in EVs released by cultured DFTD cells (89). These findings indicate a potential role EVs in metastatic processes underlying the progression of this transmissible cancer. A better understanding of transmission of cancer cells and the interplay between the transmitted cells, secreted EVs, and the host could provide valuable insights into the biology of cancer.

EVs as cancer diagnostics and therapeutics

Due to the involvement of EVs in cancer growth and development, EVs and their underlying biogenesis pathways have become targets for the development of anticancer therapeutic strategies as well as cancer diagnostic strategies (90,91). As EVs are detectable in various biofluids and their composition reflects the state of the cell from which they derive, EVs can be applied as non-invasive diagnostic markers for cancer. A recent study by Lyden *et al.* shows that the proteomic EV composition can serve as a reliable biomarker for cancer detection and determination of cancer type (92).

Sun *et al.* recently reviewed various advances in the space of EV-based anticancer therapies (93), including attenuation of neutral sphingomyelinase 2 (an element of endosomal-derived EV biogenesis) in breast cancer cells that reduces EV formation and suppression of cancer cell metastasis in xenograft mouse models (94), administration of aptamer-functionalised nanoparticles to mice which eliminate circulating cancer EVs and attenuate cancer EV-induced lung metastasis (95), and targeting immune checkpoint protein inhibitors; programmed cell death protein 1 and programmed death-ligand 1 (PD1/PD-L1) incorporated within EVs with antibodies resulting in suppressed tumour growth in a colorectal cancer model (96).

Importantly, as EVs are biocompatible, can be patient derived and have been shown to cross biological barriers (97–99), they represent a highly suitable platform for targeted therapeutic delivery. As such, over the last two decades a substantial research effort has been made to harness this potential, and particularly so with regard to cancer therapy. In a particularly high-profile use of EVs in a therapeutic context, EVs derived from human embryonic kidney 293 (HEK293) cells were modified to contain a tumour suppressing miRNA, let-7a, and express the EGFR targeting ligand GE11 peptide on their surfaces. These EVs were

administered weekly for four weeks to mice implanted with EGFR expressing breast cancer cells, causing a significant ~60% reduction in tumour size (100).

As alluded to earlier, De la Fuente et al. exploited the function of tumour derived EVs as intermediaries of tumour cell attachment and were able to redirect disseminated cancer cells. For this approach EVs purified from the ascitic fluid of ovarian cancer patients were embedded onto a 3D scaffold and the scaffolds were subsequently surgically implanted to test their efficacy in an ovarian cancer mouse model (69). The scaffolds were found to serve as a preferential site for metastasis formation and a significant increase in survival was observed. Further uses of EVs in therapeutic contexts has also been reviewed elsewhere (93,101).

The rediscovery of Darwin's gemmules

Recently, the involvement of EVs in the transfer of information from somatic cells to germ cells, and therefore their role in the processes that underpin evolution, has become a topic of interest for some within the EV field. However, this is not the first time that cell derived particles have been theorized to play a role in inheritance and evolution. In fact, the role of cell derived particles in evolution may have been first theorized by none other than Charles Darwin himself.

In 1859, Charles Darwin published his defining work *On the Origin of Species* (102). While Darwin's theory of natural selection and evolution was ground-breaking and is still revered today, there was a notable hole in his theory. Darwin could not fully explain how offspring inherited a mix of characteristics from their parents, nor how they inherited characteristics they had acquired in response to adapting to their environment; a concept proposed by Jean-Baptiste Lamarck. In his chapter in *Exosomes: A Clinical Compendium* titled *Exosomes, gemmules, pangenesis and Darwin*, Denis Noble surmises Darwin's predicament: "The problem that Darwin saw is that it is not obvious how such a process could happen in multicellular organisms possessing a separate germ line. If changes in the soma occur as a consequence of adaptation to the environment, there is no reason why this should change the germ line cells unless the adaptation is somehow transmitted to them" (103). So, when Darwin published *The Variation of Animals and Plants Under Domestication* in 1868 (104), he proposed his theory of Pangenesis to provide an answer.

Pangenesis assumed the existence of cell derived particles that Darwin referred to as gemmules. Darwin postulated that gemmules are "collected from all parts of the body to form the sexual elements". In conception, both parents' gemmules combine, and when provided with the correct nutrients these gemmules could grow and "multiply by self-division", eventually forming cells of the offspring themselves. Darwin also theorised that the reason offspring may inherit more features from one parent than the other was due to gemmules from one parent having some "advantage in number, affinity, or vigour over those derived from the other parent", thus explaining variation in inheritance. Finally, Darwin also argued that characteristics that an organism acquired during its lifespan due to environmental factors could also alter the organism's gemmules, thereby allowing acquired characteristics to be inherited by their offspring (104).

Despite Darwin's brilliance as an evolutionary biologist, his theory of Pangenesis was discredited and later proven to be incorrect. August Weismann was the first to do so in 1890, by demonstrating that mice with amputated tails could not pass this non-functional

characteristic to their offspring (105). Despite the crude nature of the experiment, this was evidence enough to convince Weissman and others that Lamarckism, in other words the inheritance of characteristics acquired in response environmental stimulation, was in fact false. Following this, Weismann's theory that germ line cells are isolated cells of the soma, the Weissman barrier, became widely accepted (103).

Although, there are elements of Pangenesis that are analogous with the modern concept of EVs. Just as Darwin describes gemmules, EVs are cell derived nanoparticles that transfer cellular information, whether proteins, nucleic acids or subcellular organelles between cells (7–9). EVs transfer information between somatic cells in both physiological and pathophysiological contexts, including cancer growth and development as described previously. Certainly, Darwin's theory of Pangenesis may at least be able to be credited as an early prediction of EV mediated intercellular communication.

However, evidence has begun to emerge that suggests that EVs may be able to breach the Weissman Barrier to deliver information from somatic cells to germ line cells.

Recent *in vitro* experiments have demonstrated that EVs derived from epididymal epithelial cells (epididymosomes) can bind and interact with spermatozoa. Epididymosomes expressing CD9 bind to live spermatozoa and are proposed to be involved in the acquisition of motility and egg recognition. On the other hand, epididymosomes lacking CD9 bind to dead spermatozoa and contain proteins involved in reactive oxygen species scavenging to help protect live sperm cells from these toxic factors (106,107). Interestingly, epididymal EVs have been shown be abundant in miRNAs. This miRNA content has also been shown to vary depending on the region of the epididymis that the EVs are derived from, suggesting differential regulation of miRNA content of epididymosomes (108). Coincubation of these miRNA enriched epididymosomes with spermatozoa has shown to result in enrichment of spermatozoa in these miRNAs, suggesting potential transfer of miRNAs to spermatozoa by EVs (106,108).

More remarkably, Rompala et al. (2018), showed that chronic intermittent alcohol exposure altered the abundance and post-translational modifications of several small noncoding RNAs in mouse spermatozoa. Furthermore, alcohol-responsive tRNA-derived small RNAs (tDRs) were also altered in epididymosomes providing evidence that small RNAs and their associated alterations are transferred primarily by epididymosomes to spermatozoa (109).

Together, this evidence challenges some of the principles of the neo-Darwinist and Modern Synthesis models of inheritance and evolution and brings some of Lamarck's and Darwin's original ideas of inheritance to the forefront. In particular, while Darwin's theory of Pangenesis was incorrect, some early evidence is beginning to show that Darwin's theory proposing that inherited characteristics can be transferred via cell derived particles to the progeny may not, in fact, be too far from the truth (108,110).

Conclusion

We have here provided a short overview of the field of extracellular vesicle research and outlined the proposed roles these membranous vesicles play in intercellular communication and cancer development and progression. As researchers start exploring the evolutionary dynamics that are postulated to be at the core of cancer, the consideration of EVs as intercellular messengers will undoubtedly prove to be significant. Moving away from

the dominant gene-centric view of cancer to allow for the incorporation of new concepts will contribute to our understanding of cancer and the development of novel therapies.

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Conflicts of Interest

S.E.B. has equity in Evox Therapeutics Ltd. and holds an ongoing contract with Evox Therapeutics Ltd. from 2017 to present. Evox Therapeutics Ltd. had no influence on the conception or realisation of this review.

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