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Proteomic profiling reveals key cancer progression modulators in shed microvesicles released from isogenic human primary and metastatic colorectal cancer cell lines

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Abbreviations

CRC, colorectal cancer; EVs, extracellular vesicles; Exos, exosomes; sMVs, shed microvesicles; SW480-sMVs; sMVs derived from SW480 cells; SW620-sMVs, sMVs derived from SW620 cells; LFQ, Label-free quantitation; CM, culture medium; MS, mass spectrometry; GeLC-MS/MS, SDS-gel liquid chromatography tandem mass spectrometry

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Abstract

Extracellular vesicles can be classified into two main classes - exosomes and shed microvesicles (sMVs). Whilst much is known about exosome cargo and functionality, sMVs are poorly understood. Here, we describe the large-scale purification of sMVs released from primary (SW480) and metastatic (SW620) human isogenic colorectal cancer (CRC) cell lines using a combination of differential ultracentrifugation and isopycnic iodixanol density centrifugation. The yield of SW480-sMVs and SW620-sMVs was 0.75 mg and 0.80 mg, respectively. Both SW480-/SW620-sMVs are heterogeneous in size (100-600 nm diameter) and exhibit identical buoyant densities (1.10 g/mL). In contrast to exosomes, sMVs are ALIX$^-$, TSG101$^-$, CD63$^-$ and CD9$^-$. Quantitative mass spectrometry identified 1295 and 1300 proteins in SW480-sMVs and SW620-sMVs, respectively. Gene Ontology enrichment analysis identified 'cell adhesion' (CDH1, OCLN, CTN families), 'signalling pathway' (KRAS, NRAS, MAPK1, MAP2K1), and 'translation/RNA related' processes (EIF, RPL, HNRNP families) in both sMV types. Strikingly, SW480- and SW620-sMVs exhibit distinct protein signatures - SW480-sMVs enriched in ITGA/B, ANXA1, CLDN7, CD44 and EGFR/NOTCH signalling networks, while SW620-sMVs are enriched in PRKCA, MACC1, and FGFR4/MTOR/ MARCKS signalling networks. Both SW480- and SW620-sMVs are taken up by NIH3T3 fibroblasts, demonstrating similar cell invasion capability. This study provides, for the first time, molecular insights into sMVs and CRC biology.

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1. Introduction

Extracellular vesicles (EVs) are heterogenous populations of lipid bilayer-membrane vesicles derived from various cell types including cancer cells [1]. EVs have been identified as crucial mediators for intercellular communication by transferring their bioactive cargo such as DNA, RNA species, oncoproteins, and lipids to a multitude of recipient cells [2-4]. EVs can be detected in bodily fluids such as blood, bile, malignant effusions and urine. Accumulating evidence shows that cells release at two main EV classes exosomes (Exos) and shed microvesicles (sMVs, also referred to as microparticles and microvesicles), and that each EV class contains subtypes [1]. Exosomes and sMVs differ in their size range and mechanism of biogenesis. Exosomes are relatively homogenous with respect size (30 to 150 nm diameter) and are formed by inward invagination of late endosomes/multivesicular bodies. On the other hand, sMVs are more heterogeneous in size (50 to ~2000 nm diameter) and originate from outward budding of the plasma membrane [1]. While the molecular cargo and functionality of exosomes has been studied extensively, our knowledge of sMVs is still in its infancy.

Previously, we reported a comparative proteome analysis of exosomes derived from the isogenic human colorectal cancer cell lines SW480 (from a primary colorectal cancer tumour) and SW620 (lymph node-metastatic colorectal cancer tumour) [5]. Here, we describe the large-scale purification of sMVs from SW480 and SW620 cell culture medium using a combination of differential ultracentrifugation and isopycnic iodixanol density centrifugation. Label-free quantitative mass spectrometry [6] was used to compare the protein profiles of SW480- and SW620-derived sMVs. Our findings reveal that SW480-sMVs are enriched in ITGA/B,

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ANXA1, CLDN7, CD44 as well as the NOTCH and EGFR signalling networks, whereas SW620-sMVs are enriched in PRKCA, MACC1, and FGFR4 as well as the MTOR and MARCKS signalling networks. Because SW480-sMVs and SW620-sMVs display distinct protein profiles and signalling networks, our study suggests an important role of sMVs in cancer progression.

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2. Materials and methods

2.1. Materials

SW480 cells were from Ludwig Institute for Cancer Research Ltd. (Parkville Branch, Melbourne) and SW620 cells were from Dr E. Vincan (Peter MacCallum Cancer Centre, Australia). All media and supplements were from Life Technologies (NY, USA). OptiPrep™ was from Axis-Shield PoC (Norway). CELLine AD-1000 Bioreactor classic flasks were from Integra Biosciences. Mouse anti-Alix, anti-CD44, rabbit anti-MET, anti-GAPDH were from Cell Signaling, Sigma-Aldrich (MA, USA), Mouse, anti-EGFR, anti-CD9, anti-CD63, rabbit anti-MET, anti-PAK1, anti-CLDN1, anti-ANXA1 and goat-anti-CLDN7 were from Santa Cruz Biotechnology (CA, USA) and Mouse anti-TSG101 was from BD Transduction Laboratories (NJ, USA).

2.2. Cell culture

Initially, SW480 and SW620 cells were cultured in 75 cm^3 flasks with RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 atmosphere. For cell lysate isolation, SW480 and SW620 cells (3 x 10^5 cells) were cultured in a 15-cm cultured plate with RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 atmosphere for 48 h.

2.3. Cell proliferation assay

SW480 and SW620 cells (5 x 10^3 cells) were seeded in 96-well plate in 100 µL RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin and then incubated for 24, 72, and 120 h, respectively. Following, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added to a final concentration of 50 µg/mL and incubated at 37°C for 4

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h. The CM was removed and further replaced with 200 µL of acidified isopropanol. The 96-well plate was shaken for 30 min and then measured absorbance at 560/690 nm using SpectraMax M5^{e} (Molecular Devices, CA, USA).

2.4. Invasion assay

50 µL growth factor reduced Matrigel™ matrix (Corning, NY, USA) (10 mg/mL) was mixed with 50 µL cell suspension which contains 20% FBS, 2% penicillin/streptomycin, and 300 SW480 or SW620 cells and incubated at 37°C and 5% CO_2 atmosphere for 2 h. Following, 100 µL 10% FBS-containing medium was added into a 96-well plate and further incubated for 120 h. Cells were observed and imaged using Zeiss AxioObserver Z1 microscope.

2.5. Purification of SW480-sMVs and SW620-sMVs

SW480 and SW620 cells (3 x 10^7 cell) were transferred into the cultivation chamber of CELLine AD-1000 Bioreactor classic flasks (Integra Biosciences) and cultured with RPMI-1640 medium supplemented with 0.6% Insulin-Transferrin-Selenium (ITS) and 1% penicillin/streptomycin at 37°C and 5% CO_2 atmosphere as described [5]. Growth medium in the upper chamber of CELLine AD-1000 Bioreactor classic flasks was replaced every 5 days. Cells in the Cultivation chamber were allowed to attach for 48 h. Then cell suspension was harvested every 24 h. The culture medium (CM) of SW480 and SW620 cells was centrifuged at 500 x g for 5 min (4 °C) and 2,000 x *g* for 10 min (4°C) and stored at -20°C. SW480- and SW620-derived CM (~30 mL/tube/cell line) was divided into 3 biological replicates (~180 mL/biological replicate/cell line). CM was centrifuged at 10,000 x *g* for 30 min to pellet crude sMVs. Both sMV pellets were resuspended with 600 µL of filtered PBS (0.2 µm). The supernatant was further centrifuged at 100,000 x *g* for 1 h to pellet crude exosomes and resuspended with 600 µL of PBS. Approximately 550 mL of crude sMVs and exosome pellet

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was subjected on top of prepared OptiPrep™ density gradient, and separation performed as previously described [5, 7]. Briefly, an OptiPrep™ (iodixanol solution) was prepared by adding 3 mL of 40, 20, 10, and 5% of iodixanol solution to 14 × 89 mm polyallomer tubes (Beckman Coulter). Dilutions were made in 0.25 M sucrose/1 M Tris (pH 7.5) solution. These tubes were topped with a layer of 2.5 mL of 40, 20, 10, 5% iodixanol solution, respectively, and centrifuged at 100,000 x *g* for 18 h at 4°C. Twelve fractions (1 mL for each fraction) were removed from the top. Twelve fractions of sMVs and Exos were washed with PBS by centrifugation at 10,000 x *g* for 30 min (4°C) for sMVs and 100,000 x *g* for 1 h (4°C) for Exos. PBS was removed and pellets were resuspended with 150 µL PBS.

2.6. Protein quantification and Western blotting

The protein content of sMV, and Exo preparations was estimated by 1D-SDS-PAGE / SYPRO® Ruby protein staining densitometry as described [8, 9]. Briefly, 5 µL sample aliquots were solubilised in SDS sample buffer (2% (w/v)) sodium dodecyl sulfate, 125 mM Tris-HCl, pH 6.8, 12.5% (v/v) glycerol, 0.02% (w/v) bromophenol blue) with 100 mM Dithiothreitol (DTT) and loaded into 1 mm, 10-well NuPAGE™ 4-12% (w/v) Bis-Tris Precast gels (Life Technologies). Electrophoresis was performed at 150 V for 45 min in NuPAGE™ 1x MES running buffer (Life Technologies) using an XCell Surelock™ gel tank (Life Technologies). After electrophoresis, gels were removed from the tank and fixed in 50 mL fixing solution (40% (v/v) methanol, 10% (v/v) acetic acid in water) for 30 min on an orbital shaker and stained with SYPRO® Ruby (Life Technologies, NY, USA) for 30 min, followed by destaining twice in 50 mL of 10% (v/v) methanol with 6% (v/v) acetic acid in water for 1 h. Gels were imaged on a Typhoon 9410 variable mode imager (Molecular Dynamics, Sunnyvale, USA), using a green (532 nm) excitation laser and a 610BP30 emission filter at 100 µm resolution. Densitometry quantitation was performed using ImageQuant software (Molecular Dynamics)

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to determine protein concentration relative to a BenchMark™ Protein Ladder standard of known protein concentration (1.7 µg/µL) (Life Technologies).

For western blotting, after electrophoresis proteins were electrotransferred onto nitrocellulose membranes using the iBlot™ 2.0 Dry Blotting System (Life Technologies). The membranes were probed with primary antibodies according to manufacturer's instructions. The secondary antibodies (IRDye 800 goat anti-mouse IgG or IRDye 700 goat anti-rabbit IgG) were diluted (1:15,000) and the fluorescent signals were detected using the Odyssey Infrared Imaging System, v3.0 (Li-COR Biosciences, Nebraska USA).

2.7. Transmission electron microscopy (TEM)

sMVs and Exos samples (1 µg in 10 µL PBS) were applied to 400 mesh carbon-coated copper grids for 2 min. Excess material was removed by blotting and samples were negatively stained with 10 µL of a 2% uranyl acetate solution for 10 min (ProSciTech, Queensland, Australia). The grids were air dried and viewed using a JEOL JEM-2010 transmission electron microscope operated at 80 kV [5].

2.8 Nanoparticle tracking analysis (NTA)

EV diameter (size) and concentration was determined by NanoSight NS300 system (NanoSight technology, Malvern, UK) equipped with a blue laser (488 nm). Briefly, sMVs and Exos were diluted in PBS (~8×10^8 particles/ml) and loaded into a flow-cell top plate using a syringe pump. Three separate technical replicates (60 sec/video) were recorded for each sample and analysed by NTA software (Build 3.1.45) [10].

2.9. GeLC-MS/MS

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sMV and cell lysate samples (15 μg) were lysed in SDS sample buffer, and proteins separated by short-range SDS-PAGE (10 x 6 mm), and visualized by Imperial Protein Stain (Invitrogen) as described [11]. The samples were excised into equal fractions (n=2), reduced with 2 mM tri(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich, C4706) at 22°C for 4 h on gentle rotation, alkylated by treatment with 25 mM iodoacetamide (Sigma-Aldrich) for 30 min, and digested with 1 μg bovine sequencing grade trypsin (Promega, V5111) at 37°C for 18 h. Subsequently, peptides were purified and extracted using reverse-phase C18 StageTips (Sep-Park cartridges, Waters, MA) in 85% (v/v) acetonitrile (ACN) in 0.5% (v/v) formic acid (FA). Peptides were lyophilised and acidified with buffer containing 0.1% FA, 2% ACN.

Proteomic experiments were performed in biological triplicate, with technical replicates (n=2), with MIAPE-compliance [12, 13]. A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to an Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded (Acclaim PepMap100, 5 mm × 300 μm i.d., μ-Precolumn packed with 5 μm C18 beads, Thermo Fisher Scientific) and separated (BioSphere C18 1.9 μm 120Å, 360/75 μm × 400 mm, NanoSeparations) with a 120-min gradient from 2-100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) (2–100% 0.1% FA in acetonitrile (2–40% from 0–100 mins, 40–80% from 100–110 mins at a flow rate of 250 nL/min operated at 55°C.

The mass spectrometer was operated in data-dependent mode where the top 10 most abundant precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 60,000 with MS/MS resolution of 15,000. Unassigned precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 1.4 Th and selected precursors fragmented by

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high-energy collision dissociation (HCD) with normalized collision energies of 25 with a maximum ion injection time of 110 msec. Ion target values were set to 3e6 and 1e5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 30 sec. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific).

2.10. Label-free mass spectrometry protein identification

Raw data were pre-processed as described [14] and processed using MaxQuant [15] (v1.6.0.1) with Andromeda (v1.5.6), using a Human-only (UniProt #133,798 entries) sequence database (May-2017). Data were searched as described [13] with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 6, with false discovery rate 1% at the peptide and protein levels, tryptic digestion with up to two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation and protein N-terminal acetylation as variable modifications, and data analyzed with label-free quantitation (LFQ) [16].

2.11. Data analysis

LFQ intensity values were normalized by protein length and fold change ratios calculated. Contaminants, and reverse identification were excluded from further data analysis. Resulting p-values were adjusted by the Benjamini-Hochberg multi-test adjustment method for a high number of comparisons [17] and statistics performed as previously described [18]. Differentially expressed proteins were identified using the criteria: ratio ≤ 2.0 (SW480), ≥ 2.0 (SW620) with $p \leq 0.05$, with identifications in at least 2 biological sample replicates.

2.12. Data visualisation

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Visualisation and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources (https://david.ncifcrf.gov/) were utilised using recommended analytical parameters [19]. DAVID and UniProt (www.uniprot.org) database resources (biological process, cellular components, and molecular function) were used for gene ontology enrichment and network analysis, respectively. Interaction mapping and protein association pathways was performed using STRING (https://string-db.org/) [20]. Venn diagrams were created from a facility from Bioinformatics & Evolutionary genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/). Heatmaps were generated using "gplots" and "RColorBrewer" packages and run in R programming language (R version 3.5.1, R: A language and environment for statistical computing, Vienna, Austria, https://www.r-project.org/). Volcano plots were generated using "mavolcanoplot" function and run in MATLAB programming language (MATLAB R2018a, MathWorks, NSW, Australia, https://au.mathworks.com).

2.13. Labelling of exosomes and uptake assay

Purified sMVs (~200 µg) in 200 µL PBS were labelled with fluorescent dye, DiO (Invitrogen) at 1 µM concentration for 15 min at room temperature, as previously described [21]. The labelled sMVs were then collected at 10 000 x *g* (30 min) and subjected to isopycnic (iodixnol-density) ultracentrifugation. NIH3T3 fibroblasts were grown on glass cover-slips (70% confluency) in 24-well plate. Cells were incubated with DiO-labelled sMVs (5 µg) at 37 °C for 2 h. Cells were then washed with PBS and subjected to imaging using fluorescent microscope Zeiss AxioObserver Z1 (Zeiss). Nuclei were stained with Hoechst stain (10 µg/mL, 30 min) prior to imaging.

2.14. Transwell-Matrigel^{TM} invasion assay

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Transwell-Matrigel™ invasion assays were performed using growth factor-reduced Matrigel™ matrix (Corning), as previously described [22]. The Transwell inserts with 8 µm pore size (Corning) were coated with Matrigel™ (100 µL of 1 mg/mL) and allowed to polymerize for 4 h (37 °C). NIH3T3 fibrobalsts (50 000 cells) in DMEM (1% Pen/Strep) were stimulated with SW480- or SW620-sMVs (30 µg/mL) or PBS alone for 2 h (37 °C). and subsequently overlaid onto Matrigel™-coated inserts. The inserts were nested onto a 24-well plate companion plate (Corning) that contained DMEM (5% FCS, 1% Pen/Strep) further supplemented with either sMVs (30 µg/mL) or PBS alone. After incubation for 16 h at 37 °C, inserts were washed, cells were fixed (4% (v/v) formaldehyde, 5 min), and the nuclei were stained with Hoechst stain (10 µg/mL). Non-invading cells were removed using cotton swab. Fibroblasts that invaded were imaged using Zeiss AxioObserver Z1 microscope.

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3. Results and discussion

3.1 SW480 and SW620 cell line characterisation

To confirm the growth and oncogenic characteristics of primary adenocarcinoma-derived SW480 cell line and metastatic tumour-derived SW620 cell line, functional assays were performed to assess cell proliferation, and invasive capabilities. In accordance with previous reports [5, 23] our data showed that SW620 cells, when compared with SW480 cells, displayed fibroblast-like morphology and higher proliferative and invasive capacities (Supplemental Fig S1A-C). In agreement with the phenotypic changes, SW620 cells were shown to have diminished expression of EGFR and CD44, and elevated expression of oncogenic MET (Supplementary Fig S1D).

3.2 Isolation and characterisation of SW480- and SW620-derived sMVs

To understand the protein composition of sMVs, we purified sMVs from SW480 and SW620 cells. A continuous cell culture approach was used CELLine AD-1000 Bioreactor classic flasks to generate ~180 mL SW480 and SW620 CM, as described [5]. The workflow used to isolate sMVs from SW480 and SW620 CM using differential centrifugation and isopycnic density (iodixanol) fractionation is outlined in Fig 1A. Briefly, CM was harvested, and differential centrifugation performed to discard floating cells (500 x *g*) and cellular debris (2000 x *g*). For sMVs the resultant supernatant was then subjected to 10,000 x *g* centrifugation. The pellet was resuspended and subsequently fractionated based upon their buoyant density into 12 fractions using iodixanol density gradient centrifugation (100,000 x *g*) [9], as outlined in Fig 1A. SYPRO©-ruby based protein densitometry/quantitation analysis of these fractions for sMVs (as described [5]) revealed that fraction 7 (buoyant density 1.10 g/mL) contained the highest protein yield (Fig. 1A, Supplementary Fig S2). For the purpose of comparative analysis, we

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also purified Exos from 10,000 x *g* supernatant using a combination of ultracentrifugation and iodixanol density gradient centrifugation (100,000 x *g*) (Fig 1A). The yield of purified SW480-sMVs and SW620-sMVs from 180 mL of CM was 0.75 mg and 0.83 mg, respectively. Western blot analysis showed that sMVs were Alix$^-$, TSG101$^-$, CD9$^-$, and CD63$^-$ [5, 7] (Fig 1B). We investigated the morphology and size distribution of purified sMVs using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) (Fig 1C-D). TEM revealed sMVs displayed round-like membranous vesicle structures 100-400 nm in size, and NTA showed particle diameters of 170-620 nm (mean 334-350 nm) which is in accordance with the typical size reported for sMVs [1, 7]. In comparison, exosomes displayed smaller size distribution range (mean 183-186 nm). Thus, sMVs released by SW480 and SW620 cells are biophysically distinct from exosomes.

3.3 Proteome analysis of SW480- and SW620-derived shed microvesicles

Label-free quantitative mass spectrometry identified [24] 1295 and 1300 proteins in SW480-sMVs (Supplementary Table S1) and SW620-sMVs (Supplementary Table S2), respectively. Inspection of these data revealed 1123 proteins are common to both datasets. Of these, 834 exhibited similar abundances ($\geq$-2 and $\leq$2 LFQ ratio) in both SW480- and SW620-sMVs (Fig 2A) (Supplementary Table S3). Common proteins include key proteins involved in sMVs formation (ARF1/6, BSG, ARRDC1 [25, 26], HSP90AA1/B1 [27], RAB22A [28], RHOA [29], and CSE1L [30]) and lipid-raft generation (ATP11C [31], TMEM30A [32], and PLSCR3 [33, 34]) (Supplementary Table S4). GO analysis revealed that the 834 common proteins are related to the following processes -"cell adhesion/adhesion molecules" (PKP2/3, CLDND1, OCLN, CTN families), "signal transduction" (KRAS, NRAS, RHOA, MAPK1/2K1), RNA binding proteins (e.g., ribonuclear proteins HNRNPK/Q/E2, YBX-1), "translation-associated proteins" (ribosomal proteins, translation initiation factors, aminoacyl tRNA ligases), and

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"transport vesicle" (VPS29/35, SNAP23) (Fig 2B,C) (Supplementary Table S4,S5). In addition, selective enrichment of proteins associated with the cytoplasm, membrane cytoskeleton, and cell surface (e.g., ARF1/4/6, ACTN1/4, and HLA-A) was common to both SW480- and SW620-sMVs (Fig 2C) (Supplemental Table S6). Interestingly, 359 of the shared 834 proteins have not been identified in exosomes derived from SW480 and SW620 cells that we previously reported, indicating that sMVs and exosomes have distinct protein profiles [5] (Supplementary Table S3).

3.4 Proteins selectively enriched in SW480-sMVs and SW620-sMVs

Inspection of the Venn diagram in Fig. 2A reveals 307 proteins in SW480-sMVs are significantly enriched (≥-2 and ≤2 LFQ ratio, $p<0.05$) and 172/307 are unique to these vesicles (i.e., not seen in SW620-sMVs). In the case of SW620-MVs, 291 proteins are significantly enriched and 177 of these are unique (not seen in SW480-sMVs). Prominent amongst the 307 proteins enriched in SW480-sMVs are regulatory components associated with signal transduction/basement membrane (e.g., integrins ANXA1, CLDN7) [35-40], cell proliferation (EGFR [41]), pro-tumorigenic signalling (CD44 [42]), angiogenesis and vascular remodelling (e.g., NOTCH1/2 [43]) (Fig 3A, Table 1). In the case of the 291 proteins enriched in SW620-sMVs most prominent were mediators of cell invasion (e.g., FGFR4 [44, 45]), metastasis (e.g., MACC1 [46]), cytoskeleton organisation/polarity (e.g., mTORC2, PRKCA [47, 48]), and signal transduction (e.g., PAK1, CLDN1, RASA1, ARHGAP1 [49-51]). Western blot analysis confirmed the differential enrichment in SW480-/SW620-sMVs of CLDN7, CLDN1, EGFR, CD44, ANAX1, and PAK1 (Fig 3B).

Signalling pathways are play a critical role in cancer such as cell proliferation, invasion/migration, angiogenesis, and metastasis [52]. Intriguingly, we found differing signalling protein networks enriched in SW480- and SW620-sMVs. For example, SW620-

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sMVs, but not SW480-sMVs, contain MTOR signalling protein network proteins (Fig 3C). MTOR is a master signalling complex that promotes cancer proliferation, differentiation, metabolism, invasion, and migration [47, 53]). mTORC2 (MTOR and RICTOR) (Supplementary Table S7) and proteins in MTOR-down-stream cascade such as PRKCA, kinases, and GTPases that promote actin organisation involved in cell invasion and migration [47, 54] were all enriched in SW620-sMVs. Importantly, we found a protein-protein network of MARCKS and PKACA in SW620-sMVs (Fig 3D). MARCKS is involved in actin cytoskeletal remodelling, cell proliferation and motility [55, 56]. Phosphorylation of MARCKS by protein kinase C releases MARCKS from plasma membrane and promotes PI3K/AKT signaling pathways [57]. In colorectal cancer, MARCKS positively regulates metastatic phenotype *in vitro* and *in vivo* [58]. The presence of these signalling networks in SW620-sMVs suggests that these vesicles may play a prominent role in signal transduction and cancer invasion/migration.

We also identified protein signalling networks in SW480-sMVs, but not SW620-sMVs. For example, SW480-sMVs contain Notch signalling pathway proteins such as NOTCH1/2 receptors which regulate endothelial cell proliferation and migration, resulting in angiogenesis [43, 59]. ADAM17 (positive angiogenic modulator that inhibita expression of anti-angiogenic factor TSP1 [60, 61]) and PSEN1 proteases that activate Notch signalling by releasing extracellular and intracellular domains of Notch receptor were also prominent in SW480-sMVs (Fig 3E) [43, 59]. Interestingly, activation of Notch directly promotes artery formation without Dll4-ligand activation, indicating effectiveness of the activated Notch receptor [62]. Importantly, Notch receptor and ITCH (E3 ligase) have been shown to be delivered to recipient cells via microvesicles, resulting in increasing NOTCH-specific gene expression (HES1/5 genes) [63] (Fig 3E). SW480-sMVs also contain EGFR (an important signalling molecule in cell proliferation, angiogenesis [64-66]) that that has been shown to associate with EPCAM

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[67], ANXA1 [68], ADAM17 [69] (Fig 3F). Inhibition of EGFR signalling causes decrease of EPCAM intracellular domain shedding, leading to attenuation of colorectal cancer progression [70]. Interestingly, EPCAM subunits such as CLDN7, CD44 were also significantly enriched in SW480-sMVs. β-catenin that binds to the intracellular domain of EPCAM to modulate Wnt signalling [71, 72] was also present in SW480-sMVs (Supplementary Table S8). Collectively, these data suggest a possible role of SW480-sMVs in cell proliferation and angiogenesis.

3.5 Identification of pre-metastatic niche factors in SW480-sMVs and SW620-sMVs

Establishment of a pre-metastatic niche is required for a survival and outgrowth of metastatic cancer cells in distinct sites [73]. Several secreted factors released from stromal and tumour cells such as soluble proteins and cargo within extracellular vesicles enable the pre-metastatic niche formation and dictate a fate of metastasised cancer cells [74]. Interestingly, interrogation of SW480-/SW620- sMVs protein profiles identified several proteins associated with pre-metastatic niche formation – these include MARCKS, S100A4, MIF, ADAM10, ANXA1, CD44, TNFRSF1B, ITGA1, and MMP14 (Table 2). Of these, ADAM10 and MIF have been reported elsewhere to be delivered by exosomes [75, 76]

ADAM10 (a disintegrin and metalloprotease) catalyses shedding of Eph [77], EGF ligands [78], and extracellular domain of Notch receptor [79, 80] to activate cell signalling pathways. ADAM10 has been shown to be delivered to from TIMP-knockout fibroblasts to breast cancer cells via exosomes and activate Notch and RhoA signalling pathways, resulting in breast cancer motility [75]. MIF or MMIF (Macrophage migration inhibitory factor) is a multifactional cytokine produced from activated T lymphocyte [81]. It plays an important role in immune responses and a production of several cytokines related to inflammation such as IL-1β/6 and TNF-α [82, 83]. An interesting study revealed that exosomal MIF derived from metastatic pancreatic ductal adenocarcinomas (PDACs) induces recruitment of bone marrow-derived

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macrophages to provide a suitable environment for PDAC metastasis [76]. Although it is evident that these key modulators identified in sMVs derived from primary and metastasis colorectal cancer cells can mediate pre-metastatic niche, functional studies both *in vitro* and *in vivo* are necessary in order to explain the role of sMVs in the tumour microenvironment.

3.6 Colon cancer biomarker identified in SW480- / SW620-sMVs

EVs are receiving much attention as possible targets for cancer diagnostics. Accumulating evidence shows that EVs and their bioactive cargo molecules, found in several bodily fluids such as blood, urine, and saliva, correlate with disease progression [84]. Although several studies indicated that exosomal DNA, RNA, and protein cargo can be serve as possible cancer biomarkers, very little is known about sMV cargo as potential cancer diagnostics. In our study, we identified several known CRC marker proteins found in sMVs. Interestingly, we identified CNN3, a marker for lymph node-metastatic colon cancer which is highly enriched in lymph node-metastatic colon cancer cell compared with primary colon cancer cell [85]. We also identified the well-known CRC markers such as CEACAM1 [86, 87] and MUC13 [88] in both SW480-/SW620-sMVs (see Table 3).

3.7 SW480-and SW620-sMVs induce invasion of fibroblasts *in vitro*

Because sMVs contain proteins implicated in signal transduction [63, 89], we next questioned whether sMVs can be taken up by recipient cells and elicit a functional response (Fig 4). To test this, we labelled SW480- and SW620-sMVs with lipophilic dye DiO and subjected the labelled sMVs (DiO) to OptiPrep^{TM}-density gradient centrifugation to remove unbound dye. We incubated NIH3T3 fibroblasts with sMVs (DiO) for 2 h. The cells were then washed and stained with DiI for their plasma membrane and Hoechst stain for their nucleus. Cells were then imaged cells using live fluorescent microscopy (Fig 4A). Compared to control NIH3T3

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fibroblasts treated with PBS vehicle alone, fibroblasts incubated with SW480- or SW620-sMVs (DiO) displayed extensive uptake of sMVs (DiO, green) as distinct puncta (Fig 4A, insets). This shows that both SW480- and SW620-sMVs were actively taken up by fibroblasts. Next, we investigated whether sMVs can elicit a functional response in fibroblasts using Transwell Matrigel^{TM} invasion assay (Fig 4B). NIH3T3 fibroblast were stimulated with SW480- or SW620-sMVs (30 µg/mL) or PBS vehicle alone for 2 h and overlaid onto Transwell-insert (pore size 0.8 µm) coated with Matrigel^{TM} matrix. The cells were incubated for 18 h to allow invasion to occur. Cells in the upper chamber were removed and the cells that had invaded to the lower side of the inset membrane were fixed and stained with Hoechst stain for their nucleus. Fluorescent microscopy revealed that compared to PBS vehicle treatment, stimulation of fibroblasts with SW480- or SW620-sMVs resulted in significantly greater levels of fibroblasts invasion across Matrigel^{TM} matrix. Although SW620-sMVs promoted greater level of fibroblasts invasion across the matrix compared to SW480-sMVs, the difference did not reach statistical significance. These data show that sMVs from CRC cells can be taken up by a recipient fibroblast cells and elicit a functional response.

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4 Concluding remarks

In this study, we developed a large-scale purification method for obtaining mg quantities of highly-purified sMVs secreted from the human isogenic colorectal cancer cell lines SW480 and SW620 to enable biochemical characterisation and functional studies. Label-free quantitative mass spectrometry was used to obtain protein profiles for SW480- and SW620-sMVs. A striking finding was that SW480- and SW620-sMVs have distinct protein signatures that distinguish one sMV from another as well as exosomes derived from the same parental cells. Gene ontology analysis revealed that SW480-/SW620-sMV proteins categorised in biological processes such 'cell adhesion', 'RNA-related', and 'signalling proteins' correlated with known cancer progression biology. Key cancer progression proteins such as MTOR, PRKCA, MACC1, MARCKS and FGFR4 were enriched in SW620-sMV (metastatic cancer cell origin). In contrast, primary cancer cell-derived sMVs (SW480-sMVs) showed a selective enrichment of integrins, ANXA1, CLDN7, CD44, and NOTCH1/2. Proteins known to modulate pre-metastatic niche formation (e.g., ADAM10 and MIF) and colon cancer markers (CEACAM1, MUC13, CNN3) were also identified in SW480-/SW620-sMVs. Collectively, our study provides, for the first time, molecular insights into sMVs and their possible role in CRC biology.

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Conflicts of interest

The authors declare no conflict of interest

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Figure Legends

Figure 1. Isolation, purification, and characterisation of EV subpopulations. (A) The experimental workflow for isolation and purification of shed microvesicle (sMV) and exosome (Exo) derived from SW480 and SW620 cell lines. SW480 and SW620 cells were grown in CELLine AD-1000 Biorector classic flask. sMVs were first isolated from the CM by differential centrifugation at 10,000 x *g*, 30 min. The supernatant was further centrifuged at 100,000 × *g* for 1 h. Following ultracentrifugation, the SW480 and SW620 pellets were fractionated using a 5–40% OptiPrep™ density gradient (100 000 × *g*, 18 h). sMVs were washed in 1 mL PBS, 10,000 × *g*, 30 min to obtain purified sMVs. Exos were washed in 1 mL PBS, 100,000 × *g*, 1 h to obtain purified Exos. Following ultracentrifugation, densities of 12 fractions from the OptiPrep™ density gradient were determined by absorbance at 244 nm using a molar extinction coefficient of 320 L $g^{-1}cm^{-1}$ (n=3). (B) Western blot analysis of classical exosomal markers (Alix, TSG101, CD9, and CD63) (Protein 20 µg per lane, n=3) (C) Transmission electron microscope images of sMV and Exo derived from SW480 and SW620 cell lines. sMV: Scale bar = 500nm. Exo Scale bar = 100 (SW480) and 200 (SW620) nm (n=1). (D) Nanoparticle tracking analysis (NTA) of sMV and Exo derived from SW480 and SW620 cell lines (mean±SEM, n=3).

Figure 2. Proteome analysis of SW480- and SW620-derived purified shed microvesicles (A) A Venn diagram of proteins identified in SW480- and SW620-sMVs, 1123 proteins were co-identified in SW480- and SW620-sMVs. Of these, 834 proteins were commonly identified using ≥-2 and ≤2 LFQ ratio cut off. (B) Gene Ontology of biological process of 834 commonly identified proteins in sMVs, data obtained from DAVID (ten lowest corrected-p-value GO terms) [19]. (C) sMVs-enriched proteins in different functional categories (normalised LFQ >50,000).

Figure 3. Enriched signalling protein in sMVs and protein networks identified in sMVs derived from primary and metastatic cell lines (STRING analysis). (A) A volcano plot of proteins in sMVs during cancer progression (291 proteins significantly upregulated in SW620-sMVs (≥2, p<0.05), 307 proteins significantly downregulated in SW620-sMVs (≤-2, p<0.05)).

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(B) Western blot analysis of enriched proteins in SW480-sMVs (EGFR, CD44, ANXA1, and CLDN7) and SW620-sMVs (CLDN1 and PAK1) (20 µg per lane, n=3). (C) SW620-sMVs MTOR. (D) SW620-sMVs MARCKS. (E) SW480-sMVs NOTCH. (F) SW480-sMVs EGFR.

Figure 4. Primary and metastatic cancer cell-derived shed microvesicles promote fibroblast invasion. (A) Live fluorescence microscopic analysis of NIH3T3 fibroblasts incubated with SW480- or SW620-sMVs (DiO, green). NIH3T3 were stained with DiI (red) and their nuclei with Hoechst (blue). Inset is the higher magnification of the image. Scale bar, 10 µm (B) Transwell Matrigel™ invasion assay of NIH3T3 fibroblasts treated with PBS vehicle alone or SW480- or SW620-sMVs (30 µ/mL). The nuclei of fibroblasts were stained with Hoechst stain (white) and images using fluorescent microscopy and quantified. Representative microscopic images are present in the lower panel (n=3).

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Table 1.

Cancer progression-related enriched proteins in sMVs derived from human colorectal cancer cells

Protein access	**Gene name**	**Protein description**	**Ratio** (SW620-sMV/SW480-sMV)[a]	**p-value**
P17252	PRKCA	Protein kinase C alpha type (PKC-A)	25484.1	3.00E-182
Q07960	ARHGAP1	Rho GTPase-activating protein 1	25042.5	2.00E-176
E9PGC0	RASA1	Ras GTPase-activating protein 1	4164.1	8.00E-162
Q6ZN28	MACC1	Metastasis-associated in colon cancer protein 1	8.5	2.00E-13
P22455	FGFR4	Fibroblast growth factor receptor 4	4.3	3.00E-07
P17612	PRKACA	cAMP-dependent protein kinase catalytic subunit alpha	3.7	4.00E-06
P11279	LAMP1	Lysosome-associated membrane glycoprotein 1	3.7	4.00E-06
Q13153	PAK1	Serine/threonine-protein kinase PAK 1	3.1	7.00E-05
P42345	MTOR	Mammalian target of rapamycin	2.7	3.00E-04
P23458	JAK1	Tyrosine-protein kinase JAK1	2.7	4.00E-04
Q14644	RASA3	Ras GTPase-activating protein 3	2.7	5.00E-04
Q13464	ROCK1	Rho-associated protein kinase 1	2.5	4.00E-04
Q68EM7	ARHGAP17	Rho GTPase-activating protein 17	2.4	2.00E-03
Q9NRY4	ARHGAP35	Rho GTPase-activating protein 35	2.3	2.00E-03
K7EP40	CLDN7	Claudin (Fragment)	-967891.5	5.00E-209
E9PEP6	NRP1	Neuropilin1	-28647.9	1.00E-188
P50281	MMP14	Matrix metalloproteinase-14	-21885.1	4.00E-179
P56199	ITGA1	Integrin alpha-1	-13793.4	1.00E-182
P49768	PSEN1	Presenilin-1	-11146.5	2.00E-167
Q08345	DDR1	Epithelial discoidin domain-containing receptor 1	-10457.1	1.00E-175
Q15582	TGFBI	Transforming growth factor-beta-induced protein ig-h3	-6738.6	4.00E-166
Q04721	NOTCH2	Neurogenic locus notch homolog protein 2	-5346.1	7.00E-180
P46531	NOTCH1	Neurogenic locus notch homolog protein 1	-4584.9	3.00E-178
A0A024R412	NRP2	Neuropilin2	-2170.5	6.00E-155
P00533	EGFR	Epidermal growth factor receptor	-1044.6	1.00E-74
P26006	ITGA3	Integrin alpha-3	-136.7	2.00E-48
P43121	MCAM	Cell surface glycoprotein MUC18	-15	3.00E-21
P16070	CD44	CD44 antigen	-11.3	4.00E-18
P78536	ADAM17	Disintegrin and metalloproteinase domain-containing protein 17	-5.3	5.00E-10
B5MCA4	EPCAM	Epithelial cell adhesion molecule	-4.4	1.10E-08
P04083	ANXA1	Annexin A1	-4.6	1.00E-08
P18084	ITGB5	Integrin beta-5	-3.2	5.00E-06
P06756	ITGAV	Integrin alpha-V	-2.5	2.00E-04
P08648	ITGA5	Integrin alpha-5	-2.2	1.00E-03

[a] Protein abundance ratio (LFQ ratio) reveals differential protein abundance between SW480 and SW620 sMVs. Positive LFQ values reflect increased protein abundance in SW620 sMVs relative to SW480 sMVs

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Table 2.

sMVs contain proteins known to modulate pre-metastatic niche in tumour microenvironment

Protein Acc.	Gene name	Protein description	SW480-sMV LFQ[a]	SW620-sMV LFQ[b]	Ratio (SW620-sMV/SW480-sMV)[c]	Target cell	Function [Reference]
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	262426	602851	2.3	Immune cells	Induce inflammation in breast cancer tissue [90]
P26447	S100A4	Protein S100-A4	2820396	4292937	1.5	T-Cell macrophage	Recruitment of T-cells, macrophage motility, promotes metastasis [91]
P14174	MIF	Macrophage migration inhibitory factor	2329768	3091043	1.3	Macrophage	Macrophage motility [76]
O14672	ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	114652	92112	-1.2	Breast cancer cell	Increase breast cancer cell motility [75]
P04083	ANXA1	Annexin A1	151668	33306	-4.6	Leukocytes	Potently and specifically inhibits the trans-endothelial migration of leukocytes [92]
P16070	CD44	CD44 antigen	409137	36076	-11.3	Pancreatic cancer cell	Metastatic niche formation [93]
P20333	TNFRSF1B	Tumour necrosis factor receptor superfamily member 1B	3182	1	-3181.6	T cells	Activates CD8 T cells [94]
P56199	ITGA1	Integrin alpha-1	13793	1	-13793.0	-	Represent cell-ECM interaction, leading to metastasis [95]
P50281	MMP14	Matrix metalloproteinase-14	21885	1	-21885.1	Osteoclasts	MMP-14, indirectly modulate TGF-β bioactivity by cleaving the ECM component [96]

[a] LFQ precursor ion intensity (normalised) (LFQ) for SW480 sMVs (n=3, averaged) (Supplemental Table S1)
[b] LFQ precursor ion intensity (normalised) (LFQ) for SW620 sMVs (n=3, averaged) (Supplemental Table S1)
[c] Protein abundance ratio (LFQ ratio) reveals differential protein abundance between SW480 and SW620 sMVs. Positive LFQ values reflect increased protein abundance in SW620 sMVs relative to SW480 sMVs
* Differential expression with p-values <0.05 as reported in Supplemental Table S1

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Table 3.

Known colorectal cancer markers identified in sMVs

Protein Acc.	**Gene name**	**Protein description [Reference]**	**SW480-sMV LFQ**[a]	**SW620-sMV LFQ**[b]	**Ratio** (SW620-sMV/SW480-sMV)[c]
Q9H3R2	MUC13	Mucin-13 [88]	1	48404	48404.3*
Q15417	CNN3	Calponin-3 [85]	33893	391418	11.6*
Q6ZN28	MACC1	Metastasis-associated in colon cancer protein 1 [97]	4772	40552	8.5
P29323	EPHB2	Ephrin type-B receptor 2 [98]	33490	78319	2.3
P01116	KRAS	GTPase Kras [99]	3199224	3595732	1.1
P13688	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 [100]	119862	118303	-1.0
P15311	EZR	Ezrin [101]	3551877	3267406	-1.1
P01111	NRAS	GTPase Nras [102]	320806	269658	-1.2
P16070	CD44	CD44 antigen [103]	409137	36076	-11.3

[a] LFQ precursor ion intensity (normalised) (LFQ) for SW480 sMVs (n=3, averaged) (Supplemental Table S1)
[b] LFQ precursor ion intensity (normalised) (LFQ) for SW620 sMVs (n=3, averaged) (Supplemental Table S1)
[c] Protein abundance ratio (LFQ ratio) reveals differential protein abundance between SW480 and SW620 sMVs. Positive LFQ values reflect increased protein abundance in SW620 sMVs relative to SW480 sMVs
* Differential expression with p-values <0.05 as reported in Supplemental Table S1