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**The emerging role of exosome and microvesicle- (EMV-) based cancer therapeutics and immunotherapy**

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**Novelty and Impact Statement:** This Mini-Review brings together the field of exosomes and microvesicles (EMVs) as drug delivery vehicles together with the burgeoning area of cancer immunotherapy. We highlight the promise of cancer immunotherapies using exosomes and RNA lipoplexes as cancer vaccines, critically assess the promising but at times controversial results using Mesenchymal stem cell-derived EMVs, discuss the possibility of therapeutic removal of EMVs and assess the use of EMVs and exosome-mimetic nanovesicles (NVs) as drug delivery vehicles.

**Keywords:** tumour microenvironment; exosomes and microvesicles (EMVs); immunotherapy; EMV-based therapy

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**Abstract**

There is an urgent need to develop new combination therapies beyond existing surgery, radio- and chemo-therapy, perhaps initially combining chemotherapy with the targeting specificities of immunotherapy. For this, strategies to limit inflammation and immuno-suppression and evasion in the tumour microenvironment are also needed. To devise effective new immunotherapies we must first understand tumour immunology, including the roles of T cells, macrophages, myeloid suppressor cells and of exosomes and microvesicles (EMVs) in promoting angiogenesis, tumour growth, drug resistance and metastasis. One promising cancer immunotherapy discussed uses cationic liposomes carrying tumour RNA (RNA-lipoplexes) to provoke a strong anti-viral-like (cytotoxic CD8<sup>+</sup>) anti-tumour immune response. Mesenchymal stem cell-derived EMVs, with their capacity to migrate towards inflammatory areas including solid tumours, have also been used. As tumour EMVs clearly exacerbate the tumour microenvironment, another therapy option could involve EMV removal. Affinity-based methods to deplete EMVs, including an immuno-depletion, antibody-based affinity substrate, are therefore considered. Finally EMV and exosome-mimetic nanovesicles (NVs) delivery of siRNA or chemotherapeutic drugs that target tumours using peptide ligands for cognate receptors on the tumour cells are discussed. We also touch upon the reversal of drug efflux in EMVs from cancer cells which can sensitize cells to chemotherapy. The use of immunotherapy in combination with the advent of EMVs provides potent therapies to various cancers.

## Introduction

For decades chemotherapy was the benchmark of cancer treatment but in recent years our understanding of tumourigenesis, angiogenesis, epithelial-mesenchymal transition (EMT), metastasis and immune modulation, has brought calls for modern, less toxic approaches with improved efficacy. Ideal approaches should not only be tailored to a given cancer but also the individual, entering the unprecedented age of personalised medicine<sup>1</sup>.

A tumour comprises cancer and stromal cells whose interplay has adapted it to evade immunity, by avoiding surveillance mechanisms or direct cell-to-cell inhibition, and also by the release of exosomes and microvesicles (EMVs) containing functional signalling elements<sup>2</sup>. Cancer cells can manipulate their surroundings, by educating tumour-infiltrating cells, to develop an environment heavily in favour of tumour growth. Host cells may include fibroblasts, myeloid-derived stem cells, mast cells, neutrophils, CD4<sup>+</sup>, CD8<sup>+</sup>, T<sub>reg</sub> T cells, natural killer (NK) cells, dendritic cells (DCs) and macrophages<sup>3-5</sup>. Together, these cells take on pro-tumour characteristics facilitating neovascularisation, matrix remodelling, growth signalling, tumourigenesis and inhibition of several key immune checkpoints, aimed to halt tumour formation<sup>6</sup>.

Emerging immunotherapies look to take advantage of existing anti-tumour immune defences, redirecting and amplifying them for more specific tumour targeting<sup>7</sup>. Host immunity is able to destroy aberrant cells in a selective manner, the most important effector cells being T cells (CD4<sup>+</sup> and CD8<sup>+</sup>), DCs and NK cells. Regular immune patrolling and surveillance allows detection of such aberrant cells. Chronic inflammation is a known risk factor of cancer and to survive cancer cells must therefore disarm immunity<sup>8, 9</sup>. To rebalance tumour microenvironments, an arsenal of immunotherapies are under investigation, including various therapeutic antibodies, DC-based vaccination, microbial vectors, adenovirus transfection and various gene therapies<sup>7</sup>.

Exosomes and Microvesicles (MVs) or EMVs represent two major classes of cell-derived vesicle. Extracellular Vesicles (EVs) is a term that encompasses EMVs and apoptotic bodies. They are

produced as part of normal cell homeostasis and functions are heavily dependent on the parental cell lineage and state of the cell at the time of their release<sup>10-12</sup>. EMVs' functions range from waste removal, to autocrine, paracrine and long distance cell-to-cell signalling. MVs are implicated in a host of functions inducing immunomodulation, thrombosis/coagulation, cell growth/apoptosis<sup>13-16</sup>. EMVs carry a multitude of functional signalling molecules, including growth factors, cytokines, genetic material and RNA transcripts, complement proteins and immune ligands<sup>17-19</sup>. This review will aim to cover our current understanding of EMVs in relation to the tumour-host interaction and their potential in novel cancer therapies.

### **Biogenesis of exosomes and microvesicles**

Exosomes and MVs have proven difficult to fully differentiate from one another, there being significant crossover of functionality and size. However they follow very distinctive pathways of biogenesis<sup>20</sup>. MVs typically 0.1-1  $\mu\text{m}$  are produced by budding from the cell surface. One proposed mechanism is via increased  $\text{Ca}^{2+}_i$ , as was reported by early complement biologists studying removal of membrane attack complex (as reviewed later<sup>21</sup>). Higher concentrations of  $\text{Ca}^{2+}_i$  activate the lipid bi-layer redistribution enzyme, scramblase<sup>22</sup>, whilst sequestering similar lipid distribution enzymes floppase and translocase (also known as flippase), that maintain normal membrane asymmetry in an ATP-dependant manner<sup>21, 23</sup>. Furthermore, cytoskeletal degradation associated with calpain and gelsolin activation leads to membrane blebbing and eventually microvesiculation. Few markers distinguish MVs from other vesicles although phosphatidylserine (PS) is known to be highly expressed on MVs due to loss of membrane asymmetry<sup>17</sup>. MVs are therefore of non-endosomal origin.

Exosome biogenesis is generally accepted to initiate with the internalisation of surface lipids and receptor-mediated endocytosis to form early endosomes. Invagination of early endosomes, results in intraluminal vesicles (ILVs), the endosomes now being referred to as multivesicular bodies or endosomes (MVBs/MVEs). The two mechanisms for exosome biogenesis (intraluminal budding) are

Endosomal Sorting Complex Required for Transport (ESCRT)-dependent and ESCRT-independent. ESCRT-I and -II complexes recruited to the outer endosomal surface mediate invagination of the endosomal membrane and ESCRT-III induces fission to release ILVs into the MVB. Protein selection that is independent of ESCRT involves ubiquitination, as for MHC class II or Epidermal Growth Factor Receptor<sup>24</sup> or sumoylation as for  $\alpha$ -synuclein<sup>25</sup>. Examples of ESCRT-independent MVB biogenesis in mammalian cells include MVBs being observed even in the absence of all ESCRT complexes (0, I, II, and III)<sup>26, 27</sup> as well as with mouse oligodendroglial cells where proteolipid protein was sorted into ILVs completely independent of ESCRT machinery, but dependent on the sphingolipid ceramide and sphingomyelinase<sup>28</sup>.

MVBs may then follow a degradative pathway fusing with lysosomes, eventually being recycled. Alternatively MVBs may fuse with the plasma membrane, whereupon ILVs are released as exosomes. These distinct MVBs, with particular cargo and fates, are both present within a cell<sup>12</sup>.

#### **Isolation and characterisation of exosomes and microvesicles**

The efforts to standardise EMV isolation and characterisation procedures have been ongoing<sup>29</sup> with continued basic research revealing new physical and biological properties. Recent reviews have detailed isolation procedures<sup>30</sup>, but to date purity of samples has remained elusive such that unless a study specifically demonstrates that samples are formed in MVBs, the vesicle populations used will likely carry a mixture of EVs derived both endosomal and non-endosomal origin.

Studies have commonly used differential centrifugation to isolate EMVs according to size from conditioned medium. Larger vesicles, typically MVs, are pelleted at 10,000-20,000 *g*. A second centrifugation step of the resultant supernatant at 100,000 *g* is then used to isolate exosomes which are typically smaller than MVs<sup>30, 31</sup>. However, the upper and lower range of exosome and MV sizes cross-over and, to confuse matters further, so do their densities<sup>32</sup>. Furthermore, although exosomes

are smaller than MVs, (50-100 nm), size may be a problematic means of distinguishing the vesicle types, as larger exosomes and smaller MVs<sup>33</sup> have been reported. Unfortunately differential centrifugation also relies on the mass of cargo being carried to pellet the vesicle, such that larger vesicles that are quite buoyant may fail to pellet in the 10,000-20,000 *g* spin, whereas smaller higher density vesicles may sediment at this force; the density of EMVs, currently stands in the range of 1.1-1.19 g/ml<sup>30</sup>.

Problems with ultracentrifugation (100,000 *g*) include extravesicular protein and nucleic acid clumping, result in inaccurately high protein measurements and numbers of exosomes. To improve purity of EMV pellets and remove contaminating extravesicular aggregates, equilibrating density gradients may be employed, sucrose mediums being preferred for EMV isolation<sup>34</sup>. Protein aggregates sediment whilst EMVs float upwards (a characteristic of their lipid membranes), separation of EMVs containing disparate densities then occurring as each EMV will float upwards until the respective forces of their density and buoyancy equilibrate<sup>20</sup>. For isolating EMVs from differing biological samples, such as saliva, it may be preferable to use other media, such as iodixanol density gradient centrifugation<sup>35</sup>. Saliva is reportedly 5-6 times more viscous than plasma<sup>36</sup>, so EMVs and proteins fail to equilibrate in appropriate fractions using sucrose gradient centrifugation, despite attempting pre-treatment filtration and sonication methods<sup>35</sup>.

Other methods for EMV isolation include size exclusion chromatography, immunoaffinity purification, polymeric precipitation and microfluidics<sup>37</sup>; commercial kits are also a growing venture. Assessment of EMV samples may use a range of techniques including electron microscopy, nanoparticle tracking analysis, atomic force microscopy, resistive pulse sensing, dynamic light scattering, flow cytometry and Western blotting.

**Future vesicle-based cancer therapies may include the use of RNA lipoplexes, MSC-derived EVs, exosome-mimetic vehicles and therapeutic removal of EVs**

The rest of this review will critically assess the various recent vesicle-based therapies, ranging from RNA lipoplex use as a candidate cancer vaccine to the sometimes controversial use of mesenchymal stem cell-derived EVs with its various anti-tumour effector mechanisms. We also touch upon therapeutic removal of exosomes and MVs where they contribute to disease pathology and assess the use of EMVs, and exosome-mimetic nanovesicles as drug delivery vehicles.

### **Cancer immunotherapies using RNA lipoplexes as cancer vaccines**

In an exciting novel approach to developing an effective cancer vaccine, nanoparticles (cationic liposomes)<sup>38</sup> carrying tumour RNA, so-called RNA lipoplexes (RNA-LPX) have been used. Such RNA lipoplexes were able to stimulate an immune response with anti-viral properties (Fig. 1). Essentially, given a slightly negative charge, the RNA-LPX were directed to DC-rich areas in mice (lymphoid tissues/spleen) and taken up by macrophages and plasmacytoid DC (both CD11c-expressing). Their uptake by macropinocytosis was more effective by pDCs, which released a first wave of IFN- $\alpha$ , which helped initial T cell priming. The mature dendritic cells (mDCs) then translated the tumour RNA and presented the tumour antigens to T cells. A second wave of IFN- $\alpha$  then fully primed the T cells, resulting in a strong and enduring anti-tumour response. Initial intravenous vaccination trials in melanoma patients showed activation of CD4 and cytotoxic CD8 T cells, as would be expected in an anti-viral response along with IFN- $\alpha$  production.

### **Mesenchymal Stem Cell-Derived Extracellular Vesicles in Cancer Therapy**

Mesenchymal stem cells (MSCs) are multipotent precursors of bone marrow stroma with the ability to differentiate into phenotypes of the mesenchymal germ layer<sup>39</sup>. Their properties in tissue differentiation and immunomodulation places them as potential therapeutic options with regard to wound healing and regeneration,<sup>40</sup> and they are particularly interesting as they locate and migrate towards damaged and inflammatory microenvironments, including solid tumours<sup>41, 42</sup>. Their targeting nature places them in good stead to deliver therapeutic agents such as therapeutic miRNA, IFN- $\beta$ , oncolytic adenovirus and anti-cancer agents<sup>43-45</sup>. Paracrine signalling seems a key mediator of



wound healing and anti-inflammatory processes utilised by MSC populations. As expected, analysis of MSC-derived EMV cargo has identified the presence of cytokines, growth factors, chemokines, mRNA and miRNA cluster groups<sup>32, 46, 47</sup>, in addition to mitochondria and mtDNA,<sup>48</sup> expanding the repertoire of communicative signalling elements associated with MSC vesicles.

Interestingly, MSCs have opposing activities *in vivo* and *in vitro*. The contrasting observations reported could be due to cell lines used (both MSC and tumour), heterogeneity of MSCs, timing of MSC treatment, choice of *in vivo* model and method of administering MSC populations<sup>49</sup>. For example, intravenous injection of homogenous MSCs reduced tumour burden whilst co-injection with tumour cells promoted angiogenesis and growth<sup>50</sup>. Nevertheless, MSCs display multi-functional activities, both with pro- and anti-tumour activity within the microenvironment. Supporting tumour growth may relate to the tissue remodelling activity MSCs perform at inflammatory sites, causing localised immunosuppression whilst harmonising multiple cell types of the stroma and endothelium<sup>51</sup>. A cancer's ability to coerce MSCs into collaboration may be vital for survival in some cancers, whilst others may utilise cells of other origins or entirely separate survival strategies for theirs, as discussed previously<sup>52-56</sup>.

In the context of the EMVs shed from MSCs, Bruno *et al.* in 2013<sup>42</sup> observed increased cell cycle arrest in phase G0-G1 and reduced proliferation of cancer cells (HEPG2 hepatoma, Kaposi sarcoma, SKOV-3 ovarian cancer cell lines) following MSC-EMV treatment *in vitro*. Furthermore, reduced tumour burdens were noted with *in vivo* models after MSC-EMV treatment. Gene expression of cell cycle proteins found the negative cell cycle regulators retinoblastoma 1 and retinoblastoma-like 1 and 2 were heightened, whilst progressive cell cycle proteins cyclin D2 (*CCND2*) and Cullin-3 (*CUL3*) displayed a 2-fold reduction following a 24h MSC-EMV incubation. In support, MSC exosomes reportedly suppressed angiogenesis by reducing VEGF expression in breast cancer cells<sup>32</sup>. Molecular analysis by qRT-PCR implicated miR-16 in blocking translation of the *VEGF* mRNA transcript,

subsequent *VEGF* mRNA expression thereby increasing following transfection with a miR-16 inhibitor. *VEGF* silencing with miR-16 was also reported by others<sup>57</sup>. Additionally, marrow stromal cell-derived exosomes expressing miR-146b arrested glioma growth<sup>58</sup>. Indeed several miRNA clusters are reported to support tumour growth, by acting as tumour suppressors or oncogenes, and which are not exclusively released by MSCs<sup>59</sup>. Anti-tumour effects that MSC-EMVs demonstrate was further observed both in *in vivo* and *in vitro* models of T24 bladder cancer tumours, using human cord blood Wharton's jelly MSCs (hWJMSC-EMVs). Specifically, hWJMSC-EMVs induced T24 apoptosis and cell cycle arrest, reportedly by upregulating caspase-3 cleavage whilst suppressing Akt phosphorylation pathways (Fig. 2A)<sup>60</sup>. Conditioned Medium derived from human liver stem cells (HLSC), expressing MSC phenotypes and several embryonic stem cell markers, have also displayed anti-tumour effects in several cell lines<sup>61</sup>. The study further reasons that it is the presence of Lefty A in the HLSC-conditioned medium that blocks tumour growth by sequestration of Nodal signalling pathways (Fig. 2B). The anti-tumour effects appear to be cell-specific, disrupting cell lines with exuberant Nodal pathways, namely HepG2, MCF7, KP6, KS and Jurkat. However, when HepG2 cells were treated with MSC-CM, lacking Lefty A, the pro-apoptotic and anti-proliferative effects were abrogated and it was even observed that there were modest increments in proliferation compared to control; others have also reported MSC-EMVs' impairment of tumour growth<sup>62,63</sup>.

Compared to MSC cell studies, MSC-EMVs have also shown heterogeneous effector mechanisms. A recent study investigated the relationship between BM-MSCs and multiple myeloma (MM) cells, where it was found that BM-MSCs from patients with MM (MM BM-MSCs) are phenotypically different from BM-MSCs from healthy donors. MM BM-MSC-EMVs were found to express elevated levels of IL-6, CCL-2 and fibronectin, whilst downregulating the tumour suppressor miR-15a, amongst other miRNAs. It was proposed that MM BM-MSCs support MM progression and dissemination<sup>64</sup>. Similar pro-tumour effects have been observed in a renal carcinoma model using hWJMSC-EMVs. After treatment with hWJMSC-EMVs, renal carcinoma cell cycles progressed to S phase and

proliferated. *In vivo*, cyclin D1 was upregulated, which supports a transition from G0/G1 to S phase. Additionally, HGF protein and mRNA expression became elevated, with EMVs seemingly involved in the delivery and stimulation of ARK and ERK1/2 pathways<sup>65</sup>. Subcutaneous co-injection of MSC or MSC-EMVs with human gastric cancer cells, SGC-7901, and human colon cancer cells, SW480, increased tumour burden and growth, as measured by proliferating cell nuclear antigen (PCNA) positive cells. Interestingly, *in vitro* proliferation was not seen, nor was there any observed difference in cell cycle when comparing controls. However, *VEGF* and *CXCR4* mRNA and protein expression was heightened *in vitro*. Therefore, it is thought that the increased tumour burden found *in vivo* is an indirect consequence of MSC-EMVs promoting an angiogenic programme, favouring tumour seeding and growth<sup>66</sup>.

Tumours have been reported to release EMVs capable of coercing MSCs into altering their activity as explored by Chowdhury *et al.*<sup>67</sup>, using prostate cancer PC3 cell-derived exosomes. Following PCa-exosome treatment, BM-MSCs differentiated into myofibroblasts expressing  $\alpha$ -SMA in a dose-dependent manner, which was thought to be facilitated by exosome-derived TGF- $\beta$ . The PCa-exosome treated BM-MSC expressed heightened VEGF, HGF, MMP-1, 3 and 13 and promoted angiogenesis of HUVECs, as demonstrated by scratch assay and CD31 labelling. Despite TGF- $\beta$  being vital to myofibroblast propagation, TGF- $\beta$  treated BM-MSCs expressed little or no  $\alpha$ -SMA, VEGF, HGF, CD31 or MMPs. The mechanism of differentiation, be it exosomal or soluble cytokine, is a key determinant in phenotype expression. A similar study also observed BM-MSC differentiation into fibroblasts expressing  $\alpha$ -SMA when treated with exosomes derived from the cholangiocarcinoma cell line, KMBC. Notable increases in IL-6, PDGF-AA, CX3CL, CXCL-1, CCL-2 and MMP-2 were found in BM-MSCs, supporting the argument for a positive feedback loop between tumours and stromal cells of the microenvironment, enhancing tumour growth and survival<sup>68</sup>.

#### **Therapeutic removal of Exosomes and Microvesicles**

Tumour microenvironments consist of a variety of mutable cell types which become educated and adapted to support the success of the primary tumour. Currently, research is littered with examples of tumour-derived EMVs tolerising and modulating their environments, promoting metastasis, angiogenesis, immunosuppression and drug resistance<sup>69</sup>. This prompted investigations into EMV removal from cancer patient sera, attempting to nullify the exertive forces of tumours on surrounding cells and tissues. A study from the late 80s explored the use of an extracorporeal hemofiltration system to this effect. Removal of low molecular weight proteins (approximately <150kDa) caused tumour shrinkage of 50% or more, for those patients that could be evaluated by a repeat biopsy, in 6 of the 16 patient sample<sup>70</sup>. Though EMVs were not identified it is thought, according to Marleau *et al.*,<sup>71</sup> that whole blood ultrapheresis removed exosomes (which would not have been known about at the time) but probably also other EVs, because of the great heterogeneity of EVs in circulation, and that this removal may have been responsible for the tumour shrinkage observed. Aethlon Medical has developed a haemofiltration system using cartridges consisting of a porous hollow fibre passage and an affinity matrix, which fit into continuous renal replacement therapy machines. The technology, named Adaptive Dialysis-like Affinity Platform Technology (ADAPT<sup>TM</sup>), separates blood components <200nm where target antigens interact with immobilised agents of the affinity matrix, such as monoclonal antibody, lectins and aptamers, therefore drawing specific components out from the blood<sup>71</sup>. Tullis et al. at Aethlon, used *Galanthus nivalis* agglutinin as an affinity substrate, which recognises high mannose glycoproteins on viral envelopes to remove virion particles from hepatitis C virus (HCV) patients.<sup>72</sup> HCV load was reduced in infected patients not receiving anti-viral drugs following three, 4-6 h dialysis treatments per week. Additionally, drug efficacy and patient response to ribavirin and pegylated interferon (PEG-IFN) therapy was also seen. The use of antibodies as affinity substrates (a type of immuno-depletion) are also being investigated<sup>73</sup>, with potential to target tumour-specific and associated antigens transported on EMV surfaces. Reportedly, antigens such as HER-2<sup>74</sup> and CD20<sup>75</sup> are present on the surface of breast cancer and B cell lymphoma-derived EMVs respectively and could be viable targets

for therapy. Other examples of immuno-depletion used in a non-clinical setting for exosome removal from cell culture supernatants, that could form the basis of adapted methods for a clinical setting have included anti-CD45-coupled magnetic bead removal following 100,000 *g* ultracentrifugation<sup>76</sup> or as our group described the removal of anti-CD63<sup>biotin</sup> complexed to exosomes using Streptavidin T1 dynabeads<sup>11</sup>.

### **Use of Exosomes and Microvesicles (EMVs) and exosome-mimetic nanovesicles (NVs) as drug delivery vehicles**

EMVs are attractive drug delivery vehicles due to their membrane composition and the adhesive proteins embedded within them, their role in cell-to-cell communication<sup>10</sup> and because exogenous cargo can be loaded into them to deliver therapeutics to tumour sites. Drug incorporation into EMVs has involved expression vectors, incubation of cells with the therapeutic in acidic conditions, electroporation,<sup>55</sup> freeze-thaw cycles, sonication, extrusion, treatment with/without saponin and permeabilization<sup>77</sup>. EMVs represent an attractive delivery method of RNA species too, given they are homeostatic carriers that can readily fuse with cells<sup>78,79</sup>.

A murine model by Alvarez-Erviti *et al.*<sup>80</sup>, utilised 'self' immature Dendritic Cell- (iDC-) exosomes. By electroporation at 400V, 150µg of siRNA against *GAPDH* (ubiquitously expressed) and *BACE1* (a target in Alzheimer's disease) could be loaded and retained in exosomes. Lamp2b is widely expressed on exosomes and was fused to rabies viral glycoprotein (RVG-exo) and ASSLNIA, a 7 amino acid muscle-specific peptide (MSP-exo) to target neurons, microglia and oligodendrocytes (by acetylcholine receptor) and muscle cells respectively (Fig. 3A). *In vivo*, naked *GAPDH* siRNA induces gene knockdown of the spleen, liver and kidney, with little apparent delivery to muscle or neurons. However, when siRNA was incorporated with RVG exosomes (RVG-exo), *GAPDH* gene knockdown in the striatum, midbrain and cortex was observed with little effect on the spleen or liver; though kidney did display knockdown of the gene it was below the threshold of significance. Furthermore,

cortical sections from sacrificed mice confirmed that *BACE1* siRNA delivered via RVG-exo, successfully reduced protein expression (62%), mRNA (61%), and  $\beta$ -amyloid 1-42 aggregates (55%). The results give reason to be hopeful for future gene and targeted therapies, though it should be noted that a report by Kooijmans SA *et al.*,<sup>81</sup> called for more effective methods of EMV loading after exploring the efficiency of siRNA uptake in EMVs, and finding retention measurements to be misleading. The peak of exogenous siRNA uptake was found at approximately 20-25%. However, the group found that without EMVs, siRNA retention remained equally detectable by nanoparticle tracking analysis (NTA) and confocal microscopy. Metal ions from the electrodes and hydroxide ions from the electroporation buffer were thought to induce the precipitation of siRNA forming detectable aggregates. EDTA, citric acid buffers and other methods successfully reduced formation of aggregates, but unfortunately this was at the expense of EMV siRNA retention resulting in likely overestimation of EMV retention of siRNA.

Of note, electrical discharge through a solution containing macromolecules, such as DNA, RNA and protein, caused significant molecule aggregation; with siRNA unlikely to be the only macromolecule affected by such forces, this may present a difficulty when loading various therapeutics into EMVs using electroporation<sup>82</sup>. Furthermore, a paper comparing methodologies of exosomal drug incorporation found that sonication, rather than electroporation, yielded greater loading of paclitaxel (PTX) into exosomes. Exosomal membrane rigidity decreased upon sonication, allowing PTX, with a hydrophobic structure, to incorporate itself between the lipid-bilayer as well as attaching itself to the surface<sup>83</sup>. An *in vivo* model was developed using pulmonary metastatic cells (3LL-M27), a drug resistant cell line which highly expresses the *MDR1* gene and *P-gp*. Mice treated with macrophage-derived PTX-loaded exosomes, displayed a two and three-fold decrease in tumour burden in comparison to taxol (50mg/kg) and exosome only treatments, respectively.

Recently the potential of exosome-mimetic nanovesicles (NVs), prepared by size extrusion of monocytes/macrophages, in the delivery of tumour chemotherapeutics, was explored<sup>84</sup>. The NVs

loaded with Doxorubicin (dox), caused, significant cell death of HUVECs pre-treated with TNF- $\alpha$  *in vitro* (Fig. 3B). Remarkably, a pre-incubation with VCAM-1, ICAM-1 and E-selectin inactivating antibodies abrogated the cytotoxic activity of dox-NVs. *In vivo*, ICAM-1 dependence was also observed for effective targeting as determined by fluorescence. Dox-NVs accumulated in tumour sites due to highly expressed ICAM-1<sup>85</sup>, whilst trypsin treatment of NVs nullified targeting capability, by removal of the extracellular domain of LFA-1. Freely administered dox diminished tumour weight in a dose-dependent manner and synergistically lowered the total number of white blood cells (WBCs). Dox-NVs displayed markedly efficient anti-tumour properties as 10 $\mu$ g of NVs, loaded with 3 $\mu$ g of dox, achieved the same level of tumour reduction as 60 $\mu$ g of free drug and avoided diminishing the WBC count. Given the targeting and efficient nature of NVs, they represent a promising future therapy.

The prognosis of patients suffering with glioblastoma multiforme (GBM) is highly dependent on the tumour's chemoresistance, and is often attributed to the expression of the drug efflux transporter P-glycoprotein (P-gp)<sup>86</sup>. A promoter of the *P-gp* gene, namely miR-9, was increased two-fold in GBM cell lines resistant to Temozolomide (TMZ).<sup>87</sup> The study sought to target this mechanism of resistance through use of MSC exosomes containing the oligonucleotide anti-miR-9, attempting to tolerate GBM cells to TMZ. Resistance was reversed as seen by a 20% reduction in cell viability compared to TMZ treatment alone and this coincided with both cleaved and uncleaved caspase 3 and the fall of P-gp surface expression.

In agreement with current literature, our group recently reported prostate cancer cells, PC3, to competently efflux docetaxel (DTX) by unloading into MVs, and thus contributing to drug resistance<sup>11</sup>. However, using a calpain inhibitor (calpeptin) or siRNA (*CAPNS1*) microvesiculation pathways can be blocked. This resulted in a 20-fold increase in docetaxel sensitivity *in vitro* (Fig. 3C), whilst *in vivo* 0.1mg/kg of DTX with 10mg/kg of calpeptin resulted in reductions in tumour growth equal to those achieved with 10mg/kg of DTX alone. Another group identified paclitaxel (PTX)

presence in MVs released from murine MSCs. Interestingly, MSC-EMVs were shown to possess anti-proliferative activity, DTX loaded MSC-MVs significantly and dose-dependently bolstering this effect on the metastatic human pancreatic cell line CFPAC-1<sup>88</sup>. A study by Saari *et al.*<sup>89</sup>, investigated which vesicle population is best suited for carrying drug cargo. Using PCa cells, separate ultracentrifugations of 20,000 *g* and 110,000 *g* were used to isolate MVs and exosomes respectively. Vesicles were loaded with high concentrations of PTX and both elicited cytotoxicity, enhancing the effects of the drug. However, at low concentrations of PTX, MVs were comparatively more potent mediators of cytotoxicity than exosomes. They further found, when drug free, that both vesicle species induced proliferation of autologous PCa cells in a dose-dependent manner.

Finally in work from Muraca's group, the differential effects of EMVs derived from MSCs of various origins were compared including umbilical cord (UC)-MSCs, bone marrow (BM)-MSCs and adipose tissue (AT)-MSCs on U87-MG glioblastoma cells<sup>88</sup>. After treating with  $25 \times 10^9$  MSC-EMVs for 48 h, interestingly UC and BM-MSC-EMVs respectively provoked a 3-fold and 2-fold increase in apoptosis (Fig. 3D), compared to controls, whilst AT-MSCs caused insignificant changes in cell viability. Further, AT-MSC-EMV treated U87-MG cells proliferated with skewed cell cycles towards S and G2/M phases. Comparably, UC- and BM-MSC-EMV treatments reduced proliferation; cells became quiescent with larger populations entering a sub-G1 phase. Apoptosis of U87-MG could be enhanced 20%-40% using UC-MSC-EMVs by loading the UC-MSC-EMVs with Vincristine (Fig. 3D), showing some potential for combination therapy.

### **Conclusions and future directions**

In this era of promising new cancer immunotherapies, vaccination strategies that prevent cancer are the ultimate goal. The use of DC-derived exosomes or 'dexosomes' to stimulate effective immunity has proved disappointing in clinical trials, but nanoparticle RNA vaccines hold huge promise. Importantly an RNA-lipoplex based vaccine overcomes the challenge of delivering vaccine antigens



to APCs. DC targeting can now be easily achieved by fine tuning the negative charge of the nanoparticles, no specific ligand being needed, and as any tumour antigen is encodable by RNA, this approach can potentially have applications in many cancers. Other promising therapies that make use of our increasing knowledge of the role of EMVs in the tumour microenvironment include immuno-depletion of particular EMVs and the use of MSC-derived EMVs.

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## Figure legends

**Figure 1. Cancer vaccines comprising novel RNA lipoplexes (RNA-LPX)** Negatively charged RNA lipoplexes (RNA-LPX) carrying tumour RNA are taken up by macropinocytosis into DCs where tumour RNA is translated and presented provoking a cytotoxic CD8<sup>+</sup> T cell response with associated waves of immunostimulatory IFN- $\alpha$ .

**Figure 2. Cancer therapy using exosomes and microvesicles (EMVs) from mesenchymal stem cells** In T24 bladder cancer, mesenchymal stem cell EMVs (blue MVs and white exosomes) induced cell cycle arrest and apoptosis by upregulating caspase-3 cleavage and suppressing Akt phosphorylation (A). EMVs from human liver stem cells (HLSC) carrying Lefty, blocked Nodal signalling in HepG2 liver cells and were pro-apoptotic and anti-proliferative (B).

**Figure 3. Exosomes, Microvesicles and Nanovesicles deliver siRNA in Alzheimer's therapy and mediate drug delivery in cancer therapy** Mouse iDC exosomes expressing a rabies viral glycoprotein (RVG) were targeted to neurons (A) where they delivered *BACE1* siRNA (a target in Alzheimer's) reducing  $\beta$ -amyloid aggregates by 55%. Monocyte derived exosome-mimetic nanovesicles (NVs) were prepared by serial extrusion through filters of decreasing pore sizes (10, 5 and 1  $\mu$ m). NVs loaded with Doxorubicin (dox), targeted tumour sites in mice producing the same tumour reduction levels as 20 times the amount of free dox; the targeting was due to NV expressed integrin LFA-1 (CD11a/CD18) affinity for tumour expressed ligand ICAM-1 (CD54) (B). The knowledge that microvesicles (MVs) can help remove chemotherapeutic agents from tumour cells was used to diminish MV-based drug efflux from prostate cancer cells pharmacologically, using calpeptin (and using *CAPNS1* siRNA) both *in vitro* and *in vivo*. As a result 100-fold lower concentrations of docetaxel could be administered with an equivalent reduction in tumour growth (C). In (D) umbilical cord-derived mesenchymal stem cell (UC-MSC) EMVs (blue MVs and white exosomes) loaded with vincristine (Oncovin<sup>®</sup>) induced double the levels of apoptosis of malignant glioma-derived, U87-MG, to 40%.

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Fig. 1

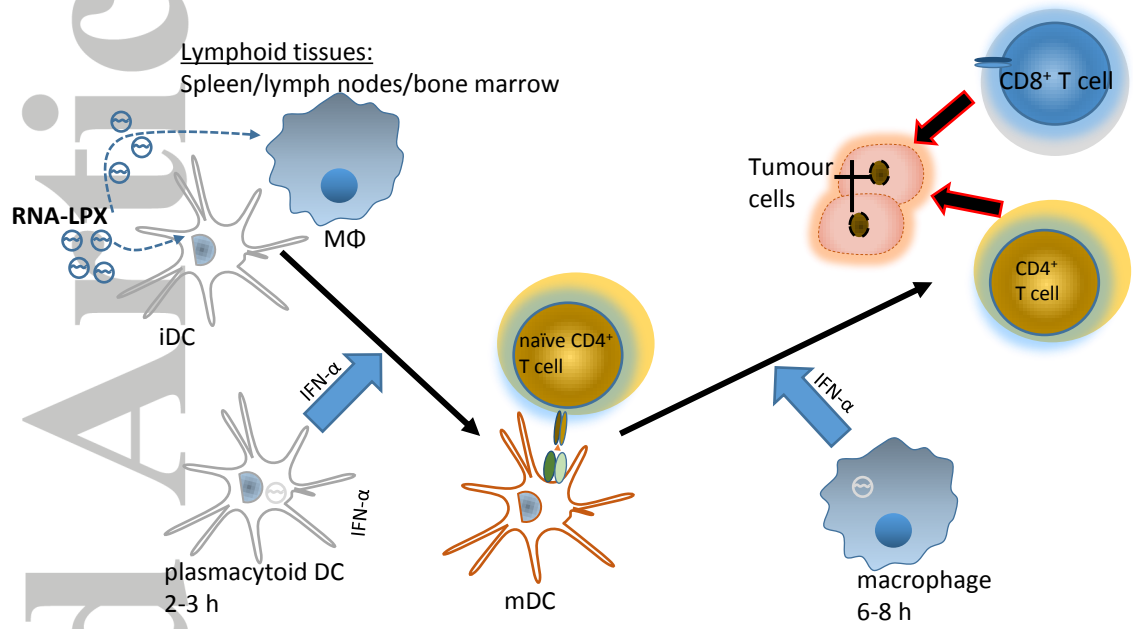
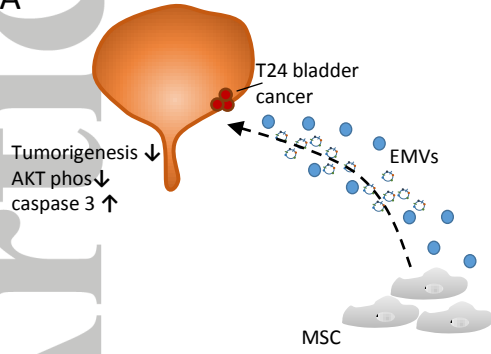
**RNA-LPX cancer vaccine**



Fig. 2

A



B

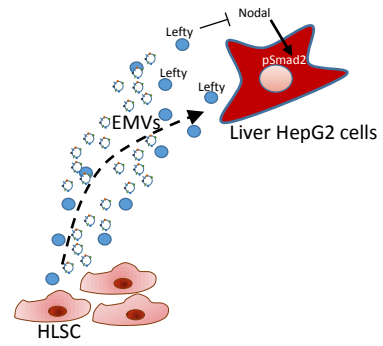


Fig. 3

