Human myeloma cell- and plasma-derived extracellular vesicles contribute to functional regulation of stromal cells

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Abstract

Circulating small extracellular vesicles (sEV) represent promising non-invasive biomarkers that may aid in the diagnosis and risk-stratification of multiple myeloma (MM), an incurable blood cancer. Here, we comprehensively isolated and characterized sEV from human MM cell lines (HMCL) and patient-derived plasma (psEV) by specific EV-marker enrichment and morphology. Importantly, we demonstrate that HMCL-sEV are readily internalised by stromal cells to functionally modulate proliferation. psEVs were isolated using various commercial approaches and pre-analytical conditions (collection tube types, storage conditions) assessed for sEV yield and marker enrichment. Functionally, MM-psEV were shown to regulate stromal cell proliferation and migration. In turn, pre-educated stromal cells favour HMCL adhesion. psEV isolated from patients with both pre-malignant plasma cell disorders (monoclonal gammopathy of undetermined significance [MGUS]; smouldering MM [SMM]) and MM have a similar ability to promote cell migration and adhesion, suggesting a role for both malignant and pre-malignant sEV in disease progression. Proteomic profiling of MM-psEV (305 proteins) revealed enrichment of oncogenic factors implicated in cell migration and adhesion, in comparison to non-disease psEV. This study describes a protocol to generate morphologically-intact and biologically functional sEVs capable of mediating the regulation of stromal cells, and a model for the characterization of tumour-stromal cross-talk by sEV in MM.

Statement of significance

Characterising the molecular content and functions of small extracellular vesicles has the potential to inform new modalities for diagnosis, risk stratification, monitoring and therapeutic intervention in MM. Here we describe MM cell line- and plasma-derived extracellular vesicles and their capacity to functionally modulate stromal cells. A key finding was the proliferative, migratory and adhesive modulation by sEV derived from both pre-malignant plasma cell disorders and MM. Our study also provides molecular insights into sEV proteomes of plasma derived from patients with MM compared to healthy individuals. These findings will direct future studies seeking to understand the mechanisms regulating pro-tumorigenic signalling by extracellular vesicles and have implications in clinical utility as next-generation liquid biopsy biomarkers in myeloma diagnosis and management.

1. Introduction

Multiple myeloma (MM) is a highly heterogenous and complex blood cancer that originates from the clonal expansion of plasma cells (PCs) in the bone marrow (BM) [1-3]. MM remains incurable despite advances in its treatment [4]. Current recommendations are observation for both MGUS (monoclonal gammopathy of undetermined significance) and smouldering MM (SMM) [5-7] with therapy usually initiated at the onset of MM-related symptoms [2, 4, 8, 9]. Although early intervention has provided survival advantage for high-risk SMM patients in clinical trials [10, 11] it is not yet routinely recommended due to a lack of sufficiently accurate and agreed upon risk stratification strategies. A better understanding of the molecular characteristics that define transition of MGUS-to-MM and to identify patients at risk of progression from SMM to MM would provide a framework for more effective treatment and improved patient outcomes.

Circulating extracellular vesicles (EVs) containing tumour-specific molecular signatures (oncoproteins, mRNAs, long noncoding RNAs and DNA fragments) have potential clinical utility as next-generation biomarkers for liquid biopsy in cancer diagnosis and management [12-17]. In the context of MM, liquid biopsies enable the characterisation of spatial heterogeneity and clonal evolution [18-24], and may represent an attractive alternative to the single-site tissue biopsies usually employed in the evaluation of MM [8, 18, 21, 22, 24, 25]. Specifically in MM, a role for large EVs (plEV; EVs shed from the plasma membrane) as predictive or prognostic biomarkers has been demonstrated from patient blood [17, 18, 26, 27], while several key studies have reported the diagnostic potential of small EVs (psEV; EVs with endosomal origin) for MM [26, 28-31].

The isolation of psEV from biofluids such as blood remains challenging [Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. Cell Mol Life Sci. 2019 Jun;76(12):2369-2382], with highly abundant proteins (HAP) such as human serum albumin (HSA) or other 'contaminants' including protein complexes and subcellular fragments often co-isolated and thus impairing the enrichment of pure low-abundant particles (i.e., psEV) [32-37]. Pre-analytical factors such as the type of anticoagulant, storage of biospecimens, blood collection procedure, and patients-related factors (e.g., medication, exercise) are also known to affect the amounts and content of psEV, which further complicates their recovery [34-36, 38-40]. It is for these reasons, that commercially available kits represent valuable tools for rapid isolation of psEV [41-43].

Tumor cell-derived small EVs (sEV) have been shown to induce phenotypic changes to non-tumor cells by transferring their bioactive cargo (e.g., proteins, nucleic acids) at both paracrine and systemic levels, thereby generating a tumor microenvironment that is permissive for tumor growth and metastasis [44-47]. sEV have also been shown to contribute to the dynamic co-evolution of MM PCs and the BM microenvironment favouring MM progression and drug resistance [46, 48, 49]. Accumulating evidence suggest a role for MM PCs-derived EVs in bone remodelling and breakdown [48, 50, 51] (a characteristic feature of MM [52]) and enhanced pro-tumoral activity of MM-BM stromal cells [49, 50, 53, 54]. However, the specific EV-driven molecular mechanisms that promote PCs homing to selected distant sites (pre-metastatic niches) during MGUS-to-MM evolution remain undefined.

In this study, we have evaluated both patient- and human MM cell line (HMCL)-derived EVs to understand their bioactivity and composition in the context of tumour and stroma crosstalk. We provide insights into plasma-derived sEV proteomes from patients with MM compared to

those from healthy individuals. The demonstration of the oncogenic potential of sEV has implications for both our understanding of MM pathogenesis and for the recognition and development of next-generation biomarkers via liquid biopsy in MM diagnosis and management.

2. Materials and methods

2.1 Cell culture

Paired HMCL contemporaneously derived from the BM and pleural effusions (PE) of 2 MM patients, KMS12BM-KMS12PE and KMS28BM-KMS28PE, were kindly provided by Dr. Takemi Otsuki, Kawasaki Medical School, Japan. HMCL were regularly authenticated by CellBank Australia with short tandem repeat profiling, in line with the standard ANSI/ATCC ASN-0002-2011 and matched publicly available data. HMCL ($2x10^5$/mL) were grown in RPMI-1640 media (Invitrogen) supplemented with 10% (v/v) EV-depleted foetal bovine serum (FBS; Invitrogen), 1% (v/v) Penicillin Streptomycin (P/S, Life Technologies) and maintained at 37 °C with 5% CO2[55]. FBS was EV-depleted by ultracentrifugation (UC) at 100,000 x g, 18 h (4 °C) [56]. The human stromal cell line HS5 (CRL-11882™), obtained from ATCC® (CRL-11882), was routinely cultured in DMEM media (Invitrogen) supplemented with 10% (v/v) EV-depleted FBS, 1% (v/v) P/S and maintained at 37 °C with 5% CO_2. Cells were passaged using Tryple Express (Thermo Fisher).

2.2 Human blood collection and plasma preparation

Blood specimens were collected from 3 healthy individuals (non-disease, ND), and 9 (3 per cohort) MGUS, asymptomatic SMM, newly diagnosed symptomatic MM patients (disease, D), following written informed consent and approval from the Alfred Hospital Research and Ethics

Committee. All samples were acquired through collection of whole blood in ethylenediaminetetraacetic acid (EDTA) or STRECK RNA Complete BCT™ (sRNA) tubes. After blood collection, tubes were immediately inverted 4–5 times and transported vertically at room temperature (RT) without agitation. To obtain platelet-free plasma (PFP), PBPL was centrifuged at 1,800 x g for 10 min, followed by 2,000 x g, 15 min (4 °C) [57]. PFP was aliquoted (1 ml) and used immediately for isolation of EVs or stored at −80°C.

2.3   Isolation and purification of cell-derived EVs

EVs were harvested from HMCL (KMS12BM and KMS12PE) cultured in 2D classic flasks (175 cm^2). Semi-confluent cells (70-80%) were washed three times with PBS and cultured for further 24 h in 35 mL of serum-free RPMI-1640 medium. Following this period, cell culture media was collected and initially processed (1200 rpm, 5 min, RT), with the supernatant further centrifuged (500 x g, 5 min followed by 2000 x g, 10 min, 4 °C) to remove residual cells and cell debris, with the supernatant stored at −20 °C [58]. Supernatant for each cell line were thawed and centrifuged at 10,000 x g for 30 min to pellet large EVs (lEV) while the supernatant was retained for sEV isolation [56]. Large EVs were resuspended in 500 μL of filtered PBS (0.2 μm) and re-centrifuged at 10,000 x g for 30 min for a total of 3 washes. The final lEV pellet was resuspended in 100 μL of PBS [58].

The supernatant was further centrifuged at 100,000 x g for 1 h to pellet crude sEV and resuspended with 400 μL of PBS [56, 58]. After one PBS wash, 400 μL of crude sEV was subjected on top of prepared OptiPrep™ (iodixanol solution) density gradient (DG), and separation performed as described [58]. Briefly dilutions were made in 0.25 M sucrose/1 M Tris (pH 7.5) solution and an OptiPrep™ density gradient was prepared by adding 3 mL of 40, 20, 10, and 5% of iodixanol solution to 14 × 89 mm polyallomer tubes (Beckman Coulter). These tubes were centrifuged at 100,000 x g for 18 h at 4 °C. Twelve fractions (1 mL for each

fraction) were collected individually from the top, and washed with PBS by centrifugation at 100,000 x g for 1 h (4 °C). PBS was removed and pellets were resuspended with 50 µL PBS. Purified sEV containing fractions were determined based on western blot analysis of exosome markers TSG101 and CD81, and fraction 7 subsequently stored at -80 °C until further use (defined as sEV) [59].

2.4   Isolation of plasma-derived EVs

EVs were isolated from 1 mL of fresh or frozen PFP collected in EDTA or sRNA tubes. If frozen PFP was utilized, samples were quickly thawed in a water bath or dry heating system at 37 °C, followed by centrifugation at 5000 x g for 15 min (4 °C) to remove cryoprecipitates [60]. plEV were isolated as described in section 2.3 above, while psEV were isolated using three different commercial methods following manufacturer's instructions. In brief, for the Purification Mini Kit (#57400, Norgen Biotek Corporation), PFP was mixed with 3 mL of Nuclease-free water, 100 µL of ExoC buffer and 200 µL of Slurry E by vortexing for 10 sec. After 5 min incubation, the mixture was centrifuged at 2000 rpm for 2 min and the supernatant discarded. The pellet was then resuspended in ExoR buffer, incubated for 5 min and centrifuged at 500 rpm for 2 min. The supernatant was transferred to a Mini Filter Spin column and centrifuged at 6000 rpm for 1 min to collect EV-eluates.

For exoEasy™ (#76064, Qiagen), PFP was filtered (0.22 µm) to exclude large particles, mixed with 1 mL XBP buffer and added into the exoEasy spin column. After centrifugation at 500 × g for 1 min at RT, the flow-through was discarded. Then, 10 mL XWP buffer was added and the column was centrifuged at 5000 × g for 5 min to remove residual buffer from the column. Next, 400 µL XE buffer was added to the column membrane followed by incubation for 1 min. The column was centrifuged at 500 × g for 5 min to collect EV-eluates.

For ExoQuick™ ULTRA (#EQULTRA-20A-1, SBI), 8 μL of highly purified thrombin (final concentration 5U/mL; #TMEXO-1, SBI) were added to 1 ml PFP to defibrinate it, making the resulting supernatant compatible with the ExoQuick™ precipitation buffer. Then 268 μL of precipitation buffer were added into thrombin-treated PFP and the mixture was incubated for 30 min at 4°C. Afterwards, the sample was centrifuged at 3000 × g for 10 min (4 °C). The supernatant was removed carefully and the pellet containing EVs was resuspended in 200 μL of Buffer B, proceeding to protein quantitation before adding 200 μL of Buffer A. The mixture or up to 4 mg of total protein content was then added into a purification column and mixed at RT on a rotating shaker for 5 min. EV-eluates were then collected by centrifugation at 1000 x g for 30 sec.

EV-eluates were aliquoted depending on intended use, and either used immediately or stored at -80 °C. Low protein binding tubes were utilized for all EV processing steps to minimize protein sample loss [56].

2.5   Depletion of albumin from plasma derived EV-eluates

HSA depletion was performed on psEV using Pierce™ Albumin depletion kit (#85160, Thermo Fisher) following manufacturer's instructions. In brief, 50 μL of psEV eluates obtained using the Norgen™ kit were added on top of 400 μL resin into a spin column and incubated for 1-2 min. The column was then centrifuged at 12,000 × g for 1 min. The flow-through was reapplied to the spin column, incubated for 1-2 min to ensure maximal HSA binding and centrifuged at 12,000 × g for 1 minute. The flow-through which contained HSA-depleted EV samples was retained and labelled as fraction 1 (F1). 50 μL of Wash-Buffer were then added to the same column to collect residual unbound EVs and centrifuged at 12,000 × g for 1 min. The flow-through (fraction 2, F2) was retained separately from F1. The wash step was repeated

to collect fraction 3 (F3). Fractions containing small EVs were then analysed separately and in combination by western blotting utilizing antibodies anti-HSA and anti-CD81.

2.6   Transmission electron microscopy

EV morphology was analysed using transmission electron microscopy (TEM) as described [61]. Briefly, EV samples (1-2 μg protein) were loaded to 400 mesh carbon-coated copper grids (#GSCU400CC, ProSciTech) for 2 min. Excess sample was removed by blotting, and 2 μL of 2% uranyl acetate solution added on the grids for 10 min to negatively stain. Grids were air dried the and images were viewed using a JEOL JEM-2010 transmission electron microscope operated at 80 kV.

2.7   Nanoparticle tracking analysis

Particle size distribution of EVs was assessed by nanoparticle tracking analysis (NTA) using both NanoSight NS300 system (NanoSight technology, United Kingdom) equipped with a 405 nm laser, sCMOS camera and syringe pump as described [61], and Zetaview PMX-120 (Particle Metrix GmbH, Germany) as described [62]. Briefly, for NanoSight, EVs (1 μg/mL in water) were injected into a flow-cell top plate by a syringe pump with detection threshold = 10, flowrate = 50 and temperature = 25 ºC. Three videos (60 sec per video) were recorded, and analyzed by NTA software (Build 3.1.45). For Zetaview, EVs were loaded into the cell and 11 cell positions were scanned with 60 frames per position were captured. Videos were analyzed by in-build ZetaView software (8.02.31).

2.8   Protein quantification and Western blotting

Protein lysates were incubated with RIPA buffer on ice for 1 h before being centrifuged at 16,000 x g for 15 min at 4 °C to remove insoluble debris. Protein concentration was measured

by microBCA assay (#23235, Thermo Fisher). Western blotting was performed using 4–15% w/v MiniProtean TGX Precast/Stain-Free Protein Gels (Bio-Rad) and Bio-Rad wet transfer system, with primary antibodies (1:1000 dilution); rabbit anti- CD63 (#ab134045, Abcam), ITGA2B (#13807S, Cell Signaling Technology), HSA (#ab207327, Abcam), and mouse anti-ALIX (#2171S, Cell Signaling Technology), TSG101 (#612696, BD Biosciences), CD81 (#7637, Santa Cruz Biotechnology), SELP (#8419, Santa Cruz Biotechnology), TUBA1A (#T9026, Sigma-Sigma-Aldrich). Secondary antibodies (Dako, Campbellfield, Victoria, Australia); anti-mouse (#P0447) or anti-rabbit (#P0217). Blots were visualised using Supersignal^{TM} west pico PLUS ECL (Pierce, Thermo Fisher) and developed with ChemiDoc^{TM} Touch Bio-Rad Imager. Digital images were analysed with the aid of Image Lab software.

2.9   Live cell multidimensional imaging by confocal microscopy

Vybrant DiO (#V22886, Invitrogen)-labelled sEV were utilized for functional studies and generated by (indirect) labelling of originating cells (HMCL) [63, 64]. HMCL were stained with DiO as per manufacturer's instructions. In brief, 5 μl of DiO solution were added to $1x10^6$ cells resuspended in 1mL serum-free medium and incubated for 20 min at 37 °C with 5% CO^2. After 3 PBS washes (1500 rpm, 5 min, 37 °C) cells were allowed to recover by resuspension in medium supplemented with 10% FBS and incubation at 37 °C for 2 h. CM was then switched to serum-free medium and HMCL were incubated for further 24 h to allow generation of DiO-labelled particles. DiO-sEV were then collected by UC after removal of cells and cell debris and isolation of lEV as described (Section 2.3).

Recipient stromal cells HS5 ($2x10^4$, final volume 100 µL) were cultured in 10-well Cellview slides (#543079, Greiner bio-one) overnight. Cells were then stained with Hoechst (nuclei; #33342, Thermo Fisher) and anti-CD10 [55] (stromal cell PE-conjugated membrane marker;

#IM1915U, Beckman Coulter). CM was then switched to serum-free medium (phenol red free RPMI, #11835030, Thermo Fisher) and three positions per well were chosen for imaging. Subsequently, DiO-small EVs (60 µg/mL; final volume 100 µL), PBS or unstained EVs (control) were added. Images were obtained by using an inverted microscope Nikon A1r confocal microscope (Tokyo, Japan) equipped with a resonant scanner, using an Apo LWD 40x WI λS DIC N2 (numerical aperture 1.15; Nikon, Tokyo, Japan). Images were sequentially acquired at a scan speed of 15 fps with a frame averaging of 8 times to improve signal to noise ratio. The dXY image resolution was 0.21µm and Z interval of 0.5 µm. The images were collected every 30 minutes for 2 h and subsequently every 2 h up to 18 h. HS5 cells were maintained at 37°C, 5% $CO2$. Images were deconvolved using 15 iterations of the TypeLandWeber algorithm using NIS Analysis Software (Nikon, Japan).

2.10   Cell proliferation assay

Cell proliferation was assessed by MTS assay (#G5430, Promega). HS5 cells ($2x10^4$) were seeded in 96-well plate and incubated for 72 h with or without the addition of increasing concentrations of HMCL-sEV (30 µg/mL) or Norgen-psEV (30 µg/mL) to a final volume of 100 µL. Then, cell proliferation was assessed following manufacturer's instructions. Briefly, 20 µL of the provided tetrazolium compound (MTS) mixed with an electron coupling reagent (phenazine methosulfate; PMS) was added to each well and incubated at 37 °C for 2 h. Cell proliferation was then determined by measuring absorbance at 490 nm using a microplate reader.

2.11   Cell migration assay

Cell migration was assessed by wound healing assay [PMID: 31414347] [PMID: 28110712]. HS5 cells ($4x10^5$, final volume 2 mL) were grown in 6-well plates with or without the addition

of 80 µg/mL Norgen^{TM}-psEV for 72 h. A wound was created by scratching (100 µL tips) the centre of the wells. After 3 washes (to remove non-adherent cells and cells debris) cells were cultured for 16 h in serum-free medium to inhibit cell proliferation,. Nuclei were then stained with Hoechst, wounds were observed and images taken with 20x Plan apo objective Nikon TiE microscope (Nikon, Japan) equipped with Zyla sCMOS (ANDOR) and analysed with NIS Analysis Software.

2.12   Cell adhesion assay

HS5 cells (4x10^5, final volume 2 mL) were grown in 6-well plates with or without the addition of 80 µg/ml Norgen-psEV for 72 h. Cultures were then washed 3 times with serum-free medium and DiO-labelled HMCL were added on top of the stromal cell layer for 2 h (1x10^6 HMCL/well). After 5 washes with serum-free medium images were taken with 20x Plan apo objective Nikon TiE microscope (Nikon, Japan) equipped with Zyla sCMOS (ANDOR) and analysed with NIS Analysis Software.

2.13   Statistical analysis

Data are presented as mean ± S.E.M. Statistical significance was determined with the aid of GraphPad Prism$^®$ 8 (p-values <0.05 were considered significant).

2.14   Mass spectrometry-based proteomics of psEV

psEV (2x from ND, heathy donor; 2x from D, MM) were solubilised in 1% (v/v) sodium dodecyl sulphate (SDS), 50 mM HEPES, pH 8.0, and quantified by microBCA. For MS-based proteomics, samples (5 µg) were normalized and prepared in 50 µL of 50 mM HEPES, pH 8.0, and reduced with 10 mM dithiothreitol (DTT) for 45 min at 50°C followed by alkylation with 10 mM iodoacetamide for 30 min at 25°C in the dark. The reaction was quenched to a final

concentration of 20 mM DTT. Sample digest was performed according to single-pot solid-phase-enhanced sample preparation (SP3) method [65]. Briefly, 1 µL of a 50 µg/µl SP3 bead stock (Sera-Mag SpeedBead carboxylate-modified magnetic particles; hydrophobic and hydrophobic 1:1 mix, GE Healthcare Life Sciences, Freiburg, Germany) were added to 50 µL of protein extract and 60 µl absolute ethanol (final concentration of 50%) and incubated for 10 min (1000 rpm) at 24 °C. Tubes were mounted on a magnetic rack; supernatants were removed and beads were washed three times with 80% ethanol (200 uL each). Beads were resuspended in 100 µl 50 mM triethylammonium bicarbonate (TEAB), pH 8.0 and digested overnight with trypsin (1:50 trypsin:protein ratio; Promega, V5111) at 37 °C, 1000 rpm. The peptide and bead mixture was centrifuged at 20,000g for 1 min at 24 °C. The supernatant was collected and acidified to a final concentration of 1.5% formic acid, frozen at -20°C for 30 min, and dried by vacuum centrifugation. Peptides were resuspended in 0.07% trifluoroacetic acid, quantified by Fluorometric Peptide Assay and normalized to 1 µg per 3 µL.

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source in positive mode as described [66]. Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) with a gradient of 2–28% acetonitrile containing 0.1% formic acid over 110 minutes at 300 nL min-1 at 55°C. An MS1 scan was acquired from 350–1,650 m/z (60,000 resolution, 3×106 automatic gain control (AGC), 128 ms injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000 resolution, 1×105 AGC, 60 ms injection time, 28% normalized collision energy, 1.3 m/z quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species were rejected and peptide

match disabled. Selected sequenced ions were dynamically excluded for 30 s. RAW data is available in ProteomeXchange (#PXD021856).

2.15   Data processing and bioinformatics pipeline of psEV

Peptide identification and quantification were performed as previously described [66, 67] using MaxQuant (v1.6.14) with its built-in search engine Andromeda [68]. Tandem mass spectra were searched against Homo sapiens (human) reference proteome (74,811 entries, downloaded 1-2020) supplemented with common contaminants. Search parameters included carbamidomethylated cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. Data was processed using trypsin/P as the proteolytic enzyme with up to 2 missed cleavage sites allowed. The search tolerance and fragment ion mass tolerance were set to 7 ppm and 0.5 Da, respectively, at less than 1% false discovery rate on peptide spectrum match (PSM) level employing a target-decoy approach at peptide and protein levels. Label free quantification (LFQ) algorithm in MaxQuant was used to obtain quantification intensity values and processed using Perseus as described [59]. LFQ intensities were log2 transformed after removing contaminants and reverse identifications. Proteins with no missing values among all sample groups are subjected to two-tail t-test with p-value adjusted at 5% permutation-based FDR. Normalized intensities were log2 transformed, with statistical analyses performed using Student's T-test or ANOVA (q-value <0.05 was considered significant). Gene enrichment functional annotation clustering analysis was performed using DAVID bioinformatics resource [69] and Gene Ontology (molecular function, biological processes). Prism and Rstudio were used for visualization of analysis.

3.   Results

3.1   HMCL secrete EVs

Culture media from cell lines KMS12PE and KMS12BM were utilized for isolation of both large and small EVs. HMCL grow in suspension in culture at a density of $2x10^5$/mL and display a round morphology typical of PCs (**Fig. 1A**). At higher densities cells may grow in clusters and/or (semi) fibroblast-like phenotype (KMS12BM) [70]. EVs were isolated using a combination of differential centrifugation and density gradient fractionation (**Fig. 1B**). Purified sEV (fraction 7, density 1.12 g/mL) expressed stereotypic EV markers TSG101 and CD81 (**Fig. 1C**) [59, 61]. Marker expression was shown to be enriched in comparison to conventional UC indicating purification of sEV using density-based fractionation (**Fig. 1C**). We further demonstrate intracellular contaminants were lowly abundant in sEV compared to parental cell lysates (KMS12PE) based on protein normalisation, including TUBA1A (α-tubulin, intracellular marker) expressed in MM cell lysates (**Fig. 1D**). We further report enrichment of exosome marker protein TSG101 in sEV in comparison to lEV from HMCL (**Fig. 1D**).

We investigated morphology and size distribution of lEV and sEV using TEM and NTA. TEM revealed a heterogenous population of lEV displaying round-like membranous vesicle structures 50–600 nm in size, and NTA revealed particle diameters of 90–340 nm (mean ~180 nm), which is in accordance with typical characteristics reported for lEV [58, 71]. sEV were cup-shaped and displayed a homogeneous size distribution by both TEM (50-170 nm) and NTA (mean ~120 nm) analysis (**Fig. 1E-F**). These data show that sEV released by HMCL are biophysically distinct from lEV. While it is important to discriminate and define the EV population of interest, for the purpose of this work observations related to sEV only will be described.

3.2   Small EVs from HMCL are taken up by human stromal cells

To gain insight into the function of HMCL-sEV, we demonstrated their uptake by human HS5 stromal cells after 18 h incubation using live cell confocal microscopy (**Fig. 2A**). DiO-labelled sEV were generated from a 24 h culture of DiO-labelled KMS12PE and KMS12BM cells. sEV were isolated as described (Section 2.3). Live cell multidimensional fluorescence microscopy revealed that sEV were taken up by HS5 stromal cells (**Fig. 2B-C**). Controls included HS5 cells with PBS or unstained sEV, where signal from sEV (green) could not be detected (**Supplementary Fig. S1**).

3.3   Small EVs from HMCL promote stromal cell proliferation

It is well known that HMCL secrete factors that induce phenotypic changes in stromal cells [72, 73], however, the role of MM-derived sEV in these actions is not well defined [49, 53, 74, 75]. Here, we examined the effects of treating human HS5 stromal cells with HMCL-sEV on cell proliferation (**Fig. 2D**). HMCL-sEV significantly increased cell proliferation after treatment with 30 μg/mL when compared to control (PBS; p=0.0166; **Fig. 2E**). These data demonstrate the involvement of sEV in MM-induced promotion of stromal cell proliferation.

3.4   Resin-based kit outperforms isolation of small EVs from frozen plasma

As HMCL-derived sEV increased the proliferation of stromal cells, we wished to determine whether MM patient-derived sEV could also be biologically active. However, isolating patient-derived sEV from plasma (psEV) represents significant challenges due to the protein dynamic range issue associated with plasma, and the concomitant co-isolation of abundant plasma proteins with sEV [35, 38, 76]. We employed several commercial kits to isolate psEV from frozen PBPL of healthy donors (ND), with each approach requiring <1 mL of PBPL. The principle of isolation of these kits include precipitation (ExoQuick™ ULTRA) [77, 78],

membrane-base affinity (ExoEasy™) [41, 79] and resin-based capture/enrichment (Norgen™) [43, 78].

The workflow for the preparation of PFP prior to EV isolation is shown in **Fig. 3A**. To ensure removal of platelet-derived EVs, blood collected was performed in sRNA tubes and initially centrifuged and cryoprecipitate removed [60]. For ExoQuick™ ULTRA, thrombin treatment was performed prior to psEV isolation as recommended by the manufacturer. For both ExoQuick™ ULTRA and exoEasy™ an additional filtration (0.22 μm) step was required. Recovery of psEV is dependent on the isolation process, where recovery rate (protein yield) differed for each approach (**Fig. 3A**). This potentially reflects co-isolation of abundant plasma proteins [37, 76], as confirmed by protein expression of HSA in psEV (**Fig. 3B**). We report selective identification of TSG101 in psEV, with Norgen™ providing marker expression in psEV (**Fig. 3B**). The size of the isolated psEV was confirmed using TEM, and typical cup-shaped membrane particles with a diameter of 50 to 80 nm were observed (**Fig. 3C**). NTA showed a size distribution of 50 to 250 nm for the 3 methods employed for isolating sEV from plasma (**Supplementary Fig. S2**). psEV isolated using the Norgen^{TM} kit (mean 85.2 nm) reflected enrichment of sEV marker protein (TSG101), despite presence of abundant plasma protein HSA, and were homogenous in size (diameter) distribution. Therefore, the resin-based Norgen^{TM} kit will be used for subsequent analysis of pre-analytical factors (namely collection tube type, storage conditions) which play a critical role in psEV isolation [57, 80, 81].

3.5   Evaluation of plasma small EVs: sRNA *versus* EDTA tube types

To evaluate the tube type in isolation of psEVs (Norgen^{TM} kit), we compared preparations isolated from frozen PFP derived from ND collected in EDTA or sRNA tubes. Importantly, heparin can reduce the EV yield, is difficult to remove and can interfere with platelet activation

(leading to release of EVs) [38, 57, 82], hence it was excluded in our analysis. For both EDTA and sRNA-derived psEV samples we observed similar protein yield (average ~1500 μg), total protein profiles (**Supplementary Fig. S3A**) and marker expression of Alix, CD63, TSG101, and CD81, normalized to total protein loaded (**Fig. 3D; Supplementary Fig. S3B-C**). For both tube types, size (mean ~80 nm; **Supplementary Fig. S3E**) and morphology of psEV were similar (**Supplementary Fig. S3D**). The level of platelet presence in psEV was determined by western blotting (ITGA2B, SELP) [83], revealing minimal contamination for either tube type of platelet-derived EVs (**Fig. 3D**). Importantly, sRNA tubes offer a significant advantage in a clinical setting based on extended processing times (up to 7 days at RT) [84] after blood collection in comparison to EDTA tubes which require immediate processing (within 2 hours) [38]. Therefore, sRNA tubes were used for downstream psEV isolation and analysis and appropriate measures were employed to limit platelet activation and thus platelet-derived EV presence in psEV analyses [57].

3.6   Evaluation of plasma small EVs: fresh *versus* frozen PFP

To determine whether banking and storage of plasma negatively affects psEV isolation, we evaluated EV purity by determination of yield and protein expression of EV markers prior to and after freezing of plasma derived from ND. Both sRNA and EDTA sources were evaluated. No significant differences were observed for protein yield (average ~1400 μg) or total protein profiles (**Supplementary Fig. S3A**). Fresh samples displayed a higher EV-protein expression profile (Alix, CD63, TSG101, CD81) compared to frozen preparations, normalized to total protein loaded (**Fig. 3D; Supplementary Fig. S3B**). The expression of platelet EV markers (ITGA2B, SELP) was also shown to be very low for both fresh and frozen psEV (**Fig. 3D**). No observable difference in size or morphology was noted by TEM for fresh or frozen psEV (**Supplementary Fig. S3D**) or size distribution based on NTA (mean ~82 nm; **Supplementary**

Fig. S3E). A lower background was observed at TEM analysis for fresh samples indicating psEV preparations with less contaminants. Thus, we suggest that psEV yield can be influenced by storage conditions, and suggest fresh samples are used were possible.

3.7   MM patient-derived psEV isolation

We next applied the isolation of sEV from PFP derived from patients with MM. Fresh and frozen plasma conditions for psEV for disease (D1-3) and non-disease (ND1-2) were assessed, revealing no significant differences based on total protein profiles and normalised TSG101 protein expression (**Fig. 3E-F**), sEV size and morphology (**Supplementary Fig. S4A-B**). Further, minimal protein expression of platelet marker ITGA2B was confirmed (**Fig. 3E**).

3.8   MM patient-derived plasma small EVs induce stromal cell proliferation and migration

It is known that MM-derived secreted factors contribute to stromal cell reprogramming through cell-to-cell contact and/or secretion of factors such as cytokines and EVs [49, 85, 86]. In this study, we examined whether psEV derived from patients with progressive MM (i.e., MGUS, SMM, MM) could contribute to cell proliferation or migration. We treated HS5 cells with MM patient-derived psEV (Norgen™ kit) (**Fig. 4A**) to reveal significantly increased stromal cell proliferation, in comparison to cells treated with EV-elution buffer only and to healthy donors (ND)-derived psEV (**Fig. 4B**).

We next assessed whether psEV from MM disease subtypes could influence stromal cell proliferation, however no significant difference was observed with MGUS (pre-malignant condition) or SMM (asymptomatic stage) derived psEV (**Fig. 4B**). This suggests that psEV derived from different disease stages of MM have selected functional effects on stromal cells.

When studying cell migration, MM-derived psEV significantly increased human stromal cell migration (**Fig. 4C-D**). psEV derived from MGUS and SMM patients could induce significant stromal cell migration (**Fig. 4C-D**). These data indicate that psEV derived from MM patients are biologically-active and induce cell proliferation and migration in stromal cells.

3.9 Plasma small EVs regulate tumour-stromal cell adhesion

As we demonstrated the ability of MM-derived psEV to promote stromal cell proliferation and migration, we next wanted to investigate whether cell adhesion could be induced between tumour cells (HMCL) and stromal cells. MM cells adhesion to the stromal compartment is one of the key features of MM and is partly a result of the attraction by stromal cells [87]. Several circulating factors released from tumour cells including cargo within EVs are known to re-program recipient cells enabling mechanisms involved in pre-metastatic niche formation favouring migration and adhesion, important means of cancer cell homing and outgrowth [59, 88-90].

DiO-labelled HMCL were co-cultured with HS5 cells in presence or absence of psEV from ND or D (MGUS, SMM, MM) patients. A significant increased HMCL adhesion to stromal cells pre-treated with MM-psEV was observed compared to ND-psEV or elution buffer only (**Fig. 4E-F**). Importantly, a significant increase in HMCL adhesion was also observed when HS5 cells were pre-treated with psEV derived from both MGUS and SMM patients suggesting that not only MM-psEV but also psEV derived from early stages of disease (MGUS, SMM) can induce changes in stromal cells to support tumorigenic cell adhesion. In addition, significantly increased cell adhesion was observed for both BM-derived and PE-derived HMCL (**Fig. 4F**) which represent different stages of MM [70, 91]. This was validated using an

additional pair of HMCL (KMS28BM-KMS28PE). These results confirm the crucial role of MM-derived psEV in inducing cell adhesion between tumour cells (MM) and stromal cells.

3.10 Proteomic profiling of MM-derived psEV reveals factors implicated in cell migration and adhesion

To gain an insight into the molecular changes in malignant MM-derived psEV in comparison to ND psEV, we performed quantitative proteomic profiling. To overcome limitations with plasma proteome analyses using mass spectrometry, HSA was depleted from psEV (**Fig. 3B, Supplementary Fig. S5A**). Based on stringent informatics, a total of 305 proteins were identified (n=2 for each sample group, correlation matrix 0.93 within sample group) (**Supplementary Fig. S5B**). For MM-derived psEV (D), 124 proteins were identified, while 181 proteins were identified in ND psEV **(Fig. 4G)**. A cursory inspection of the data set revealed 18 proteins reported highly enriched in EV marker databases (ExoCarta/ VesiclePedia; e.g., ANXA2, PRDX2, ACTG1, TFRC, PKM; **Supplementary Fig. S5C**). Comparative analysis of psEV between MM (D) and ND revealed 24/305 proteins were unique or upregulated proteins in MM-derived psEV (log2 fold change $\geq$2.0; **Fig. 4G, Supplementary Table S1; Table 1**). Interestingly, several proteins were associated with cell adhesion and migration (i.e., MYH4, CD166, CD44), and pre-metastatic niche formation (e.g., ANXA2, FN1), described previously in EV-mediated pro-tumoral stromal remodelling [77, 92, 93]. Enrichment map analysis (using DAVID bioinformatics resource) revealed that these biological processes associated with subset were significantly (p<0.05) associated with 'cell migration' and 'cell adhesion' (**Supplementary Tables S2-3**), with CD166 and CD44 associated with both annotations (**Fig. 4H**).

Taken together, these results show that psEV derived from MM patients are enriched in specific proteins associated with cell migration and adhesion. Further studies are required to implicate specific proteins identified in psEV on stromal cell function.

Discussion

As the potential of sEV for biomarker discovery and clinical application (e.g. liquid biopsy) increases in complex cancers such as MM, it is crucial to have standardized protocols for sEV isolation, purification and analysis but such standardized protocols [33, 38, 39, 94] in MM are still lacking [95]. Specifically, no consensus exists on the type of starting material (e.g., plasma *versus* serum) and isolation protocol [Toward Standardization of Mesenchymal Stromal Cell-Derived Extracellular Vesicles for Therapeutic Use: A Call for Action. Proteomics. 2019 Jan;19(1-2):e1800397], with most studies focused on RNA-based downstream applications [30, 31, 85, 96] compared to functional or proteomic-based studies [77, 97]. UC and precipitation are the most widely adopted methods in MM [31, 46, 50] despite their proven inadequacy for the removal of 'contaminants' including HAP [37] [95], with very few reports describing density-based fractionation (DG-UC) as a purification method [46, 51, 77, 95]. In this study, we provide a detailed optimized framework for isolation and comprehensive characterisation of sEV derived from both HMCL and human plasma.

Consistent with several reports [56, 59, 98], we show that DG-UC, employed to enrich and purify sEV from CM, improved sEV-purity compared to the commonly utilized UC. DG-UC is not applicable to clinical samples due to the high starting plasma volumes required [56]. Furthermore, commercially available kits have been proven to isolate sEV suitable for a number of downstream applications [31, 43, 46]. In this study, we assessed sEV isolated from

1 mL of plasma derived from healthy donors using three different commercial methods which have been successfully utilized in several reports [31, 41, 43]. The resin-based (Norgen[TM]) kit provided the highest enrichment of TSG101 and psEV with typical morphology associated with sEV when compared to precipitation and membrane-base affinity, in which a high TEM background has been described by other groups [41, 99]. Hence, the Norgen[TM] kit was utilized for further evaluation of pre-analytical factors (i.e., storage conditions and tube type) that play a critical role in psEV isolation [38, 39, 81]. Fresh samples provided psEV with higher purity compared to frozen counterpart, consistent with previous reports [35]. Despite EDTA and sRNA tubes providing comparable purity, plasma collected in sRNA tubes was chosen for the further investigation of psEV from MGUS and MM patients as the sRNA tubes have the added advantage of not requiring immediate processing. Albumin is a frequent contaminant of sEV isolated from blood and is known to dramatically affect proteomic-based downstream applications [37, 100]. Thus, the significant depletion of albumin we achieved, as demonstrated by immunoblotting, was important for the subsequent downstream mass spectrometry-based characterization of psEVs.

Accumulating evidence indicates that sEV are important mediators in the cross-talk of MM PCs with their microenvironment [85, 86]. However, the role of tumour-derived sEV in MM pathogenesis and MGUS-to-MM progression has not yet been thoroughly investigated. Here, we show that HMCL-sEV are functional and readily taken up by recipient stromal cells. Endocytosis has been described as the primary route of uptake of MM-sEV by stromal cells and its blockade can disrupt the alterations induced by sEV in target cells, revealing the importance of this route in MM cell-stromal cell cross-talk [74]. Further, Purushothaman et al [77] have demonstrated the heparan sulfate-fibronectin axis as the mediator of sEV interaction with target (endothelial) cells impacting their behaviour. For the purpose of this work the

mechanisms of MM-sEV uptake and its blockade were not evaluated but further investigation to inform its translational application in MM is warranted. Several studies have shown the involvement of sEV derived from both MM-derived tumor and stromal cells in cell proliferation [46], angiogenesis [86, 101], immunosuppression [86], fibroblast reprogramming [85], bone lysis [48, 50, 51]. Consistently, in response to their interaction with both HMCL-sEV and MM-psEV, (non-MM) human stromal cells acquired pro-proliferative and pro-migratory phenotypes. In turn, increased HMCL adhesion to pre-treated stromal cells was observed. Interestingly, our proteome analysis of psEV revealed upregulation of key molecules involved in these mechanisms in MM-psEV compared to plasma from healthyindividuals. This is an important finding – supporting the fact that changes observed in psEV reflect differences in the underlying oncogenic pathology (**Table 1**). A key finding from our proteomic analysis revealed important changes to the psEV proteome of MM associated with tumorigenesis, including cell proliferation, invasion, migration, angiogenesis, epithelial-mesenchymal transition [102-104]. Future studies are focused on expanding the limited number of patient samples analysed in this preliminary study, providing further insights into the pathogenesis of MM, and potentially defining a signature of psEV associated with MM. Along this line, we report several factors, including proteins involved in both cell-migration and cell-adhesion [105-107] previously implicated in the biology and/or clinical outcome of cancers including MM [97, 105, 106, 108], thus supporting our observations. Harshman et al [97] has shown that sEV derived from corticosteroid resistant HMCL and the serum of MM patients are enriched for CD44. The authors describe the potential of sEV-CD44 as a predictive biomarker of overall survival in MM. In support of our findings, CD44 has also been described in cell adhesion-mediated drug resistance in MM [106], and sEV-CD44 has been identified as a key regulator of metastatic progression and chemoresistance in solid tumors [109, 110]. Of note, CD166 has been shown to induce osteotropism and survival of tumor cells in the bone in prostate cancer

by increasing cell migration and cell adhesion [111, 112]. Importantly, Xu et al [105] have described the expression of CD166 in both HMCL and primary MM cells isolated from BM and that CD166 promoted MM cell homing to the BM compartment and osteolysis. Our preliminary findings therefore suggest a possible new modality of HMCL adhesion to stromal cells through enrichment of key molecules in psEV.

It has been shown that sEV derived from early stages of solid tumors are able to remodel stromal cells at the pre-metastatic niche promoting tumor cell homing and outgrowth [59], not inconsistent with our observation of enhanced cell migration and adhesion mediated by psEV derived from MGUS and SMM which represent earlier stages of MM disease. It has been demonstrated that potentially more aggressive genetic PC sub-clones are already present in MGUS and SMM [113]. It is possible that these more aggressive sub-clones may favour cell migration and adhesion through the release of specific factors enriched in psEV to ultimately promote PCs outgrowth and disease progression. Accumulating evidence suggests that treatment of patients with high risk SMM provides a survival advantage [10, 11], thus highlighting the need for improved patient stratification and a better understanding of MGUS-to-MM evolution and biology.

The pathogenesis of MM PCs growth outside the BM (extramedullary disease) is still poorly understood. Of note, we observed that both BM- and PE-derived HMCL which reflect different stages of disease and harbour the same translocation status, increase their ability to adhere to pre-educated stromal cells. Thus, we speculate that the observed enrichment of specific markers in MM-psEV may represent a driver for adhesion to target cells not only for less aggressive MM cells but also for cells with more aggressive phenotypes. Therefore, both the

genomic landscape of MM PCs and the EV-mediated cross-talk between tumor-stromal compartments could participate in conjunction to favour the homing and possibly the outgrowth of phenotypically aggressive PCs.

We acknowledge that psEV preparations may contain other vesicular and non-vesicular particles known to contribute to the above-mentioned mechanisms. Further optimization of psEV purification, larger cohort of samples, and *in vitro/in vivo* investigation are needed to confirm if the observed phenotypic changes are specifically relevant to psEV rather than a combination of factors present in plasma. A recent report by Lyden et al [114] shows that small EVs and particles (i.e., exomeres; EVPs) with a unique tumour-related proteomic signature are detectable only in plasma from patients with cancer, and confirms that protein packaging is heterogeneous across tumor types and reflects tumor biology. The authors also indicate that EVPs reflect the systemic effects of cancer where the tumor microenvironment is a major contributor to cancer-associated EVPs in plasma. This report highlights the importance of defining methods for capturing and characterizing tumour-derived EVs and particles from body fluids for a direct and specific liquid biopsy approach.

Summary

In summary, we demonstrate that biologically-active and morphologically intact sEV can be isolated from HMCL culture media and human plasma derived from MGUS, SMM and MM patients. We also show that psEV derived from both pre-malignant and malignant stages of MM induce phenotypic changes in target stromal cells, further demonstrating their importance in tumor-to-stroma cross-talk. We identified factors involved in cell migration and cell adhesion in MM-psEV in comparison to those from healthy individuals, potentially supporting

the functional involvement of oncogenic sEV. These findings will enable future studies seeking to characterize the underlying mechanism regulating pro-tumorigenic signalling by EVs, and have implications in clinical utility as next-generation biomarkers for liquid biopsy in MM diagnosis and management.

Conflict of interest

No potential conflicts of interest were disclosed.

Abbreviations: MM, multiple myeloma; SMM, smouldering myeloma; MGUS, monoclonal gammopathy of undetermined significance; EVs, extracellular vesicles; sEV, small extracellular vesicles; lEV, large extracellular vesicles; HMCL, human myeloma cell line; ND, non-disease; D, disease; PBPL, peripheral blood plasma; psEV, plasma-derived small EVs; PFP, platelet-free plasma; sRNA, STRECK RNA tube; CM, culture medium; DG-UC, density gradient ultracentrifugation; HSA, human serum albumin; HAP, highly abundant proteins.

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Figure Legends

Figure 1. Isolation and characterization of small EVs from HMCL CM. (A) Morphology of the human MM cell line (HMCL) KMS12PE analyzed by bright-filed microscopy. Scale bar, 10 µm. (B) Workflow for isolation of small EVs from CM derived from HMCL (KMS12PE and KMS12BM) using a combination of differential centrifugation and buoyant density gradient (iodixanol/OptiPrep^{TM}) centrifugation. (C) Western Blot analysis of OptiPrep^{TM} fractions compared to UC with small EV-markers TSG101 and CD81 (KMS12PE; n=3). (D) Lysates of small EVs are compared to lysates of large EVs and originating HMCL using anti-TSG101 and anti-TUBA1A (KMS12PE; n=3). (E) TEM images of HMCL-derived small EVs and large EVs; representative of >10 images (KMS12PE; scale bar, 200 nm). (F) Size distribution of HMCL-derived small and large EVs evaluated by NTA (KMS12PE; n=3).

Figure 2. Small EVs purified from CM of HMCL are taken up by human stromal cells and induce cell proliferation. (A) Uptake of DiO-labelled small EVs (white arrows), isolated from HMCL, by recipient human stromal cells (HS5) captured by fluorescence live cell confocal microscopy after 18 h of incubation. Representative deconvolved live cell images (KMS12BM; n=3). Upper panel: orthogonal view is displayed; scale bar, 10 µm. Lower panel: 3D reconstruction of merged channels. Middle (z) of cells is shown. (B) Live cell confocal microscopy analysis of MM cell-small EVs uptake by HS5 cells at different time points. Representative middle (z) ofcell images are shown (KMS12BM; n=3). Scale bar, 10 µm. (C) Quantification of small EV-uptake by NIS software (KMS12BM; n=3). (D) Schematic representation of proliferation assay. (E) HS5 cells were treated with increasing concentrations of MM cells (KMS12BM)-derived small EVs for 72 h and cell proliferation was determined

by MTS assay. Data represent the percentage of PBS-treated cells expressed as the mean ± S.E.M. (* p=0.0166, 30 µg/mL; * p=0.0219, 60 µg/mL; n.s.= not significant; n=3).

Figure 3. Isolation and characterization of human plasma-derived small EVs. (A) Workflow for preparation of platelet free PBPL (PFP) and isolation of plasma small EVs (psEV) using commercial kits. Protein yields are also indicated for each kit (n=3, non-disease, ND-psEV). (B) Comparison of commercial kits by western blot analysis of small EV-lysates isolated from frozen PFP, and collected in sRNA tubes, derived from healthy donors (non-disease, ND). Anti-TSG101 and anti-HSA were utilized (representative of n>3). (C) TEM images of small EVs obtained from the same ND-frozen PFP sample isolated with 3 commercial kits; representative of >10 images (scale bar, 200 nm). (D) Western blot analysis of psEV isolated with the Norgen[TM] kit using EV markers (TSG101, CD81, ALIX, CD63) and platelet markers (ITGA2B and SELP). Lysates of psEV derived from frozen ND-PFP are compared to lysates of psEV derived from fresh PFP. sRNA collection tubes and EDTA tubes are also compared (n=3). (E) Western blot analysis of total PFP- and psEV-lysates derived from ND- and D-PFP collected with sRNA tubes. Frozen and fresh samples are also compared (n=3). (F) Data represent protein expression intensity for TSG101 (normalized to total protein load). ND- *versus* D-psEV and fresh *versus* frozen samples are compared. Data are presented as mean ± S.E.M. (** p=0.0217, ****p=0.0068; n=3).

Figure 4. MM plasma-derived small EVs promote cell proliferation, migration and adhesion *in vitro*. (A) Schematic representation of functional assays (left panel, proliferation; lower panel, migration; right panel, adhesion). (B) HS5 cells were treated with 30 µg/mL psEV (Norgen[TM]) derived from ND and D (MGUS, SMM, MM) patients or EV elution buffer

(control) for 72 h and cell proliferation was determined through the MTS cell proliferation assay. Data represent the percentage of control expressed as the mean ± S.E.M. (** p=0.0098, MM-psEV; n>6). (C) Representative images of wound healing assay showing migration of HS5 cells into the wound area when cultured for 72 h in the absence (elution buffer, control) or presence of 80 µg/mL psEV derived from ND and D patients (scale bar, 100 µm). (D) Quantification of cells by nuclei count (Hoechst – blue) within the region of interest (ROI=middle of images delimited by red lines) by NIS Analysis Software. Wound area (ROI) was selected from control images (time 0) and utilized for analysis. Note, functional effects of psEV derived from patients with earlier stages of disease (MGUS, SMM) are similar to MM-psEV. Data are presented as mean ± S.E.M. (*** $p=0.0005$, ****$p<0.0001$; n=5) (E) BM-dependent (KMS12BM, KMS28BM) and BM-independent (PE; KMS12PE, KMS28PE) MM cells were labelled with DiO and added for 2 h to cultures of HS5 untreated (elution buffer) or pre-treated (72 h) with 80 µg/mL of psEV derived from ND and D patients. After 5 washes, images were taken via fluorescence microscopy (scale bar, 100 µm). (F) Quantification of MM cell count (DiO – green) by NIS Analysis Software. Data are presented as mean ± S.E.M. (****$p<0.0001$; n=5). (G) A Venn diagram of identified proteins in 2xND vs 2xD (MM) HSA-depleted psEV. 120 proteins were co-identified in D and ND. Of these, 24 proteins were upregulated and 80 were downregulated in D vs ND ($p<0.05$; log2 fold change ±2). (H) D-psEV upregulated proteins associated with cell migration and adhesion.

Table 1 - Proteins in MM psEV (uniquely identified or enriched) in comparison to non-disease.

Supplementary Figure and Table Legends

Supplementary Figure S1. Uptake of DiO-labelled MM cell-small EVs by HS5 stromal cells. (A) Representative deconvolved live cell images of stromal cells HS5 treated with control (PBS or unstained small EVs derived from MM cells-KMS12BM) for 18 h (n=3). Merged channels: nuclei, Hoechst – blue; MM cell-small EVs, DiO – green; membrane stromal cell marker, CD10 PE – red. Orthogonal view is displayed; scale bar, 10 μm. (B) Confocal microscopy analysis of MM cell (KMS12BM)-DiO small EVs uptake by HS5 cells at 18 h. Representative wide view images (n=3). Merged channels: nuclei, Hoechst – blue; MM cell-small EVs, DiO – green. Scale bar, 100 μm.

Supplementary Figure S2. Size distribution of psEV isolated utilizing commercial kits evaluated by NTA. (A) Norgen™. (B) ExoEasy™. (C) ExoQuick™ ULTRA (* Zetaview; ** Nanosight; n=3).

Supplementary Figure S3. Comparison between ND-psEV isolated using the Norgen™ kit derived from frozen- or fresh-PFP and sRNA- or EDTA-tubes. (A) Total protein analysis by stain free gel (n=3). (B) Data represent protein expression intensity for each EV-marker normalized total protein loaded. Fresh PFP *versus* frozen PFP for both sRNA and EDTA tubes. Data are presented as mean ± S.E.M. (*$p<0.05$; ****$p<0.0001$; n=3). (C) Data represent protein expression intensity for each EV-marker normalized total protein loaded. sRNA *versus* EDTA collection tube for both frozen and fresh PFP. Data are presented as mean ± S.E.M. (n=3). (D) Representative TEM images (scale bar, 200 nm). (E) Size distribution of psEV evaluated by NTA (n=3).

Supplementary Figure S4. Comparison between ND-psEV and D-psEV derived from frozen PFP and sRNA collection tubes, isolated using Norgen^TM kit. (A) Representative TEM images (scale bar, 200 nm). (B) Size distribution of psEV evaluated by NTA (n=3).

Supplementary Figure S5. MS-based proteomic analysis of psEV. (A) Western blot analysis of albumin (HSA) depletion from ND-psEV eluates utilizing anti-CD81 and anti-HSA. Lysates of HSA-depleted fractions (F1 and F2+3) are compared to psEV- and total PFP-lysates. Total protein profiles by stain free gel are also displayed (n>3). (B) Left panel: Multi-scatter plot analysis of protein intensities and R2 values for 2 biological replicates per cohort (D1-D2 vs ND1-ND2). Diagonal in the matrix is distribution of data. Right panel: Quality control pairwise comparison plots of median protein MS intensities between the cohorts. (C) List of EV-proteins identified by MS-proteomic profiling in psEV that are reported in top100 EV markers in ExoCarta and Vesiclepedia databases.

Supplementary Table S1. psEV proteome from heathy donor (ND) and disease (D; myeloma) patients. Positive and negative Fold Change values indicate factors that are upregulated and downregulated respectively in D-psEV.

Supplementary Table S2. Factors identified in psEV proteome (D vs ND) associated with cell migration.

Supplementary Table S3. Factors identified in psEV proteome (D vs ND) associated with cell adhesion.