Breast cancer-derived exosomes reflect the cell-of-origin phenotype

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Abstract

A manner in which cells can communicate with each other is via secreted nanoparticles termed exosomes. These vesicles contain lipids, nucleic acids and proteins, and are said to reflect the cell-of-origin. However, for the exosomal protein content, there is limited evidence in the literature to verify this statement.

Here, we use proteomic assessment combined with pathway-enrichment analysis to demonstrate that the protein cargo of exosomes reflects the epithelial/mesenchymal phenotype of secreting breast cancer cells. Given that epithelial-mesenchymal plasticity is known to implicate various stages of cancer progression, our results suggest that breast cancer subtypes with distinct epithelial and mesenchymal phenotypes may be distinguished by directly assessing the protein content of exosomes. Additionally, our work is a substantial

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step towards verifying the statement that cell-derived exosomes reflect the phenotype of the cells-of-origin.

Statement of significance of the study

Breast cancer remains the leading cause of cancer-related death in women worldwide. Despite improved clinical management, the heterogeneity of the disease and plasticity of the tumour microenvironment present major challenges that impact patient survival. This study uses comprehensive proteomic assessment combined with gene set enrichment analysis to evaluate breast cancer cells and their secreted extracellular vesicles (exosomes). We found that the protein cargo in exosomes closely reflect that of originating breast cancer cells, and can inform on the epithelial or mesenchymal phenotype of the cancer cells. The phenotypic depolarisation of epithelial cells to elongated mesenchymal cells has profound impacts on all stages of cancer progression, in particularly with metastatic spread and resistance to current therapies. The findings of this study highlight the potential use of exosomes as surrogate biomarkers to understand phenotypic heterogeneity of breast cancer, permitting more targeted/personalised therapeutic approaches.

Introduction

Cell-derived nanovesicles (30-150 nm) originating from multi-vesicular bodies and late endosomes are termed exosomes ^[1]. The ability of these membrane-bound vesicles to package lipids, nucleic acids and proteins have implicated them as key mediators in cargo trafficking and intercellular communication ^[2]. All exosomes contain common core proteins that reflect its biogenesis such as tetraspanins (CD9, CD63 and CD81) and TSG101 ^[1]. Additionally, they contain numerous other proteins that are postulated to reflect the phenotypic and physiological state of the cell that secretes them. For this reason, exosomes are highly heterogeneous and may contain proteomic information that informs on the pathological processes of many diseases, including cancer. Indeed, several studies have reported the utility of assessing cancer-derived exosomes as biomarkers for the diagnosis and prognosis of cancer ^[3-5]. While exosomes hold great promise in the setting of cancer, few studies to date have directly compared the proteome signature of cancer exosomes to its

parent cell. Specifically, it remains unclear to what extent cancer exosomes reflect the roles of its parent cell in cancer initiation, progression and metastasis.

During cancer progression, epithelial cancer cells can display a wide array of phenotypes, some of which are caused by genetic or epigenetic alterations ^[6]. Some of these changes are due to oncogenic transformation, whereas others seem to be an adaptation to altered environmental cues ^[7-9]. Several genetic and environmental causes result in an adaptation of a mesenchymal-like phenotype ^[10]. This transition from an epithelial to mesenchymal stage, termed epithelial-to-mesenchymal transition (EMT), has profound impacts on all stages of cancer progression from initiation, growth and metastasis, to resistance to therapy ^[11]. Generally, mesenchymal-like cancer cells are reported to be migratory, less sensitive to chemotherapies, and show low proliferative ability ^[12-14]. The characteristics of these mesenchymal-like cancer cells are thought to be a common cause for metastatic spread ^[15]. In the setting of breast cancer, key studies previously demonstrated that induction and maintenance of EMT via transcription factors such as Twist and NF- κ B is essential for tumour metastasis in mouse model systems ^[16, 17]. These key transcription factors can work to repress E-cadherin expression, which is a central component in mediating stability of cell adhesion and polarity. Consequently, a loss of E-cadherin expression can increase breast cancer cell motility and invasiveness. We recently demonstrated that exosomes released by mesenchymal lung cancer cells can confer chemoresistance and mesenchymal traits to recipient epithelial lung cancer cells ^[13]. However, just how closely the protein content of breast cancer-derived exosomes reflects the epithelial and mesenchymal state of parent cancer cells remains inconclusive.

In this study, we present a proteomic evaluation of two commonly used C57BL/6-syngeneic murine breast cancer cell lines (E0771 and PyMT-WT) and its secreted exosomes. Gene set enrichment analysis of the cell proteome show distinct mesenchymal and epithelial signatures, in E0771 and PyMT-WT cell lines, respectively. Importantly, we demonstrate that the proteome of secreted exosomes is reflective of the mesenchymal (E0771-derived) and epithelial (PyMT-WT-derived) status of the cell. Together, this data demonstrates that the secretome of exosomes reflects the epithelial/mesenchymal phenotype of secreting cancer cells. Additionally, our study highlights the potential use of exosomes to understand phenotypic heterogeneity of breast cancer, permitting more targeted therapeutic approaches.

Materials and Methods

Cell culture

The murine C57Bl/6 breast cancer lines E0771 ^[18, 19] and PyMT-WT mammary carcinoma cell lines ^[20] were maintained and generated as previously described. In brief, cells were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% foetal bovine serum and 1% (w/v) penicillin/streptomycin in a humidified incubator at 37°C and 5% CO₂. Cells were confirmed to be negative for mycoplasma before being used for experimentation, and retested every three months thereafter.

Exosome isolation

Exosomes were purified from cell culture supernatants by ultrafiltration and size exclusion chromatography as previously described ^[21]. Briefly, cells were cultured to 70% confluency, then washed three times in phosphate buffered saline (PBS) and grown for an additional 24 hours in serum-free media. Conditioned cell media was harvested and centrifuged (300 x g; 10 minutes) to remove detached cells, followed by filtration using a 0.22-µm filter to remove contaminating apoptotic bodies and cell debris. The resulting cell-free medium was concentrated by ultrafiltration using the Centricon Plus-70 Centrifugal Filter (Ultracel-PL Membrane, 100 kDa, Merck Millipore) at 3500 x g, 4°C to an approximate volume of 500 µL. Exosomes were then purified from concentrated media by overlaying on qEV size exclusion columns (Izon Science, Ltd Christchurch, New Zealand), and further concentrated in Amicon® Ultra-4 (10 kDa nominal molecular weight; Merck Millipore) centrifugal filter units to a final volume of 200 µL. Cell numbers were quantified by counting (Countess II FL Automated Cell Counter, Thermo Fisher Scientific) at time of conditioned media collection.

Transmission electron microscopy

Exosomes were visualised using transmission electron microscopy as previously described ^[22]. Briefly, purified exosome preparations were fixed with paraformaldehyde and transferred to Formvar-carbon coated electron microscopy grids. Grids were transferred to 1% (v/v) glutaraldehyde, followed by water washes. For contrast, grids were negatively stained with 1% (w/v) uranyl-oxalate solution, pH 7 for 5 minutes before transferring to methyl-cellulose-UA (a mixture of 4% uranyl acetate and 2% methyl cellulose) for 10 minutes. Excess fluid

was removed and exosomes imaged with a JEOL 1011 transmission electron microscope at 60kV.

Western blotting

Equal amounts of protein from exosome preparations or total-cell lysates (quantified by standard Bradford Assay or BCA assay) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes using semi-dry transfer. Membranes were blocked in 5% non-fat powdered milk in PBS-T (0.5% Tween-20) and probed with primary antibodies: mouse mAb anti-flotillin-1 (Cat #610821; BD Biosciences, USA), goat pAb anti-TSG101 (Cat #sc-6037; Santa Cruz Biotechnology, USA), rabbit mAb anti-CD9 (Cat #ab92726; Abcam, UK), rat mAb anti-PDGF Receptor β (Cat # 14-1402-82; Thermo Fisher Scientific), rabbit mAb anti-claudin-3 (Cat #83609; Cell Signalling Technology), rabbit mAb anti-E-cadherin (Cat #3195; Cell Signalling Technology), rabbit mAb anti-vimentin (Cat #5741; Cell Signalling Technology), mouse mAb anti- α -tubulin (Cat #T9026; Sigma-Aldrich), and rabbit anti-GM130 (Cat #ab52649; Abcam, UK). HRP-conjugated goat, rabbit, rat and mouse secondary antibodies were used and the protein bands visualized using X-ray film and enhanced chemiluminescence reagent (Amersham ECL).

Tunable resistive pulse sensor (TRPS)

Particle concentration and size distribution were analysed by TRPS technology with the Izon qNano system (Izon Science, Ltd Christchurch, New Zealand). Particle quantitation was standardised with 70 nm carboxylated polystyrene calibration beads at a concentration of 1.5×10^{11} particles/mL (CPS70) and NP100 nanopores.

Protein analysis using Liquid Chromatography-Mass Spectrometry

Five independent exosome preparations and total-cell lysates were used for proteomic analysis using liquid chromatography-mass spectrometry. Samples were reduced/alkylated and digested as described ^[23]. Briefly, samples were reduced using 10 mM dithiothreitol in 2% SDS, 50 mM Tris.HCl pH 8.5 containing 1 x protease inhibitors (P8340, Sigma-Aldrich, MO, USA) and alkylated by the addition of iodoacetamide to 25 mM.

Exosome preparation

Samples were methanol co-precipitated with trypsin ^[23] at a ratio of 1:100 (trypsin:sample), resuspended in 40 mM ammonium bicarbonate 10% acetonitrile and digested for 2 hours at 37°C. Further trypsin (1:100) was added and the digest continued for a further 8 hours at 37°C. Samples were acidified with the addition of 1% TFA and 2 µg of sample analysed on a hybrid LTQ Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer with a nanospray flex source (Thermo Fisher Scientific, Bremen, Germany) and nanoAcquity nano-uHPLC (Waters, MA, USA). Peptides were injected on a 5µ Symmetry C18 180µm x 20 mm (Waters, MA, USA, PN-186006527) trap column at 15 µL/min for 3 minutes and separated on a 1.7µm BEH130 C18, 75µm x 200mm column (Waters, MA, USA, PN-186003544) using a gradient from 2% B (A-0.1% formic acid; B-Acetonitrile 0.1% formic acid) to 5% B @ 5 min, 30% B @ 140 min, 50% B @ 150 min, 95% B @ 152 min and hold for 6 minutes. Data was collected using CID as described in ^[24].

Whole cell lysate preparation

Samples were methanol precipitated, LysC/trypsin digested and analysed as per Dave *et al.* 2014 ^[25] (Protein Fractionation-free Workflow) except for the following changes. Data was acquired on a hybrid Orbitrap Velos-Pro instrument with the survey scan in the orbitrap at 60 000 resolution (m/z 400) and the top 15 ions were selected for fragmentation. Ion transfer capillary temperature 275°C and maximum injection time for MSMS was 70 ms.

Database processing and protein identification

Peak lists for the 20 .RAW files were generated using MaxQuant (^[26]; version 1.5.1.2) and were searched using Andromeda ^[27] against the reference proteome for Mouse (50821 canonical and isoform sequences, downloaded from www.uniprot.org on 27 August 2013). Reversed sequences and the MaxQuant contaminant database were also searched. The instrument type was set to Orbitrap and default precursor and fragment ion mass tolerances were applied (i.e., precursor mass tolerance of 20 ppm for the first search, 4.5 ppm for the main search and fragment ion mass tolerance of 0.5 Da). Enzyme specificity was set to trypsin/P and two missed cleavages were allowed. Carbamidomethyl cysteine was specified as a fixed modification and acetylation of the protein N-terminal, deamidation of asparagine/glutamine and oxidation of methionine were specified as variable modifications. A minimum peptide length of 7 was specified and identification of second peptides was

enabled. For identification, the peptide-spectrum match (PSM) and protein false discovery rates (FDRs) were set to 0.01. Default settings were applied for all other parameters.

Protein group identifications and PSM counts were extracted from the MaxQuant results file “evidence.txt”. The MaxQuant reported protein groups were further collapsed using a previously described procedure^[25] so that protein sequences arising from the same gene were reported as a single protein group. The total number of PSMs for each protein group was determined for each replicate, where PSMs matching more than one protein group were excluded. Protein groups reported as potential contaminants were removed. To ensure only confidently identified protein groups were retained for downstream analyses, the following filters were applied for each comparison: i) protein groups were identified by at least two peptides with distinct amino acid sequences and ii) protein groups were reliably observed (i.e., at least one PSM in a minimum of three out of five replicates of E0771 exosomes, E0771 total cell, PyMT-WT exosomes or PyMT-WT total cell).

Protein groups identified in both E0771 and PyMT-WT exosomes were visualised as a heat map (Figure 2B). The heat map was generated in R (version 2.15.1) using heatmap.2 from the gplots package (version 2.11.0). The PSM counts for each protein group were $\log_2$ transformed, scaled and centred to give a mean of zero and unit variance (z-score). Prior to the $\log_2$ transformation, a pseudocount of 1 was added to all PSM counts. Protein groups and replicates in Figure 2B were then clustered using hierarchical clustering with Euclidean distance and complete linkage.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA)^[28], was used to identify enriched pathways in exosomes and cells as previously described^[29]. Protein spectral counts for each cell line were analysed using the Molecular Signatures Database (MSigDB). Analysis was performed using custom gene sets of cellular proteins that were at least 2 fold different between E0771 and PyMT-WT cells, and the C2 curated gene sets database (version 6.1), Signal2Noise ranking metric, 1000 phenotype permutations, and a weighted enrichment statistic. Gene sets with a FDR of < 0.05 were considered significant.

Results

Exosomes were firstly isolated from the conditioned medium of two commonly used C57BL/6-syngeneic murine breast cancer cell lines, E0771 and PyMT-WT (Figure 1). Exosome preparations showed the typical double-layered spherical structure, as assessed by transmission electron microscopy (Figure 1A). Exosomes were also positive for known core protein markers CD9, Flotillin-1, TSG101, and negative for the cis-Golgi apparatus marker, GM130 (Figure 1B). Using TRPS, the size distribution of exosomes was confirmed to be at the expected diameter of 30 to 150 nm (Figure 1C). Quantification of exosomes secreted by both lines showed that the PyMT-WT line produces less exosomes per cell than the E0771 line (Figure 1D).

To determine if the proteomic content of isolated breast-cancer exosomes reflects its cell of origin, label-free quantitative proteomics analysis was performed. Equal amounts of protein from five independent exosome preparations and total-cell lysates from E0771 and PyMT-WT cell lines were analysed. MaxQuant analysis resulted in the acceptance of 620963 PSMs, which corresponded to 57712 peptide sequences (peptide identification FDR of 0.01) and 5883 protein groups (at least one peptide exclusively matched the protein group and protein identification FDR of 0.01).

Proteins identified in E0771 and PyMT-WT exosomes were firstly compared to investigate if breast cancer cells with differing metastatic potential would secrete altered exosome proteomes. It is known that the development of PyMT-WT primary mammary tumours in both spontaneous and transplanted syngeneic mouse models is slow, and metastasis generally limited to a small number of lung nodules ^[19, 20, 30]. In contrast, E0771 tumour growth is quicker (median doubling time of 5-6 days), and display a higher metastatic potential ^[19, 31]. While a majority of proteins were found to be common between E0771 and PyMT-WT exosomes (1609 proteins), 325 proteins were uniquely detected in E0771 and 613 proteins in PyMT-WT exosomes (Figure 2A). A heat map generated from counts of shared proteins between E0771 and PyMT-WT (1609) demonstrated a significantly different proteomic signature, and suggests breast cancer subtypes may be distinguished by assessing exosomes (Figure 2B).

Of particular interest, GSEA analysis of total cell proteins determined the EMT gene set to be significantly enriched in E0771 exosomes when compared to PyMT-WT exosomes (Figure 3A, B; Supplementary Table 1). Accordingly, western blot analysis also confirmed the reduction of epithelial marker E-cadherin, but conversely increased mesenchymal protein, PDGFR β in E0771 exosomes when compared to PyMT-WT exosomes (Figure 3C). EMT has been extensively implicated in promoting the initial stages of metastatic outgrowth^[11, 32], and one may speculate that a bias towards this pathway as an indicator of tumour metastatic potential. Furthermore, GSEA analysis showed that unlike E0771, PyMT-WT exosomes were significantly enriched in proteins involved in cell adhesion, and stem cell-like phenotype (Figure 3D; Supplementary Table 2). Consistent with GSEA results, PyMT-WT-derived exosomes demonstrated an increased expression of both claudin-3 tight junction protein and EpCAM, compared to E0771 exosomes as assessed by western blot (Figure 3C).

Next, we tested the hypothesis that exosomes are representative of the cancer cell from which they were derived. This concept is particularly important as the proteomic information contained in tumour-derived exosomes hold great promise as diagnostic and prognostic biomarkers. To investigate this concept, we evaluated if breast cancer-derived exosomes have a similar and comparable EMT gene set enrichment when compared with the cell of origin. Approximately 4000 proteins were identified in both E0771 and PyMT-WT whole cell lysates, of which a total of 267 proteins were found uniquely in E0771 cells (Figure 4A; Supplementary Table 3). Another 366 proteins were exclusively detected in PyMT-WT cells (Figure 4A; Supplementary Table 4). Similar to E0771-derived exosomes, GSEA analysis revealed the EMT pathway is indeed also up-regulated in E0771 cells (Figure 4B). Distinct mesenchymal and epithelial proteomic signatures were observed in E0771 and PyMT-WT cell lines, respectively (Figure 4C). Interestingly, most of the proteins found to be in greater abundance in E0771 exosomes were also enriched in the cells of origin (Figure 4C). Consistent with these observations, the epithelial cell marker E-cadherin was only detected in PyMT-WT cells while vimentin, which is present in mesenchymal cells, was expressed in E0771 cells (Figure 4D).

Furthermore, we then used GSEA analysis to confirm the observation that the protein profile in exosomes reflect that of originating cells. Cellular proteins with at least 2-fold increase in either E0771 or PyMT whole cell lysates were used to generate custom gene sets. The

exosome proteomes were then examined to see if they are also enriched in proteins from the custom gene sets. Indeed, GSEA analysis confirmed that the overall proteome of exosomes do reflect cell of origin (Figure 5). Additionally, proteins highly enriched ($\geq \log_2$ ratio threshold of >2) in E0771 and PyMT-WT exosomes compared to cell of origin were subjected to ontology analysis using Enrichr software. The top five biological process, molecular function and cellular component generated from analysis is listed in Supplementary Tables 5 and 6, respectively. Enriched proteins in exosomes compared to cell of origin may indicate specific packaging during exosome production. Taken together, our results suggest breast cancer subtypes with distinct epithelial and mesenchymal phenotypes may be distinguished by directly assessing the protein content of exosomes.

Discussion

Breast cancer remains the second highest cause of cancer mortality in women worldwide^[33]. It is a heterogeneous disease reflected by the various clinical and molecular classifications. A key contributor to this phenotypic heterogeneity is the initiation of epithelial-to-mesenchymal transition (EMT). EMT results in a more aggressive cancer phenotype through increased migration and invasion^[11, 15], as well as chemoresistance^[12-14]. EMT is a key process in which the dissemination of epithelial breast tumour cells intravasate into the circulation, home and extravasate at metastatic sites, and eventually form macrometastatic deposits. Current clinical management of breast cancer is directed by histopathology and molecular characteristics of primary breast tumours. However, expression of biomarkers for classification of breast tumours can be highly variable even within an individual tumour. This variability, along with small biopsies, often limits the understanding of the extensive heterogeneity present in a breast tumour. Moreover, histological and molecular subtyping using a small single biopsy is unlikely to contain a true reflection of the tumour heterogeneity, and epithelial-mesenchymal state.

Extensive research on understanding tumour heterogeneity in clinical practice has revealed that circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) in patient blood can be utilized as liquid biopsies to understand the phenotypic and genetic heterogeneity of breast cancer. Other components in the blood that can provide information about the primary tumour are secreted extracellular vesicles such as exosomes. Together with previously

published studies, our current findings provide further evidence that exosomes can provide information on the protein content of the cell of origin ^[28, 29]. Here, we demonstrate using proteomic assessment combined with GSEA that the proteomic content of exosomes can reveal the epithelial-mesenchymal phenotype of breast cancer cells. Specifically, we showed that the epithelial-mesenchymal protein enrichment profile of exosomes closely mimicked the mesenchymal phenotype of E0771 cells and the less metastatic luminal phenotype of PyMT-WT cells. Our conclusions mirror those from a recent study which demonstrated that when human squamous cell carcinoma (SCC) cells were stimulated to undergo EMT-like changes, secreted extracellular vesicles also exhibit a similar proteome reprogramming ^[34]. It is unclear from the current study whether exosomes enriched in mesenchymal proteins from more metastatic mesenchymal breast cancer cells are capable of inducing EMT in recipient epithelial cancer cells. Given that inducers of EMT, including TGF β ^[35], β -catenin ^[36] and Wnt-signalling molecules ^[37] are associated with exosomes, one may speculate they do have this ability. Supporting this idea, fibroblast-derived exosomes were previously shown to utilise Wnt-PCP signalling to stimulate breast cancer cell activity, motility and invasion ^[37]. Future mechanistic work to better understand the functional role of breast cancer-derived exosomes on EMT transformation is required. Nevertheless, studies have previously shown that cancer-derived exosomes have the ability to accelerate tumour progression by passing malignant properties that increase oncogenic potency ^[38, 39], induce pre-metastatic niche formation ^[31, 40] and reduce efficacy of cytotoxic drugs ^[41, 42]. For example, we previously demonstrated that exosomes secreted by highly metastatic breast cancer cells (4T1 and E0771 cells) can condition a favourable microenvironment that promotes metastatic colonization by reducing immune surveillance ^[31]. Similarly, pancreatic cancer exosomes can also initiate pre-metastatic niche formation in the liver by altering liver macrophages to subsequently increase liver metastatic burden ^[40]. Importantly, this study further highlights exosomes as key contributors to cancer progression and tumour heterogeneity.

While exosomes may reflect their cell of origin, they are also enriched in other specific proteins, lipids and nucleic acids compared with overall cellular levels ^[43-45], suggesting the presence of well-controlled systems that modulate exosome packaging. Indeed, although our study confirmed via GSEA analysis that proteins in E0771 and PyMT-WT exosomes do reflect cell of origin, specific sets of proteins are enriched in exosomes. For example, we noted that the MAPK signalling pathway appeared as a key biological process possibly

involved in E0771 exosome function; a pathway known to be important in initiation of TGF- β -induced EMT^[46, 47]. Various mechanisms have been implicated in the specific sorting of proteins into exosomes, including ESCRT (endosomal sorting complexes required for transport)-dependent and independent systems, tetraspanins and lipid-dependent mechanisms^[48, 49]. More recently, post-translational modifications, specifically ubiquitin and ubiquitin-like modifiers are postulated to alter exosomal protein activation and increase proteome diversity^[50]. Currently however, protein sorting mechanisms are only partially understood; thus warranting further investigation as exosome composition will ultimately determine the outcome of cell-cell communication. A better understanding of this process, together with profiling the proteomic content of exosomes offers an exciting avenue to ultimately allow for more precise treatment tailored to the phenotypic heterogeneity within a breast tumour.

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Figure Legends

Figure 1. Isolation and characterisation of breast cancer exosomes.

A) Morphological characterisation of exosomes isolated from the conditioned media of E0771 and PyMT-WT breast cancer cell lines by transmission electron microscopy. The scale bar indicates 100 nm. **B)** Detection of exosome marker proteins TSG101 (47 kDa), CD9 (25 kDa), Flotillin-1 (70 kDa) and golgi marker GM130 (112 kDa) in cell lysates (C) and exosomes (E) derived from E0771 and PyMT-WT cells by western blotting. **C)** Size distribution and quantification of E0771- and PyMT-WT-derived exosomes as measured by Tunable Resistive Pulse Sensing. **D)** Quantification of vesicles smaller than 100 nm per 10^6 cells secreted by both lines (n=3 independent repeats/line). Results are presented as mean $\pm$ s.e.m. and analysed by parametric t-test. A p-value<0.05 was considered statistically significant.

Figure 2. Comparison of proteins from E0771 and PyMT-WT-derived exosomes as identified by label-free mass spectrometry.

(A) Venn diagram depicts the distribution of common and unique proteins with a false-discovery rate of 1% in exosomes secreted by E0771 and PyMT-WT exosomes. (B) A heat map was generated from counts of proteins shared between E0771 and PyMT-WT secreted exosomes using generated in R (version 2.15.1) using heatmap.2 from the gplots package (version 2.11.0). Each row represents a protein and each column an independent preparation of the E0771 or PyMT-WT exosomes. The PSM counts for each protein group were log2 transformed, scaled and centered to give a mean of zero and unit variance (z-score). Prior to the log2 transformation, a pseudocount of 1 was added to all PSM counts. Protein groups and replicates were then clustered using hierarchical clustering with Euclidean distance and complete linkage.

A

B

Figure 3. Confirmation of epithelial to mesenchymal transition (EMT) enrichment in E0771-derived exosomes.

(A) Gene set enrichment analysis identified the EMT gene set as significant enriched in E0771 exosomes when compared to PyMT-WT exosomes. (B) Heat map generated from protein counts implicated in the EMT pathway. (C) Exosomes derived from E0771 and PyMT-WT cells were analysed for mesenchymal (PDGFR β), epithelial (E-cadherin), adherence (EpCAM), and tight junction (claudin-3) proteins. CD9 served as loading control. (D) Gene set enrichment analysis identified cell adhesion, and stem cell-like phenotype as significantly enriched gene sets in PyMT-WT exosomes compared to E0771 exosomes.

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Figure 4. Cancer cells express a similar and comparable EMT gene set enrichment compared with exosomes.

(A) Venn diagram depicts the distribution of common and unique proteins with a false-discovery rate of 1% in E0771 and PyMT-WT whole cell lysates. (B) Gene set enrichment analysis identified the EMT gene set as significant enriched in E0771 cells compared to PyMT-WT cells. (C) Heat map generated from protein counts implicated in the EMT pathway. (D) E0771 and PyMT-WT cells were analysed for epithelial (E-cadherin) and mesenchymal (vimentin) proteins. α -tubulin served as loading control.

A

B

C

D

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Figure 5. Proteins in exosomes reflect the cancer cell of origin.

Gene set enrichment analysis identified that proteins enriched in the cell of origin are also enriched in (A) E0771 and (B) PyMT-WT exosomes. For this analysis, cellular proteins with at least 2-fold increase in either E0771 or PyMT-WT whole cell lysates were used to generate custom gene sets. The exosome proteomes were then examined to assess if they are also enriched in proteins from either cell line.

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