Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct

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Abbreviations: Exos, exosomes; sMVs, shed microvesicles; EVs, extracellular vesicles; CM, culture medium; CCM, concentrated culture medium; PDCD6IP/Alix, programmed cell death 6 interacting protein; TSG101, tumour susceptibility 101; ESCRT, endosomal sorting complex required for transport; UC, ultracentrifugation; DC, differential centrifugation; SCUF, sequential centrifugal ultrafiltration; PVDF, hydrophilic polyvinylidene difluoride; MVB, multivesicular body; DLS, dynamic light scattering; cryo-EM, cryo-transmission electron microscopy.

ABSTRACT

Secretion and exchange of extracellular vesicles (EVs) by most cell types is emerging as a fundamental biological process. Although much is known about EVs, there is still a lack of definition as to how many naturally occurring EV subtypes there are and how their properties and functionalities might differ. This vexing issue is critical if EVs are to be fully harnessed for therapeutic applications. To address this question we have developed and describe here a sequential centrifugal ultrafiltration (SCUF) method to examine, in an unbiased manner, what EV subtypes are released *in vitro* into cell culture medium using the human colon carcinoma cell line LIM1863 as a model system. Using the culture medium from ~7.2×10^9 LIM1863 cells SCUF was performed using hydrophilic PVDF membranes with low protein binding properties (Millipore Durapore™ Ultrafree-CL filters with 0.1, 0.22, 0.45 and 0.65 µm pore size). EV particle sizing was measured using both dynamic light scattering and cryo-electron microscopy. Comparative proteome profiling was performed by GeLC-MS/MS and qualitative protein differences between EV subtypes determined by label-free spectral counting. The results showed essentially two EV subtypes; one subtype (fraction Fn1) comprised heterogeneous EVs with particle diameters of 30-1300 nm, the other (fraction Fn5) being homogeneous EVs of 30-100 nm diameter; based on cryo-EM both EV subtypes were round shaped. Western blot analysis showed Fn 5 (SCUF-Exos) contained traditional exosome marker proteins ($Alix^+$, $TSG101^+$,$CD81^+$,$CD63^+$), while Fn1 (SCUF-sMVs) lacked these protein markers. These findings were consistent with sMVs isolated by differential centrifugation (10,000*g*, DC-sMVs) and exosomes (100,000*g* EVs depleted of 10,000g material). The buoyant density of sMVs determined by OptiPrep™ density gradient centrifugation was 1.18-1.19 g/mL and exosomes 1.10-1.11 g/mL. Comparative protein profiling of SCUF-Exos/-sMVs revealed 354 and 660 unambiguous protein identifications,

respectively, with 256 proteins in common. A salient finding was the first report of 350 proteins uniquely identified in sMVs may of which have the potential to enable discrimination of this EV subtype from exosomes (notably, members of the septin family, kinesin-like protein (KIF23), exportin-2/chromosome segregation like-1 protein (CSE1L), and Rac GTPase-activating protein 1 (RACGAP1)). We report for the first time that both SCUF-Exos and SCUF-sMVs isolated from LIM1863 colon cancer cells induce invasion of recipient NIH3T3 cells. Interestingly, the SCUF-sMVs promote invasion to a significantly greater extent (3-fold) than SCUF-Exos. This analytical SCUF method for fractionating EVs is potentially scalable using tangential flow filtration, thereby providing a solid foundation for future in-depth functional studies of EV subtypes using diverse cell types and functional assays.

1. INTRODUCTION

Secretion and exchange of extracellular vesicles (EVs), nano- to micrometer-sized membranous organelles, by most cell types is emerging as a central paradigm for intercellular communication [1,2]. While EVs have been primarily studied *in vitro* using cell culture media as a source material, they are also found *in vivo* in diverse body fluids, such as semen, synovial fluid, saliva, urine, breast milk, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, and blood [1,3]. EVs are thought to modulate recipient cell behaviour by transfer of their intrinsic cargo constituents such as oncogenic proteins [4-6], infectious proteins [7,8] malarial proteins [9], miRNAs/mRNAs [10], DNA [11], and lipids [12]. There is now an increasing awareness that EVs play a critical role in the development of diverse pathologies such as cancer (e.g., of pre-metastatic niche formation [13], neurodegenerative disorders [8], and infectious diseases (e.g., malaria [9], bacterial infection [14,15]). Collectively, these studies have engendered great interest in harvesting EVs for therapeutic applications such as regenerative medicine [16,17], vaccination against infectious disease [18,19], and EV vaccines for possible cancer treatment [20-22]. These studies have led to several clinical and pre-clinical investigations of EV-based therapies [2,23-25].

Two seminal reports in 2008 revealed that EVs isolated from cultured tumour cells contain cargo information that parallels blood-EV information obtained from disease patients. Skog et al., reported that isolated EVs from cultured glioblastoma cells obtained from resected human tumours contained transcripts (e.g., EGFR variant III mRNA) and miRNAs that mirrored serum-EV information from patients with glioblastoma [4], while Taylor and colleagues demonstrated that specific miRNA signatures from ovarian cancer cell line EVs (and lung cancer cell lines) correlated with miRNA profiles of EpCAM-immunocaptured-

EVs from blood obtained from ovarian cancer (and lung cancer) patients [26,27]. These studies highlight the potential use of serum-EV information to provide tumour diagnostic biomarkers and assist in the design of therapeutic strategies.

EV annotation is a vexed question. They can be classified based on their cellular origins and/or biological functions or on their biogenesis [2]. These concerns are widely discussed in the international EV community (International Society for Extracellular Vesicles (ISEV)) [28]. In broad terms there are thought to be two EV subtypes: 100-1000 nm diameter microvesicles (shed microvesicles, sMVs; membrane blebs) and 30-150 nm diameter exosomes [29,30]. sMVs are generated by directly outward budding from the plasma membrane [31,32], while exosomes are generated in the early/late endosomal pathway by the inward budding of multivesicular bodies (MVBs) luminal membranes to form intraluminal vesicles (ILVs); MVBs then traffic to and fuse with the plasma membrane whereupon they release their ILV contents into extracellular space (exosomes) [30]. Given that most EV functional studies are performed using ill-defined EV preparations, it is very difficult to ascribe function to a specific EV subtype – this has ramifications when designing EV therapeutics, especially when determining possible EV-subtype side-effects in clinical investigations [25]. These issues have engendered great interest in establishing how many naturally occurring EV subtypes there are and improving methodologies for EV isolation.

Effective methods for the isolation and characterisation of EVs remain challenging [33,34]. Current strategies include differential centrifugation (DC) [35], filtration using hydrophilic polyvinylidene difluoride (PVDF) membranes of different pore sizes [36,37], high performance size-exclusion chromatography (SEC) [38], ultrafiltration with SEC [34],

immunocapture [39,40], differential density gradient ultracentrifugation [33], tangential flow filtration [41], field flow fractionation [42], and microfluidic isolation [43]. Additionally, there is now a plethora of commercial "easy isolation kits" – designed essentially to precipitate EVs from body fluids. However, these one-step kits are designed for diagnostic purposes and do not enable separation of EV subtypes one from another or distinguish EVs from macromolecular aggregates [30]. A further development in clinical diagnostics is the use of blood-based EV antibody arrays for multiplexed phenotyping of EV subtypes [44].

In the present study, we set out to determine the number of EV subtypes secreted by the human colon carcinoma cell line LIM1863. To this end we developed a sequential centrifugal ultrafiltration (SCUF) method that relies on microfiltration through a series of hydrophilic PVDF membranes of different pore sizes (0.1-0.65 μm). We show that >95% of the total EVs released from LIM1863 cells into culture media are exosomes (45%) and sMVs (50%). Using GeLC-MS/MS we identify for the first time 350 proteins that are selectively enriched in sMVs (in comparison with exosomes), many of which have not been previously described in EVs; we expect that many of these identifications will form the basis for definitive sMV protein markers that will enable their distinction from exosomes. Importantly, we demonstrate for the first time, that LIM1863 colon cancer cell-derived SCUF-Exos/-sMVs display differential invasive activities on recipient fibroblast cells.

2. Materials and methods

2.1. Preparation of concentrated culture medium (CCM) from human colon carcinoma LIM1863 cells

Human colon carcinoma LIM1863 cells [45] were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) containing 5% FCS, 0.1% insulin-transferrin-selenium (ITS) (Life Technologies, Carlsbad, CA), 60 µg/mL benzyl penicillin and 100 µg/mL streptomycin (P/S) supplementary, and incubated at 37 ºC and 10% CO_2 atmosphere [39]. LIM1863 cells (7.2×10^9 cells) were washed four times with 30 mL of RPMI-1640 medium, and cultured in 900 mL RPMI-medium supplemented with 0.6% ITS and P/S for 24 h. Culture medium (CM) was collected (900 mL) and centrifuged at 4 ºC at 480*g* for 5 min / 2,000*g* for 10 min to remove floating cells and cell debris. CM was concentrated to 22 mL (CCM) using 8 individual Amicon Ultra-50, Ultracell centrifugal filter devices (3K NMWL) (Merck Millipore, MA, USA) and used immediately for EV isolation. Protein content of samples was estimated by 1D-SDS-PAGE/SYPRO™ Ruby protein staining densitometry, as previously described [33].

2.2. Isolation of sMVs and exosomes by differential centrifugation (DC)

CCM (1 mL, ~1.89 mg protein) was centrifuged at 10,000*g* (TLA-55 fixed angle rotor, Optima™ MAX-XP Ultracentrifuge, Beckman Coulter) for 30 min at 4 ºC to sediment sMVs (DC-sMVs). The supernatant solution (sMV-depleted) was then centrifuged at 100,000*g* (TLA-55 fixed angle rotor, Optima™ MAX-XP Ultracentrifuge) for 1 h at 4 ºC to recover crude exosomes (DC-Exos). For isolation of total EVs (DC-EVs, i.e., mixture of sMVs and

exosomes), CCM (1 mL) was centrifuged at 100,000*g* (TLA-55 fixed angle rotor, Optima™ MAX-XP Ultracentrifuge) for 1 h at 4 °C. The supernatant, which contains soluble-secreted proteins (DC-soluble secretome, DC-SS) was carefully harvested using an Eppendorf pipette and transferred to a fresh micro-tube for future use. All EV preparations (DC-sMVs, DC-Exos and DC-EVs) were washed by re-suspension in 1 mL PBS (Gibco®, Life Technologies, Carlsbad, CA) and centrifuged at either 10,000*g* (DC-sMVs), or 100,000*g* (DC-Exos, DC-EVs). EV preparations were resuspended in 200 µL PBS (Gibco®, Life Technologies) for future use. From 1 mL CCM (i.e., ~3.2 × 10^8 LIM1863 cells) EV yields (protein) were - DC-EVs, ~338 µg; DC-sMVs, ~187 µg; DC-Exos, ~ 219 µg; and DC-SS, ~1.55 mg. All EV preparations were used either immediately or stored at -80 °C.

2.3. Isolation of sMVs and exosomes by sequential centrifugal ultrafiltration (SCUF)

EVs were fractionated using a series of different pore-sized PVDF ultrafilters (Durapore™ Ultrafree-CL, Merck Millipore) ranging from 0.65, 0.45, 0.22, and 0.1 µm. CCM (20 mL) was firstly centrifuged at 3,000*g* using 10 individual 0.65 µm ultrafilters (until ~50 µL remained in the combined filters), the filters were subsequently washed with a total of 500 µL PBS. The combined rentenates (SCUF-fraction >0.65 µm, Fraction 1 (Fn1)) from this step were transferred into a fresh micro-tube with 0.5 mL PBS and harvested by centrifugation at 10,000*g* for 30 min. The filtrate (i.e., <0.65 µm) was sequentially filtered through 0.45 µm, 0.22 µm, and 0.1 µm filters. The last filtrate (EVs passed through 0.1 µm filter), with remaining Fractions 2-5 (Fns 2-5) collected using Eppendorf pipettes and individually harvested by ultracentrifugation at 100,000*g* for 1 h. SCUF- <0.1 µm EVs, Fraction 5 (Fn5) were washed with 0.5 mL PBS and re-centrifuged at 100,000*g* for 1 h. All EV preparations

were resuspended in 500 µL PBS. Protein yield of EV preparations: SCUF-Fn1, 836 µg; SCUF-Fn5, 1328 µg.

2.4. Western blot analysis

All EV preparations (15 µg protein) were lysed 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) and incubated with 50 mM DTT (where necessary), heated for 5 min at 95 ^{o}C and subjected to electrophoresis using precast NuPAGE™ 4-12% (w/v) as previously described [39]. Membranes were probed with primary mouse anti-TSG101 (BD Biosciences; 1:1000), mouse anti-Alix (Cell Signaling Technology; 1:1000), mouse anti-CD81 (Santa Cruz Biotechnology; 1:1000), mouse anti-KIF23 (Santa Cruz Biotechnology; 1:200), mouse anti-CSE1L (Santa Cruz Biotechnology; 1:200), mouse anti-CD63 (non-reducing) (Santa Cruz Biotechnology; 1:1000), mouse anti-EPCAM (non-reducing) (Santa Cruz Biotechnology; 1:1000). Membranes were further incubated with secondary antibody IRDye 800 goat anti-mouse IgG (1:15,000, Li-COR Biosciences). All antibody incubations were carried out using gentle orbital shaking at RT. Western blots were washed three times in TTBS for 10 min after each incubation step and visualised using the Odyssey Infrared Imaging System, version 3.0 (LI-COR Biosciences, Nebraska USA).

2.5. Dynamic light scattering (DLS)

For DLS analyses 50 µL CCM or 50 µL (10 µg protein) of each EV preparation was shaken (Titertek shaker, Flow Laboratories, Inc.,) at 4 ^{o}C for 20 min to dissociate possible EV aggregates. Disposable micro cuvettes (ZEN0040, Malvern Instruments Ltd., UK) were used

and DLS measurements conducted at 20 ^{o}C using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK), operated at 633 nm and back scattering at 173^{o}. Light scattering was recorded for 200 s with 10 replicate measurements. DLS signal intensity was transformed to volume distribution [volume (%)], assuming a spherical shape of EVs, using the Dispersion Technology Software v.5.10 (Malvern Instruments Ltd., UK). Each EV preparation is representative of three technical replicates.

2.6. Cryo-transmission electron microscopy (cryo-EM)

Cryo-EM imaging of EV preparations was performed essentially as described [46], but with minor modifications. Briefly, EV preparations (~2 µg protein, non-frozen samples prepared within 2 days of analysis) were transferred onto glow-discharged C-flat holey carbon grids (ProSciTech Pty Ltd). Excess liquid was blotted and grids were plunge-frozen in liquid ethane. Grids were mounted in a Gatan cryoholder (Gatan, Inc., Warrendale, PA, USA) in liquid nitrogen. Images were acquired at 300 kV using a Tecnai G2 F30 (FEI, Eidhoven, NL) in low dose mode. Size distribution of vesicles (range 30->1000 nm) was calculated for the 5 fields of view (~200 different vesicles for each EV preparation).

2.7. OptiPrep™ density gradient (DG) analysis of DC-Exos and DC-sMVs

Discontinuous iodixanol gradients, containing 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol were prepared by diluting a stock solution of OptiPrep™ (60% (w/v) aqueous iodixanol (Axis-Shield PoC, Norway) with 0.25 M sucrose/10 mM Tris, pH 7.5 [33]. Each EV preparation (DC-Exos: 500 µL, 578 µg protein; DC-sMVs: 500 µL, 893 µg) were individually overlaid on the gradient, and centrifugation performed at 100,000*g* for

18 h at 4 ºC. Twelve individual 1 mL gradient fractions (with increasing density) were collected manually. Fractions were diluted with 2 mL PBS and centrifuged at 100,000*g* for 3 h at 4 ºC followed by washing with 1 mL PBS, and resuspended in 200 µL PBS. To determine the density of each fraction, a control OptiPrep™ gradient containing 1 mL of 0.25 M sucrose/10 mM Tris, pH 7.5 was run in parallel. Fractions were collected as described, serially diluted 1:10,000 with water, and the iodixanol concentration determined by absorbance at 244 nm using a molar extinction coefficient of 320 L $g^{-1}cm^{-1}$ [47].

2.8. GeLC-MS/MS

EVs preparations (10 µg protein) were lysed in SDS sample buffer, electrophoresed by SDS-PAGE (approximately 15 mm into the gel) and visualized using the Imperial™ Protein Stain (Thermo Fisher Scientific). Each gel lane was excised and individual gel pieces subjected to *in-gel* reduction, alkylation and trypsinization [39,46]. Briefly, the gel pieces were reduced with 10 mM DTT (Calbiochem, San Diego, USA) for 30 min, alkylated for 20 min with 25 mM iodoacetic acid (Fluka, St. Louis, USA), and digested with 500 ng trypsin (Promega Sequencing Grade, Wisconsin, USA, Freehold, USA) for 16 h at 37 °C. Generated tryptic peptides were extracted from the gel pieces [48] and subjected to GeLC-MS/MS using a nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) coupled on-line to a high-resolution linear ion trap (Orbitrap Elite) mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific). Tryptic peptide samples were first loaded on a pre-column (Acclaim PepMap100 C18 5 µm 100Å, Thermo Fisher Scientific) and separated using a VYDACMS C18-reversed phase column (25 cm length, 75 µm inner diameter, 3µm 300Å, Grace, Hesperia, CA) with a 120-min linear gradient from 0 to 100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) at a

flow rate of 250 nL/min. MS data were acquired using a data-dependent Top 20 method dynamically choosing the most abundant precursor ions from the survey scan (300–2500 Th) using CID fragmentation. Survey scans were acquired at a resolution of 120,000 at m/z 400. Unassigned precursor ion charge states as well as singly charged species were rejected and peptide match was disabled. The isolation window was set to 3 Th and fragmented with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 ms and 60 ms respectively and the ion target values were set to 3E6 and 1E6, respectively. Selected sequenced ions were dynamically excluded for 90 s. Data were acquired using Xcalibur software v2.1 (Thermo Fisher Scientific).

2.9. Database searching and protein identification

Raw data were processed using Proteome Discoverer (v1.4.0.288, Thermo Fischer Scientific). MS2 spectra were searched with Mascot (Matrix Science, London, UK; v 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, v 1.4.0.288), and X! Tandem (v 2010.12.01.1) against a database of 125,803 ORFs (Uniprot Human, 2014_07). Peptide lists were generated from a tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines and protein N-terminal acetylation as variable modifications. Precursor mass tolerance was 10 ppm, product ions were searched at 0.6 Da tolerances, min peptide length defined at 6, maximum peptide length 144, and max delta CN 0.05. Peptide spectral matches (PSM) were validated using Percolator based on q-values at a 1% false discovery rate (FDR) [49,50]. With Proteome Discoverer, peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1% FDR [51]. Scaffold (Proteome Software Inc., Portland, OR, v 4.3.4) was employed to validate MS/MS-based peptide and protein identifications from database searching. Initial

peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm [52]. Protein probabilities were assigned by the Protein Prophet algorithm [51]. Protein identifications were accepted, if they reached greater than 99% probability and contained at least 2 identified unique peptides. These identification criteria typically established <0.01% false discovery rate based on a decoy database search strategy at the protein level. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Contaminants, and reverse identification were excluded from further data analysis. UniProt was used for protein annotation (cellular compartment, subcellular location, molecular function, transmembrane regions).

2.10. Semi-quantitative label-free spectral counting

Significant spectral count normalised (Nsc) and fold change ratios (Rsc) were determined as previously described [33,39,46,53]. The relative abundance of a protein within a sample was estimated using Nsc, where for each individual protein, significant peptide MS/MS spectra (i.e., ion score greater than identity score) were summated, and normalised by the total number of significant MS/MS spectra identified in the sample. To compare relative protein abundance between samples the ratio of normalised spectral counts (Rsc) was estimated. Total number of spectra was only counted for significant peptides identified (Ion score ≥ Homology score). When Rsc is less than 1, the negative inverse value was used. The number of significant assigned spectra for each protein was used to determine whether protein abundances between the four categories (DC-sMVs, DC-Exos, SCUF-sMVs, SCUF-Exos). For each protein the Fisher's exact test was applied to significant assigned spectra. The

resulting p-values were corrected for multiple testing using the Benjamini-Hochberg procedure [54].

2.11 Transwell-Matrigel™ invasion assay

Invasion assays were performed as previously described [55] with modifications. Transwell inserts (8.0-µm pore size, Corning) were coated with 1 mg/mL of Growth-factor-reduced Matrigel matrix (Corning) and allowed to solidify for 4 h at 37 °C. NIH3T3 cells (50,000) in DMEM (P/S) were stimulated with SCUF-sMVs (30 µg/mL), SCUF-Exos (30 µg/mL) or vehicle (PBS) alone for 2 h at 37 °C. The cells were collected and overlaid onto the Matrigel-coated Transwell inserts. The inserts were placed onto wells of a 24-well plate that contained DMEM (5% FCS, P/S) supplemented with either SCUF-sMVs (30 µg/mL), SCUF-Exos (30 µg/mL) or vehicle alone. Invasion chambers were incubated for 16 h at 37 °C to facilitate invasion. Cells were fixed in 4% formaldehyde at RT for 5 min and nuclei stained with Hoechst (10 mg/mL) for 20 mins. Non-invasive cells were removed from the upper side of the filter using cotton swabs. Nuclei of the invaded cells were imaged using Zeiss AxioObserver Z1 microscope. Five fields of view were obtained per insert (n=3 biological replicates). Images were quantified using Image J software v 1.49e. Error bars represent s.e.m (*** $p<0.0005$).

3. Results

3.1. Characterisation of EVs isolated by differential centrifugation (DC)

As a first step towards understanding how many types of naturally-occurring EVs there are, and how they differ from one producer cell type to another, we used the traditional method of DC to isolate and characterise shed MVs (sMVs, also referred to as 'microvesicles') and exosomes from the same cell culture medium. Because of our extensive interest in the human colon carcinoma cell line LIM1863 [33,39,56], we used the culture medium from this cell line as a model for this study. LIM1863 cells, established at the Ludwig Institute in Melbourne in 1987 from a resected invasive human colon carcinoma, grow as free-floating suspension clusters that contain differentiated columnar cells [45]. LIM1863 cells (~7.2 × 10^9 cells) were grown in serum-free media supplemented with 0.6% ITS to minimise FCS protein contaminants – under these conditions the viability of LIM1863 cells is >96% [39]. The CM (900 mL) was depleted of intact cells and cell debris by low-speed centrifugation and then concentrated to 22 mL (CCM) using several 3K NMWL membrane filters (Fig. 1A). The protein concentration of CCM was ~1.9 mg/mL comprising ~15% EVs and ~85% soluble-secreted proteins. The yield of total EVs (100,000*g* pellet, DC-EVs) was ~338 μg/mL, sMVs (10,000g pellet, DC-sMVs) ~187 μg/mL, and the yield of exosomes (100,000*g* pellet minus 10,000*g* pellet, DC-Exos) was ~219 μg/mL (Fig. 1A). Western blot analysis of DC isolated EVs revealed the presence of 'accepted' exosome markers Alix, TSG101, CD63 and CD81 in the DC-Exos preparation, low expression levels of Alix and CD81 but undetectable CD63 in DC-sMVs; TSG101 is present in both preparations but clearly at levels significantly lower in DC-sMVs (Fig. 1B). Morphological analysis of the DC-purified EVs is shown in Fig. 1C. These data revealed that both vesicle subtypes were round-shaped. By

contrast, DC-sMVs are heterogeneous in size. Internal diameter measurements of EVs observed in the cryo-EM were made manually (5 fields of view, ~200 individual EV measurements) (Fig. 1D). These observations revealed DC-Exos to be essentially homogeneous with diameters in the range 30-100 nm; by contrast, DC-sMVs were observed to be heterogeneous in size ranging from 30-1300 nm (~30% of sMVs, 30-100 nm; ~65% 100-650 nm; and ~2-3% of sMVs >650 nm diameter).

3.2. Characterisation of EVs isolated by sequential centrifugal ultrafiltration (SCUF)

To shed light on how many types of naturally occurring EVs are released from LIM1863 cells we next employed an unbiased method that minimises the use of high centrifugal (*g*)-force centrifugation [57]. This method, namely sequential centrifugal ultrafiltration (SCUF), exploits the ability of a series of hydrophilic PVDF membrane filters of different pore size to fractionate EVs. Fig. 2A outlines the strategy for purifying LIM1863-derived EVs sequentially using membrane filters with pore-sizes 0.65 μm, 0.45 μm, 0.22 μm and 0.1 μm. Briefly, the retentate from membrane 1 (pore size 0.65 μm) was harvested and washed with PBS (Fraction 1, referred to as SCUF-Fn1, >0.65 μm). The filtrate from the 0.65 μm flow-through was further passaged sequentially though 0.45 μm, 0.22 μm, and 0.1 μm membrane filters. (Retentates from these filtration steps are referred to as SCUF-Fn2 (>0.45 μm), -Fn3 (>0.22 μm) and -Fn4 (>0.1 μm) and EVs harvested using ultracentrifugation at 100,000*g* for 1 h; the filtrate from the 0.1 μm membrane filter step was further centrifugation at 100,000*g* for 1 h, the pellet is referred to as SCUF-Fn5 (0.1 μm flow-through), and was washed with 0.5 mL PBS. Protein estimation of SCUF-Fns 1-5 revealed that Fn1 accounted for ~27% of EVs, Fn5 ~69% and Fns2-4, 3-4% (Supplementary Fig. 1). Given the low EV yields in Fns 2-4, these SCUF fractions were not characterised further. Western blot analyses of SCUF-Fn1

and -Fn5 (Fig. 2B) reveal that Fn5 is consistent with exosomes ($Alix^+$, $TSG101^+$, $CD63^+$, $CD81^+$, compare with DC-Exos in Fig. 1) and Fn1 with sMVs (compare with DC-sMVs in Fig. 1). Interestingly, there was no detectable Alix, TSG101, CD63 and CD81 in Fn1. For these reasons, we refer to SCUF-Fn5 as exosomes (SCUF-Exos) and Fn1 as sMVs (SCUF-sMVs). Morphological analysis using cryo-EM showed that both SCUF-Exos and SCUF-sMVs were round-shaped (Fig. 3A, B). Similar to DC-Exos, the SCUF-Exos were essentially homogeneous with internal diameters <100 nm and SCUF-sMVs were heterogeneous in size; selective cryo-EM images of SCUF-sMVs reveal particles with diameters over a broad range (30-1300 nm). Indeed, a small number of large EVs (~1300 nm diameter) were observed and morphological features consistent with large oncosomes reported by the Di Vizio laboratory [58]. We next determined the hydrodynamic radius of SCUF-Exos and -sMVs by dynamic light scattering (Fig. 3C). These data reveal three peaks in the starting material (CCM) with particle diameters in the range 30-80 nm, 100-200 nm, and ~350-1500 nm. Two EV subtypes (50-80 nm and 120-200 nm diameter) were observed in the SCUF-Exos fraction and essentially one broad EV peak (450nm-1300 nm) in the SCUF-sMVs fraction. Manual measurement of particles observed in the cryo-EM images (from Fig. 3A, B; 5 fields, ~200 measurements) showed that the size distribution of EVs in the SCUF-Exos preparation to be essentially homogeneous (range 30-100 nm) while EVs in the SCUF-sMVs preparation exhibited EVs with a broad spectrum of particle diameter (30-1300 nm diameter) (Fig. 3D).

3.3. Protein profiling of EVs released from LIM1863 cells

To gain insights into the protein cargo of DC- and SCUF-purified EVs released from LIM1863 cells we performed GeLC-MS/MS proteome profiling analyses. A total of 354 proteins were found in SCUF-Exos compared with 606 proteins in SCUF-sMVs. An

inspection of these datasets reveals 256 proteins common to both datasets, while 98 and 350 proteins are uniquely enriched in SCUF-Exos and SCUF-sMVs, respectively (Fig. 4A; protein lists are given in Supplementary Table S1). The most abundant proteins, based upon number of spectra identified, common to both EV subtypes are the cytoskeletal-related proteins (actin, EIF3C, EIF2S3, PFN1, CFL1, TM9SF2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1A). Those proteins uniquely-enriched in SCUF-Exos include PTGFRN (prostaglandin F2 negative regulator protein, CD9P1/CD315), exosomal marker proteins (TSG101, CD44, FLOT1, PROM1), enzymes/proteases/peptidases (ATP1A2, MEP1A, ADAM10, XPNPEP2), and receptors (EPHB3, GPRC5C) (Table 1B). Of the 350 proteins uniquely enriched in SCUF-sMVs the most abundant include endoplasmin (a molecular chaperone that functions in the processing and transport of secreted proteins), Na/K ATPase (ATP1A2), several mitochondria-associated-proteins (LRPPRC, IDH2, ATP5A1, HSPA9, HSD17B4, SORD), cytoskeletal proteins (SPTBN1, SPTAN1, ACTN1, CKAP4, KIF23), and members of the non-clathrin vesicular coat protein (COP) family (Table 1C). Interestingly, we observe a significant enrichment of mitochondrial-associated proteins in SCUF-sMVs when compared with SCUF-Exos, (Fig. 4B, and Supplementary Table S2). The recovery of LIM1863-derived SCUF-sMVs and DC-sMVs, compared with DC-sMVs/-Exos, is shown in Supplementary Fig. 2A and B.

To establish the relative abundance of a particular protein within SCUF-Exos and -sMVs we performed label-free spectral counting analysis of the data. In Fig. 4C we show that proteins associated with exosome biogenesis (e.g., ESCRTs, Rab GTPases, syntenins) are enriched in SCUF-Exos relative to SCUF-sMVs, while cytoskeleton-related proteins (microtubules, septins, actin binding proteins, microtubule binding proteins, motor proteins (e.g., kinesin-

like protein 23, KIF23) and intracellular trafficking/sorting proteins (COPI, COPII, AP-1 complex, Arp2/3 complex, Exportins (e.g., chromosome segregation 1-like protein (CSE1L/exportin-2), importins are selectively enriched in SCUF-sMVs). Extended lists of the above-mentioned protein family categories selectively enriched in SCUF-sMVs (Table 2A-D) or SCUF-Exos (Table 3). Confirmation of the selective enrichment of two proteins - KIF23 and CSE1L – in sMVs relative to exosomes was shown by western blot analysis of DC-purified samples (Fig. 5). Of note was the removal of low levels of CSE1L in DC-Exos by further purification using OptiPrep™ density gradient analysis.

3.4 SCUF-sMVs and SCUF-Exos are functionally distinct in the Transwell invasion assay

EVs are reported to have roles in modulating recipient cell invasion in cancer progression [25]. Functional differences between Exos and sMVs remain unclear [59]. To address this question we asked whether SCUF-sMVs and SCUF-Exos are functionally distinct in modulating cell invasiveness of recipient fibroblast cells. Mouse fibroblast NIH3T3 cells were stimulated with SCUF-sMVs and SCUF-Exos and invasion through a layer of Matrigel™ matrix was quantified using Transwell invasion assay. Both SCUF-sMVs and SCUF-Exos promote significant invasion of non-invasive NIH3T3 cells (Fig. 6). However, compared to SCUF-Exos, SCUF-sMVs promote significantly greater invasion (~3-fold) of NIH3T3 cells (Fig. 6). Moreover, the differential enrichment of invasion, migration, and motility-related components in SCUF-Exos and SCUF-sMVs (Supplementary Table S3) suggest that these EV sub-populations may promote invasion using different mechanisms.

4. Discussion

Membranous EVs released from most cell types provide a vehicle for intercellular communication by transfer of their protein/ miRNA/ mRNA/ lipid cargoes to recipient cells [2]. In the last 5 years it has become evident that miRNA signatures and transcripts contained in tumour-derived EVs [56,60] can serve as potential diagnostic biomarkers of glioblastoma [4], ovarian [26], colorectal [61], prostate [62] and lung cancers [27]. To date, most EV functional and diagnostic/therapeutic studies have been undertaken using impure, heterogeneous and poorly characterised material. Hence, a central question in EV biology is how many naturally-occurring EV subtypes are released from host cells and where they differ in their cargo content – is this reflected in differing functionalities? To provide an integrated overview of the number and properties of EV subtypes released from LIM1863 cells we have extended these studies to include an isolation method that minimises the use of high *g*-force (i.e., traditional differential centrifugation), which is known to influence purity and yield of EVs , but rather relies on sequential low *g*-force centrifugal ultrafiltration (SCUF).

Our current findings show that only two EV subtypes are released from LIM1863 cells – one EV subtype, is characteristic of exosomes being relatively homogeneous in size (30-100 nm diameter), whereas the other subtype is characteristic of sMVs (also referred to as plasma membrane blebs [63,64], microparticles [9], oncosomes [65], and microvesicles [31]). Our findings show sMVs to be heterogeneous in diameter, exhibiting a broad spectrum of (30-1300 nm diameter). Both EV subtypes released from LIM1863 cells are round-shaped, as assessed by cryo-EM. Interestingly, a small percentage (~1-2%) of the sMV EVs exhibit

large ~1300-nm diameters, characteristic of large oncosomes reported by the Di Vizzio laboratory [66]. Further studies are warranted to determine whether these large sMVs might be released by other colon tumour cells, and have functionalities distinct from other EV subtypes. Interestingly, the morphology and size of EVs released from LIM1863 cells into cell culture were consistent with the cryo-EM findings reported by the Brisson laboratory for EVs identified in the blood of healthy subjects (platelet-free plasma prepared 4 h after collection) [67]. In agreement with this study, we also observed spherical EVs by cryo-EM ranging in diameter from 30 to 1000 nm (~80% between 50-500 nm). However, unlike Aurraud et al., [67], we do not observe any tubular EVs in our DC-sMVs/ SCUF-sMVs preparations. The morphology and size (25-260 nm diameter, mean 30 nm) of EVs reported in fresh EDTA-plasma from healthy individuals by Yuana and colleagues [68] is also consistent with our findings for colon tumour LIM1863 cell-derived sMVs and exosomes.

We next examined the protein profiles of EVs isolated by SCUF methodology and compared these data with EVs from LIM1863 cells isolated by traditional differential centrifugation (DC). Western blot analysis showed both DC-Exos and SCUF-Exos to be $Alix^{+}$, $TSG101^{+}$, $CD63^{+}$ and $CD81^{+}$. In the case of sMVs, DC-sMVs were clearly $CD63^{-}$ and showed low but detectable levels of Alix, TSG101 and CD81. Strikingly, SCUF-sMVs were negative for Alix, TSG101, CD63 and CD81 indicating that the low levels of these marker proteins can be most likely ascribed to DC sMVs preparations being contaminated with low levels of exosome as a consequence of ultracentrifugation [39]. Using a very rigid identification threshold (Mascot, Sequest, and X! Tandem search algorithms, 1% protein FDR, and 5% peptide probability) GeLC-MS/MS identified 354 and 606 proteins in SCUF-Exos and SCUF-sMVs, respectively (Supplementary Table S1). There are 256 proteins common to both EV subtypes; 98 and 350

proteins are uniquely enriched (i.e., peptides found exclusively in one EV subtype but not the other) in SCUF-Exos and SCUF-sMVs, respectively. We observe a significant number of proteins that are associated with mitochondria (based upon GO ontology) – 67 in total, of which 14 are common to both EV subtypes and 48 and 5 exclusively observed in SCUF-sMVs and SCUF-Exos, respectively (Fig. 4B, a detailed list of these mitochondrial proteins is given in Supplementary Table S2). Whilst we have not focused on these proteins in this analysis due to the likelihood that they are contaminants, we cannot rule out the possibility that mitochondrial proteins/mRNA (mitochondrial mRNA is also found in LIM1863-derived EVs, unpublished findings) may in some instances be EV constituents taken up by recipient cells. For example, it has been reported that mitochondria and mitochondrial DNA can be actively transferred between cells and shown to rescue aerobic respiration – however, it is not clear how this process occurs [69]. Needless to say, further work is required to establish whether EVs are implicated in this dynamic process.

We next focused on the 98 proteins uniquely enriched in SCUF-Exos. As shown in Table 1B/Table 3 these proteins can be categorised according to their role in exosome biogenesis (see Fig. 4C (i)), trafficking (sorting and release), receptors (EPHB3, GPRC5C, PTGFRN), tetraspanins (e.g., CD63 (Rsc 14.5, LAMP3), CD82 (Rsc 13.2), CD151 (Rsc 5.5), TSPAN6 (Rsc 5.5) - CD9 (Rsc 3.0) and TSPAN8 (Rsc 2.5)), are also present in SCUF-sMVs but enriched in SCUF-Exos), enzymes/ proteases/ peptidases (ATP1A2, MEP1A, ADAM10, XPNPEP2) as well as (including membrane, and integral membrane proteins, Supplementary Table S4). These data are consistent with previously published protein profiles for LIM1863-derived exosomes isolated from CM by immunoaffinity-capture technology [39] and include exosome marker proteins Alix, TSG101, CD63, CD81, Flotilin-1 and Prominin-1.

We next examined the protein profile of SCUF-sMVs (Table 1C, Table 2). Of the 606 proteins identified by GeLC-MS/MS, 350 are selectively enriched (Fig. 4A, Table 1C). Conspicuously, the ATP-binding cassette protein ABCE1, an inhibitor of endoribonuclease activity [70] and member of the ABC reporter protein family, is selectively enriched in SCUF-sMVs while other members of the ABC reporter family (e.g., ABCB1, ABCG2) are found in EV subtypes; by contrast, ABCC2 is selectively enriched in SCUF-Exos. It is interesting to note that while human urinary exosomes are enriched for the ABC transporter proteins ABCB1, ABCC9, and ABCB11 [71], sMVs from human breast MCF-7 cells [72] and rat NHI6F Tu28 pancreatic beta cells [73] lack this family of proteins. Of note was the finding by Bebwary and colleagues that sMVs from drug (doxorubicin)-resistant MCF-7 cells are selectively packaged with P-gp/ABCB1 and cytoskeletal elements that might be implicated in P-gp stabilisation within sMVs [73]. Along these lines, it is noteworthy that SCUF-sMVs are selectively enriched with cytoskeletal elements [74] such as microtubules (e.g., TUBB (Rsc -1.9), TUBB2C (Rsc -1.8), TUBA4A (Rsc -1.7), TUBA1B (Rsc -1.4), and septins (e.g., SEPT2 (Rsc -7.1), SEPT9 (Rsc -2.1), SEPT11 (Rsc -3.1)), along with their associated binding partners (Table 2A). A salient finding was the exclusive identification of septins-2, -9, and -11 in SCUF-sMVs. Septins form oligomeric assemblies that are multifunctional – for example, they can act as scaffolds at the plasma membrane to accumulate proteins (e.g., receptors, transporters) and promote their functional interaction, and act as diffusion barriers to compartmentalize membrane proteins to specific cellular domains [74]. To our knowledge, this is the first report of septins in sMVs – and given their absence in SCUF-Exos warrant further investigation as potential makers of sMVs. Likewise, the kinesin-like protein KIF23 [75] and chromosome segregation 1-like protein/exportin-2

(CSE1L/XPO) [76] which are uniquely enriched in SCU-sMVs based on GeLC-MS/MS (and confirmed by western blot analysis, Fig. 5) are potential markers of sMVs. That some select cytoskeleton/cytoskeleton-associated/motor proteins enriched in the human platelet plasma membrane/cytoskeleton and platelet-derived MPs, are also selectively enriched in SCUF-sMVs (e.g., Actin, Actinin, Dynamin, Myosins, Tubulin, VDAC1/2, Septins), but not all (KIF23, CSE1L) [77,78]. Interestingly, Cav-1, which was identified as oncosome cargo [79], and a circulating EV marker of metastatic prostate cancer [80], was not identified in either LIM1863 SCUF-Exos or -sMVs but seen in colon cancer SW620 cell-derived exosomes [53].

We next looked at vesicle internalisation-associated proteins exclusively enriched in SCUF-sMVs (Table 2C). Of these, the most prominent were the clathrin adaptors (AP2A1, AP2G1, AP2M2, and AP2B1) and cargo-specific adaptors. We further report the identification of the endocytic adaptor phosphatidylinositol-binding clathrin assembly protein (PICALM, Rsc -2.1), and cargo-specific adapters PCSK9 (Rsc -10.1) and PPP2R1A (Rsc -3.6) in sMVs. Although exosome internalisation by recipient cells has been reported to occur via multiple processes such as phagocytosis , clathrin-mediated endocytosis , macropinocytosis , receptor-mediated , and direct fusion , further studies are required to improve our understanding of underlying mechanisms of sMV recipient cell recognition, internalisation and uptake. In this regard, it has been suggested that internalized microparticles occur within a few hours through a process involving anionic phospholipids, lactadherin and αvβ3 integrin.

We find several key components in our LIM1863 SCUF-sMV/-Exos datasets that are implicated in colorectal cancer (Supplementary Table S5) – for example, carcinoembryonic antigen (CEACAM1, CEACAM5) [81] and cadherin-17 (CDH17) [82]. In comparison, these proteins were found enriched in both SCUF-EV subtypes. Several tumour suppressor proteins (e.g., RAB7A [83], N-myc Downstream Regulated Gene 1 (NDRG1) [84]) were identified in both SCUF-EV subtypes. To our knowledge, this is the first report of NDRG1 in sMVs.

One of the important questions in the EV field is whether Exos and sMVs have biologically distinct activities. While previous reports have demonstrated that tumour-derived exosomes promote invasiveness of recipient breast cancer cells [55] and fibroblast differentiation into tumour-promoting stromal myofibroblasts [85,86], breast cancer-derived sMVs mediate breast cancer invasion [87], and breast cancer cell-derived Exos/sMVs in macrophage-induced cancer cell invasiveness [59], interpretation of these data are confounded by the lack of vesicle purity (i.e. possible heterogeneous mixtures of sMVs/ Exos). To address this question we asked whether SCUF-sMVs and SCUF-Exos are functionally distinct in modulating cell invasiveness of recipient fibroblast cells. In our study, while both SCUF-sMVs and SCUF-Exos showed invasive activity in recipient NIH3T3 cells (Fig. 6), SCUF-sMVs exhibited greater invasive activity (~3-fold) than SCUF-Exos. Differential enrichment of invasion, migration, and motility-related components in SCUF-Exos and SCUF-sMVs (Supplementary Table S3) suggest that these EV sub-populations may promote invasion using different mechanisms.

Finally, do our observations provide any clues to how many types of naturally occurring EVs there are, and how they differ from one another? Using DLS we show that there are two classes of EVs released from LIM1863 cells into culture media – one subtype that is essentially homogeneous in size (30-100 nm diameter), the other heterogeneous in size (30-1300 nm diameter). These two EVs represent >95% of all EVs released from LIM1863 cells. We were able to isolate these two EV subtypes using sequential centrifugal ultrafiltration in analytical amounts sufficient to perform MS-based proteome analysis. In comparison, the 30-100 nm diameter subtype (SCUF-Exos) contained exosomal marker proteins (Alix, TSG101, CD81, CD63) whereas the 30-1300 nm diameter subtype (SCUF-sMVs) lacked these markers; both EV subtypes were round shaped. Protein profiling of SCUF-Exos and -sMVs by GeLC-MS/MS identified 98 and 350 proteins to be selectively enriched in these two EV categories, respectively. The 98 proteins identified in SCUF-Exos were in agreement with our previous findings [33]. Our findings for SCUF-sMVs reveal the most comprehensive protein dataset to date (350 uniquely enriched in sMVs in comparison with Exos) for this EV subtype and provide the basis for identifying specific protein markers for sMVs. Further, this study provides a novel methodology that results in the isolation of highly-purified EVs from LIM1863 colon cancer cells that are functionally distinct. The analytical SCUF method we have developed is potentially scalable using tangential flow filtration and provides a solid foundation for future in-depth functional studies of EV subtypes from diverse cell types and an increased range of functional assays.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Isolation and characterization of EVs using differential centrifugation (DC) (A) DC was employed to isolate EVs (sMVs and Exos) from human colon cancer LIM1863 cell culture medium (CM). Cells were grown in serum-free medium with insulin-transferrin-selenium (ITS, 0.6%) for 24 h, and 900 mL CM collected, centrifuged, and concentrated (CCM) to 22 mL; protein yield 41.6 mg. To prepare 'total EVs' 1 mL of CCM was centrifuged at 100,000*g* for 1 h (338 μg). For DC-sMVs and DC-Exos 1 mL of CCM was centrifuged at 10,000*g* for 30 min (DC-sMVs, 187 μg) and sequentially ultracentrifuged at 100,000*g* for 1 h (DC-Exos, 219 μg). (B) For Western blotting, DC-sMVs and DC-Exos (15 μg protein load) were separated by 1D-SDS-PAGE, electro-transferred, and membrane probed with anti-mAbs directed to Alix, TSG101, CD63, CD81 and EPCAM. (C) Cryo-electron micrographs of DC-sMVs and DC-Exos; the scale bar represents 100 nm. (D) Size distribution of DC-sMVs and DC-Exos measured manually from cryo-electron micrographs (~200 measurements from 5 fields of view).

Fig. 2. Isolation and characterization of EV subtypes using sequential centrifugal ultrafiltration (SCUF). (A) SCUF was employed to isolate EVs (sMVs and Exos) from human colon cancer LIM1863 CCM (20 mL, 37.8 mg). CCM was fractionated based on a combination of molecular pore-sized ultrafilters (Durapore™ Ultrafree-CL, Merck Millipore) ranging from 0.65, 0.45, 0.22, and 0.1 μm. SCUF-sMVs were isolated using 0.65 μm membrane filter (Fn 1, 836 μg). Following sequential ultrafiltration of the <0.65 μm filtrate, SCUF-Exos were isolated using a 0.1 μm membrane filter (Fn 5, 1328 μg). (B) For Western blotting, SCUF-Exos and SCUF-sMVs (15 μg) were separated by 1D-SDS-PAGE, electro-

transferred, and membrane probed with anti-mAbs to Alix, TSG101, CD63, CD81 and EpCAM.

Fig. 3. Characterization of SCUF-EVs using DLS and cryo-EM. (A) SCUF-Exos and (B) SCUF-sMVs were visualised by cryo-EM. Scale bar shown for individual size ranges. (C) Size distribution of SCUF-Exos and SCUF-sMVs (manually measured from ~200 cryo-electron micrographs from 5 fields of view. (D) CCM, SCUF-sMVs and SCUF-Exos were analysed by dynamic light scattering. Mean hydrodynamic diameter of EVs was calculated by fitting a Gaussian function to the measured size distribution.

Fig. 4. Proteomic characterisation of SCUF-Exos and SCUF-sMVs. (A) A two-way Venn diagram of SCUF-Exos and SCUF-sMVs reveals 256 proteins commonly identified, while 350 and 98 proteins were uniquely identified in SCUF-sMVs and SCUF-Exos, respectively (Supplementary Table S1). (B) A two-way Venn diagram of datasets with mitochondrial-associated proteins identified in SCUF-Exos (Mito) and SCUF-sMVs (Mito). UniProt annotation (e.g., cellular compartment, subcellular location) identified mitochondrial-associated proteins (these mitochondrial-associated proteins from the SCUF-sMV and –Exos datasets are given in Supplementary Table S1). (C) Semi-quantitative normalized spectral count ratios (Nsc) of selected proteins including (i) exosome markers, (ii) cytoskeleton network components, and (iii) cargo trafficking and sorting proteins. For each individual protein, significant peptide MS/MS spectra were normalized by the total number of significant peptide MS/MS spectra identified in the sample. The ratio serves an indicator of proteins abundance, i.e. the higher the ratio, the more abundant the protein within the sample.

Fig. 5. Validation of SCUF-sMVs proteins KIF23 and CSE1L. Western blotting was performed (15 μg protein) for DC-sMVs, DC-Exos, OptiPrep™ density gradient separated (DG)-sMVs (1.19 g/mL fraction) and DG-Exos (1.10 g/mL fraction), and probed with anti-mABs directed to KIF23, CSE1L, and TSG101.

Fig. 6. SCUF-sMVs from LIM1863 colon cancer cells induce higher invasive activity in recipient fibroblast cells than SCUF-Exos. NIH3T3 cells stimulated with serum-free DMEM medium containing SCUF-sMVs, SCUF-Exos or vehicle alone were harvested and overlaid onto Matrigel-coated Transwell inserts (Materials and methods). The lower chamber contained DMEM (5% FCS, P/S) supplemented with SCUF-sMVs, SCUF-Exos or vehicle alone. Post 16 h incubation, cells were fixed, stained with Hoechst and the numbers of invading cells at the bottom of Transwell inserts were imaged and quantified using Image J software v 1.49e. Error bars represent s.e.m (*** $p<0.0005$). Data representative of 3 independent experiments. Scale bar = 50 μm.

Fig. 1. Isolation and characterization of extracellular vesicles (EVs) using differential centrifugation

(color only online)

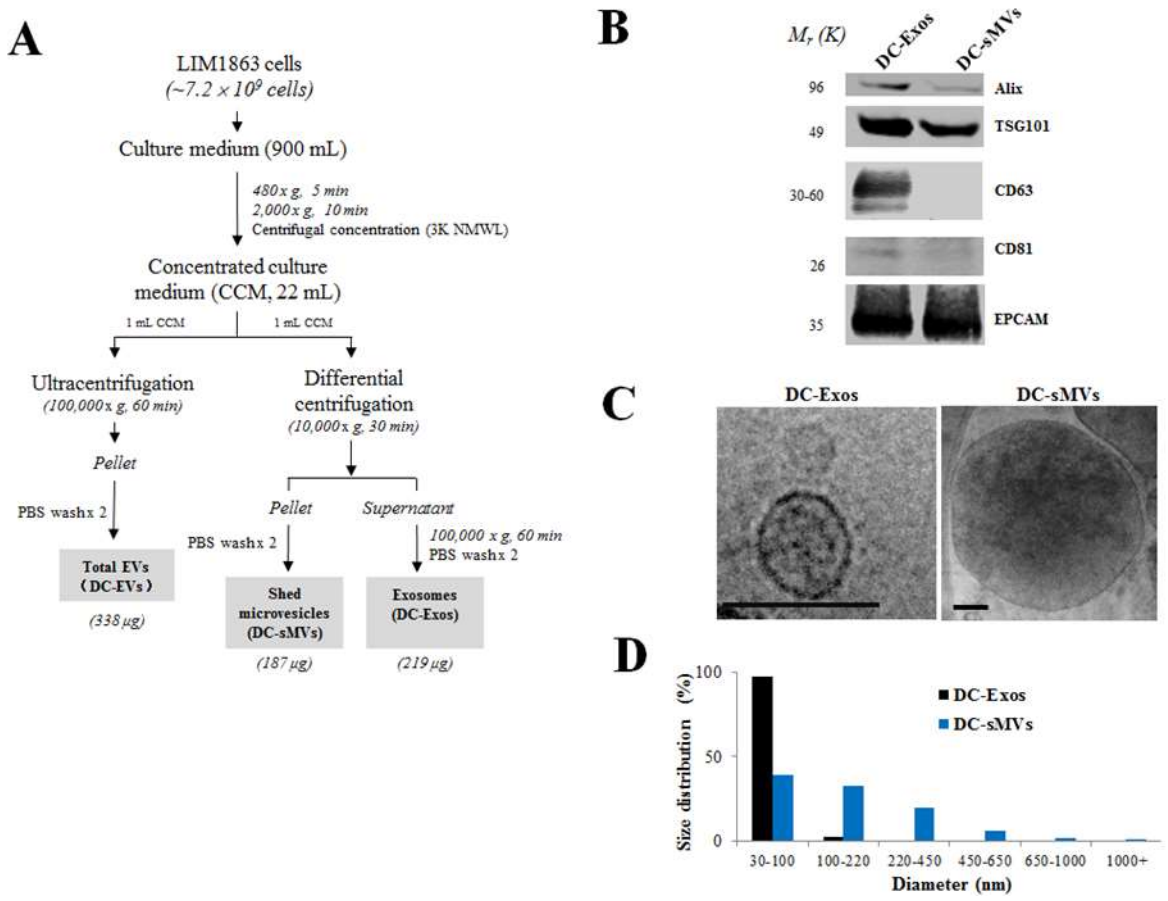


Fig. 2. Isolation of distinct EVs using sequential centrifugal ultrafiltration (SCUF)

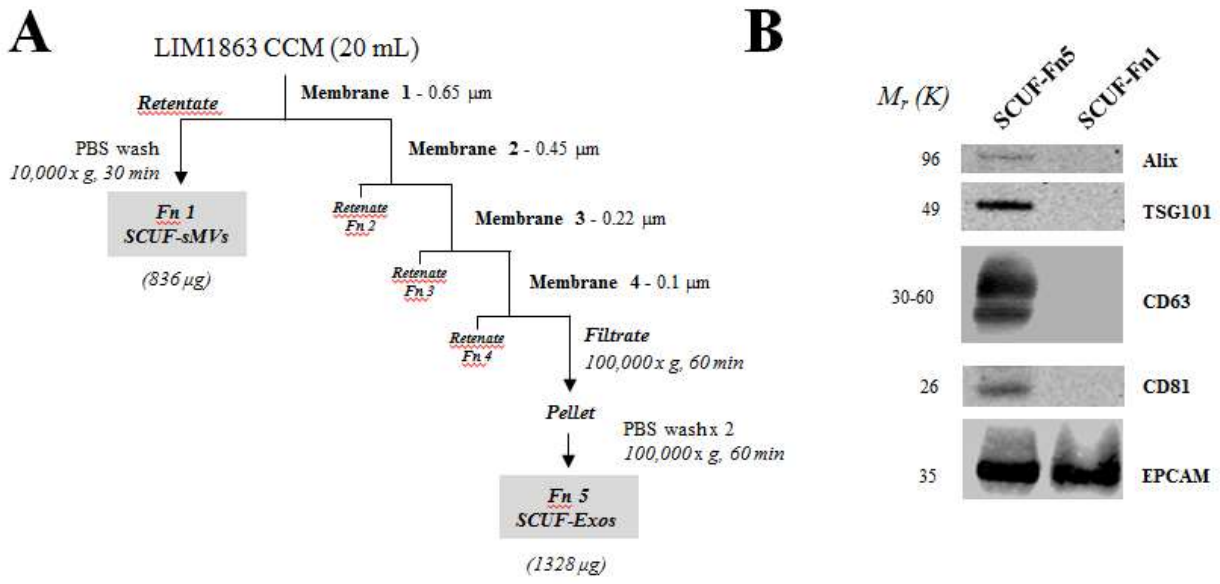
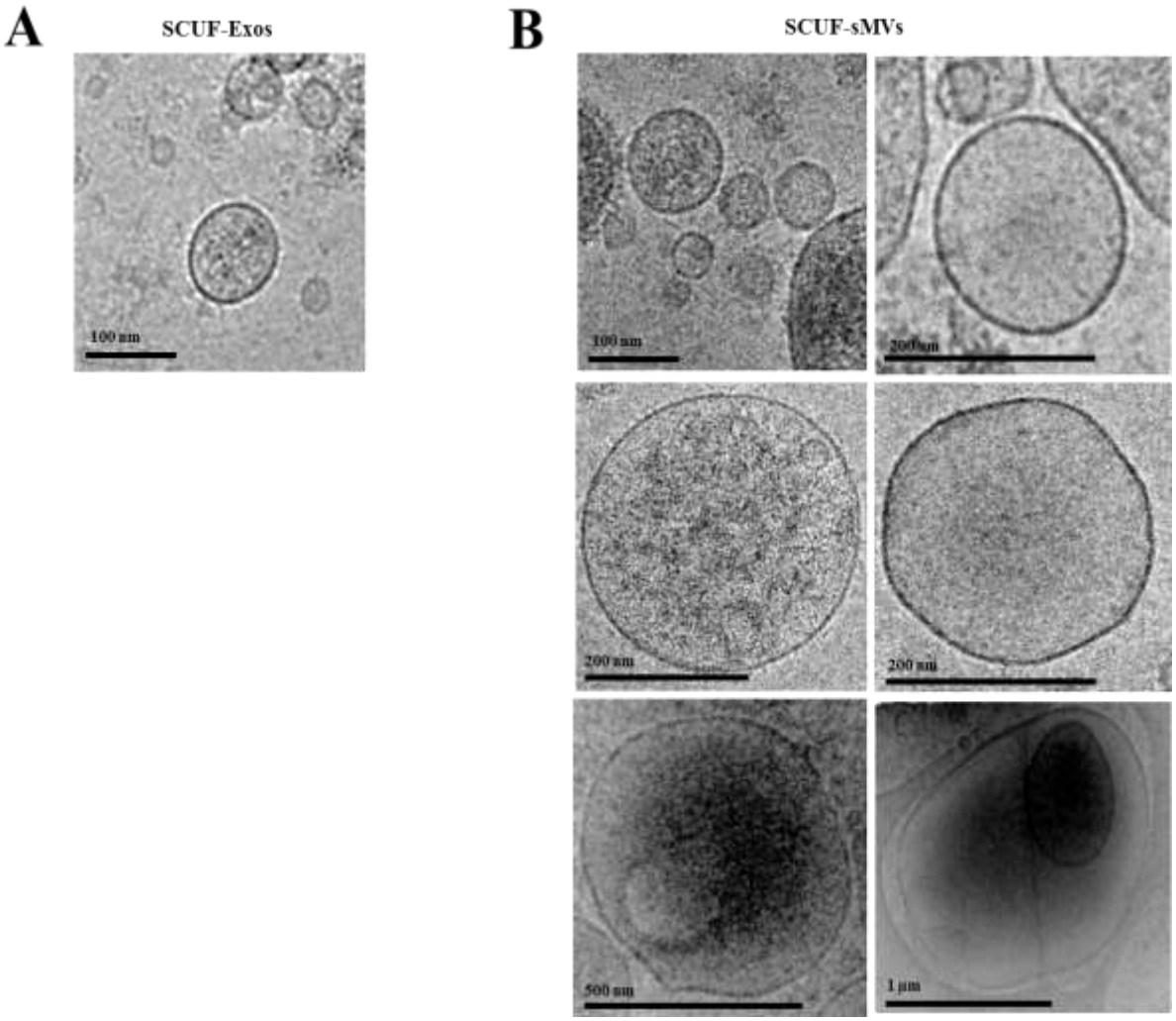


Fig. 3. Characterization of distinct EVs isolated by sequential centrifugal ultrafiltration



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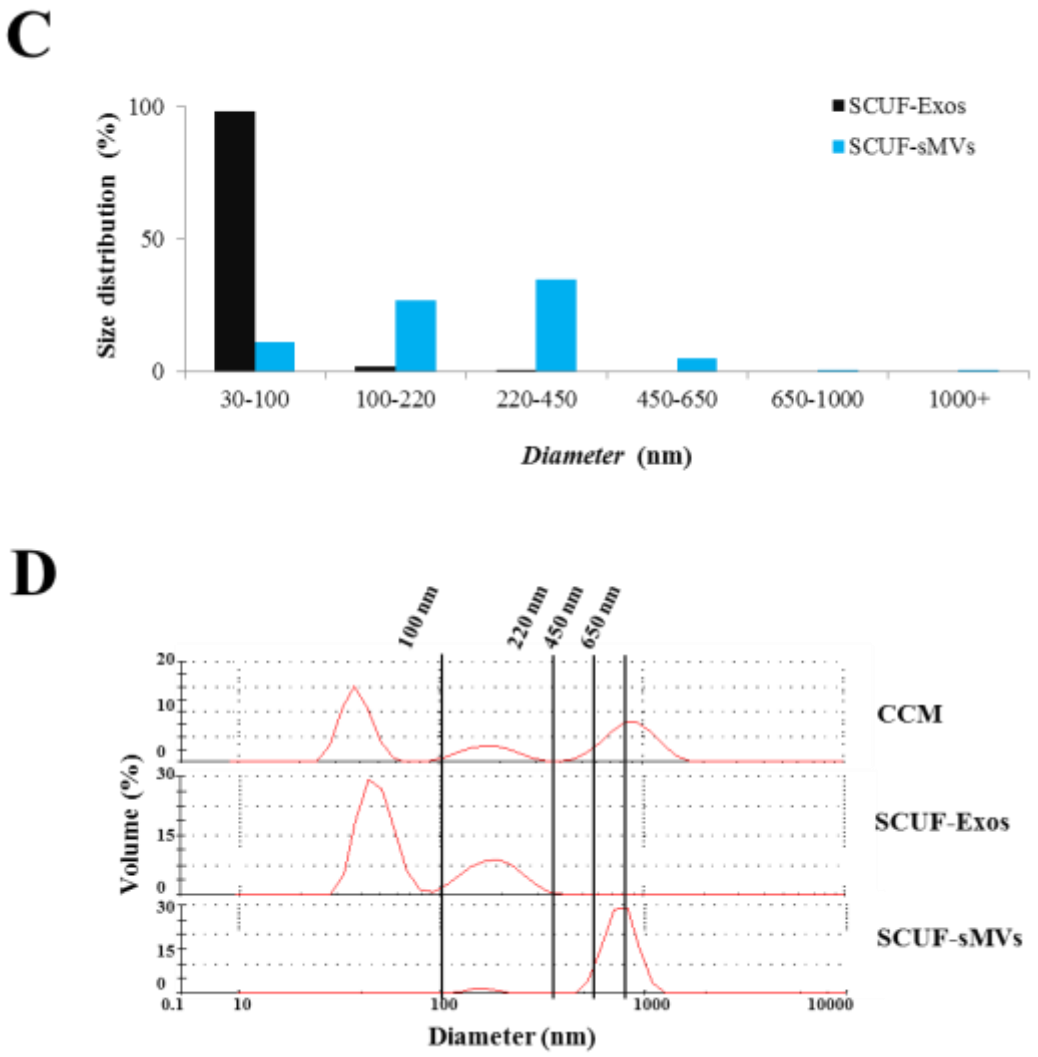
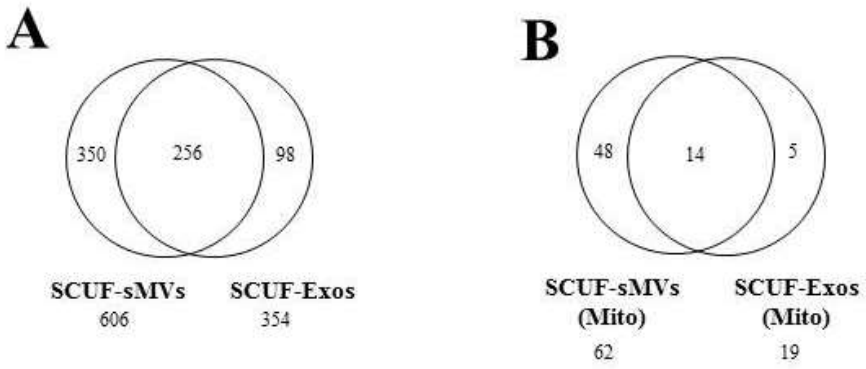


Fig. 4. Proteome analysis of LIM1863 cell derived SCUF-Exos and SCUF-sMVs.



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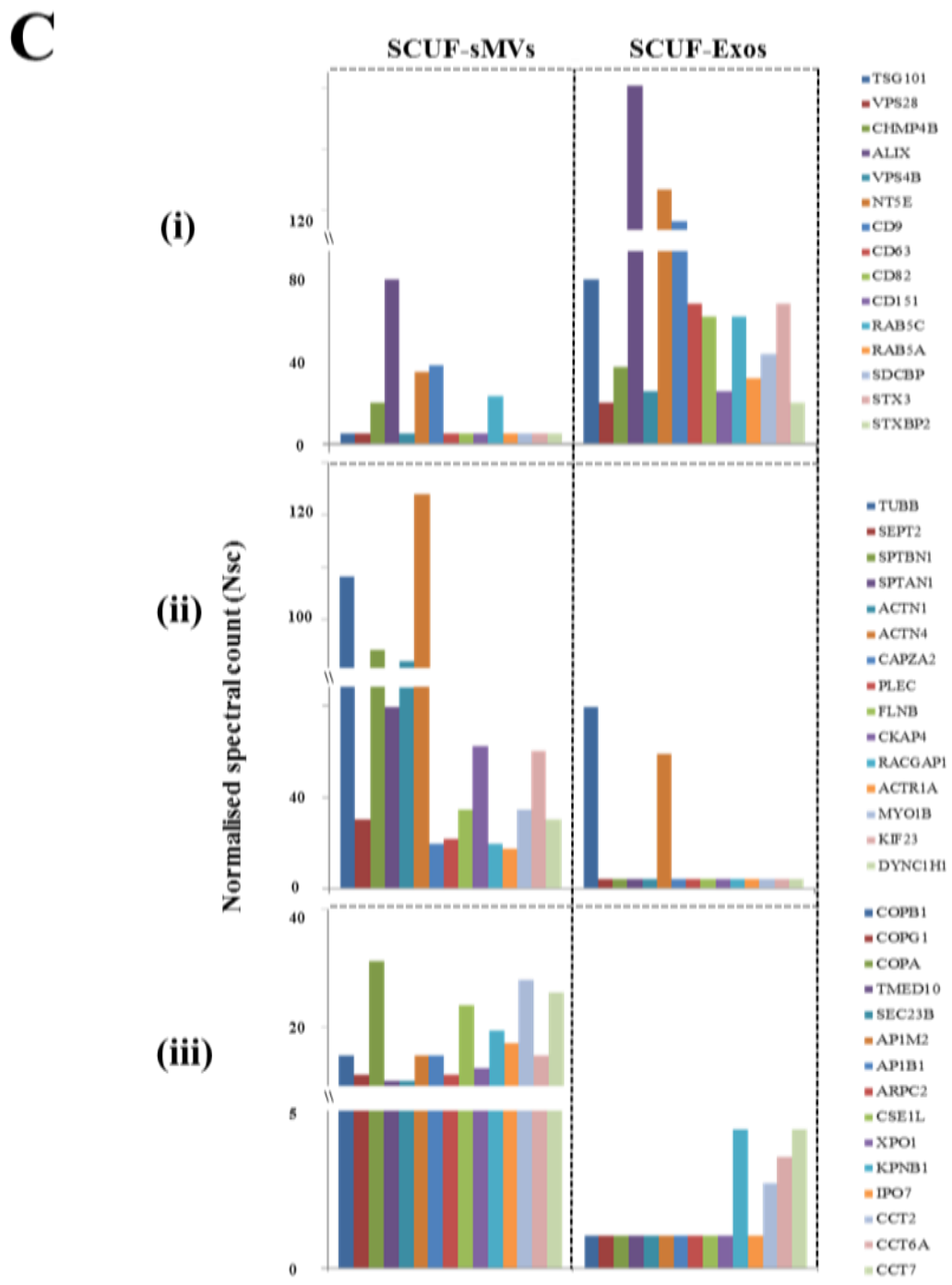

Fig. 5. Validation of sMVs markers: KIF23 and CSE1L.

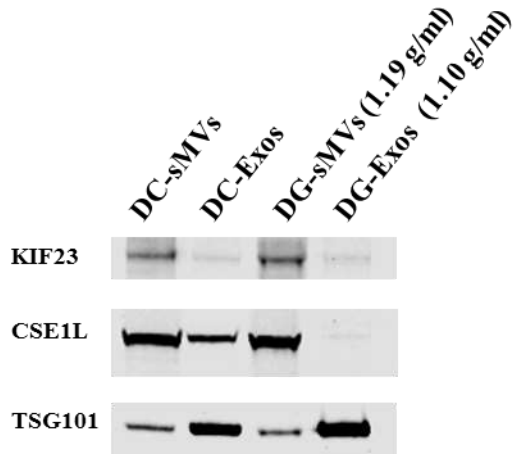

Fig. 6. SCUF-sMVs and SCUF-Exos promote invasiveness

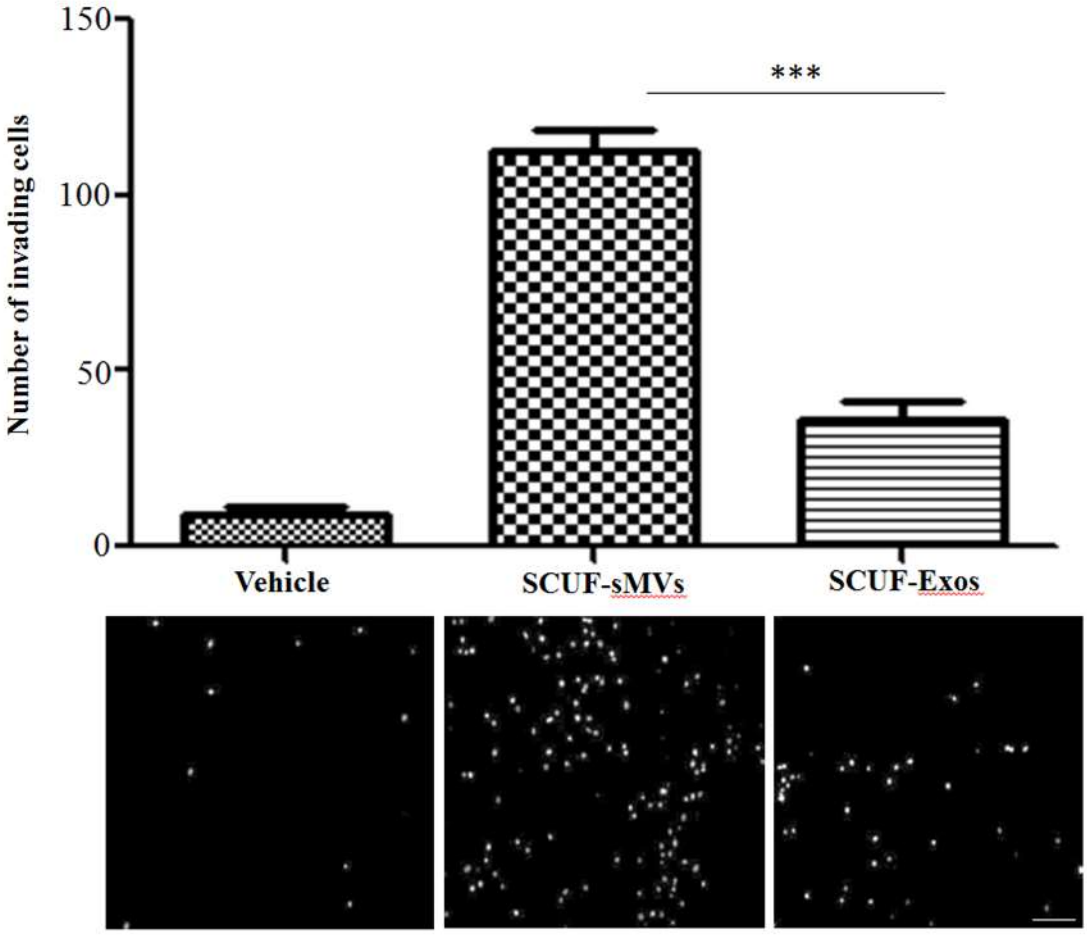

Table 1 - Relative quantification by label-free spectral counting of selected proteins from LIM1863 cell-derived EV subtypes

Table 2 - Categorization of selected proteins enriched in SCUF-sMVs

Table 3 - Categorization of selected proteins enriched in SCUF-Exos