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Author/s:

Shafiq, A;Suwakulsiri, W;Rai, A;Chen, M;Greening, DW;Zhu, HJ;Xu, R;Simpson, RJ

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Transglutaminase-2, RNA-binding proteins and mitochondrial proteins selectively traffic to MDCK cell-derived microvesicles following H-Ras-induced epithelial-mesenchymal transition

Adnan Shafiq¹, Wittaya Suwakulsiri¹, Alin Rai^{1,2}, Maoshan Chen^{1,3}, David W. Greening^{1,2}, Hong-Jian Zhu⁴, Rong Xu^{1,5*}, and Richard J. Simpson^{1*}

¹*Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria 3086, Australia*

²*Baker Heart and Diabetes Institute, Melbourne, Victoria 3004, Australia*

³*Myeloma Research Group, Australian Centre for Blood Diseases, Monash University/ The Alfred Hospital, Melbourne, Victoria 3004, Australia*

⁴*Department of Surgery (Royal Melbourne Hospital), The University of Melbourne, Parkville, Victoria 3050, Australia*

⁵*NanoBiotechnology Laboratory, Australian centre for Blood Diseases, Central Clinical School, Monash University, Melbourne, Victoria 3004, Australia*

***Correspondence:**

Professor Richard J. Simpson
La Trobe Institute for Molecular Science (LIMS)
LIMS Building 1, Room 412
La Trobe University, Melbourne, Victoria 3086
Australia
Tel: +61 03 9479 3099
E-mail: Richard.simpson@latrobe.edu.au
and Dr Rong Xu
E-mail: Rong.Xu@monash.edu

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RNA-binding proteins/ mitochondrial proteins

Abbreviations: **SMVs**, shed microvesicles; **EMT**, epithelial-mesenchymal transition; **CM**, culture media; **ECM**, extracellular matrix; **Exos**, exosome; **MDCK cells**, Madin-Darby Canine Kidney cells; **21D1 cells**, H-Ras-transformed MDCK cells; **TGM2**, tissue transglutaminase-2

Abstract

Epithelial-mesenchymal transition (EMT) describes an evolutionary conserved morphogenic process defined by loss of epithelial characteristics and acquisition of mesenchymal phenotype, and altered patterns of intercellular communication, leading to functional changes in cell migration and invasion. In this regard, we have previously reported that oncogenic H-Ras induced EMT in MDCK cells (21D1 cells) trigger changes in the protein distribution pattern in cells, exosomes, and soluble protein factors (secretome) which modulate the tumour microenvironment. Here, we report that shed microvesicles (also termed microparticles/ ectosomes) secreted from MDCK cells following oncogenic H-Ras-induced EMT (21D1-sMVs) are biochemically distinct from exosomes and parental MDCK-sMVs. The protein spectra of RNA-binding proteins and mitochondrial proteins in 21D1-sMVs differ profoundly to those of exosomes, likewise proteins associated with suppression of anoikis. We show that 21D1-sMVs promote cell migration, confer anchorage-independent growth, and induce EMT in parental MDCK cells. An unexpected and novel finding was the selective sorting of tissue transglutaminase-2 (TGM2) into 21D1-sMVs; there was no evidence of TGM2 in MDCK-sMVs. Prior treatment of 21D1-sMVs with neutralizing anti-TGM2 or anti-FN1 antibodies attenuates the invasive capability of fibroblasts. These finding suggest that microvesicle-associated TGM2 may play an important contributory role in the EMT process and warrants further investigation. (199/ 200 words).

Significance

Epithelial-mesenchymal transition (EMT) is an evolutionary conserved biological process whereby highly-polarized immotile epithelial cells convert to motile mesenchymal cells. The last decade has witnessed a growing awareness of the contribution of extracellular vesicles (EVs), notably exosomes, to the EMT process. It is now recognised that another major class of EVs – referred to as shed microvesicles (sMV) or microparticles/ ectosomes - exists. Whereas exosomes (typically 30 – 150 nm in diameter) are of endocytic origin, sMVs (typically 50 – 1,300 nm in diameter) form by direct budding from the plasma membrane. sMVs and exosomes are biophysically and molecularly distinct. Here, we dissect the MS-based protein profile of sMVs released from MDCK cells following oncogenic H-Ras-induced EMT, and examine the impact of these re-programmed sMVs on their functional behaviour in recipient parental MDCK cells and fibroblasts upon uptake. We define proteins selectively trafficking to MDCK cell-derived sMVs following oncogenic H-Ras transformation – notably, tissue transglutaminase-2 (TGM2), RNA binding proteins and mitochondrial proteins. This work will open avenues for future studies aimed at furthering our understanding of the contribution of sMVs in the EMT programme, and the targeting of EV subtypes as potential druggable entities for therapeutic application.

1. Introduction

Epithelial–mesenchymal transition (EMT) is an evolutionary conserved biological process enabling polarized immotile epithelial cells to convert to motile mesenchymal cells ^[1,2]. This highly-regulated program is essential for both physiological and pathological processes and can be grouped depending on biological context: for example, early embryogenesis, wound healing and tissue regeneration, and cancer invasion and metastasis ^[1-5]. Multiple cellular regulatory networks have been implicated in triggering EMT such as tyrosine kinase receptors (e.g., epidermal growth factor (EGF), fibroblast growth factor (FGF), connective tissue growth factor, platelet-derived growth factor (PDGF), insulin-like growth factor etc), and signalling pathways involving integrins, Wnt, nuclear factor κ -B, and transforming growth factor β (TGF- β) ^[1,2,6]. A common feature of these regulatory networks is that they induce EMT master transcription factors including Snail, Zeb-1, and Twist which inhibit the expression of genes associated with the epithelial state (e.g., E-cadherin, ZO-1, claudins, occludins, α 6 β 4 integrins etc) and induce the expression of genes associated with the mesenchymal state (e.g., N-cadherin, vimentin, fibronectin, MMPs, β 1 β 3 integrins ^[1,2,5,7]. Transcriptional re-programming during the EMT process involves remodelling of cell–cell and cell–extracellular matrix interactions leading to detachment of highly-polarized epithelial cells from each other and the underlying basement membrane, and promotion of spindle-shaped mesenchymal morphology ^[7]. In the context of cancer progression, EMT is essential for amplifying tumour-initiating capability and metastatic potential of cancer cells, especially cells at the leading tumour edge ^[2]. A critical mechanism of EMT is resistance of tumour cells to undergo *anoikis* (cell-detachment-induced-apoptosis) ^[8].

Over the past decade considerable effort has been directed towards understanding the contribution of soluble factors (proteins/ peptides) secreted into the extracellular space (the secretome) and how they might impact on cells undergoing EMT. For example, using the Madin-Darby Canine Kidney (MDCK) cell line model for EMT and a combination of mass spectrometry-based protein-profiling (Orbitrap technology and label-free quantitation) we identified several proteins secreted from oncogenic H-Ras-transformed MDCK cells (21D1 cells) into the extracellular space that are known to mediate EMT ^[9,10]. Secretome-based proteomic profiling identified many extracellular effectors that coordinate biological response during H-Ras-induced EMT that enhance cell mobility – notably, dysregulated cell-cell contact and cell-matrix adhesion proteins. For example, down-regulated proteins included desmocollin 2, clusterin, collagen XVII and transforming growth factor-beta induced protein ig-h3 (Beta ig-h3), while up-regulated secretome proteins included proteases and factors that promote cell migration (e.g., MMP-1, kallikrein -6/-7, TIMP-1, and

S100A4/metastasin)^[10]. In another study proteomic profiling identified proteins involved in extracellular matrix (ECM) remodelling – for example, diminished expression of basement membrane constituents (collagen type IV, laminin 5) and up-regulation of ECM constituents (SPARC, collagen type I, fibulins -1 and -3, biglycan, and decorin) following EMT^[9]. More recently, we found that H-Ras-induced EMT of MDCK cells resulted in extensive reprogramming of the protein repertoire of exosomes (secreted membranous extracellular vesicles^[11]) in favour of selected uptake of cargo proteins known to promote metastatic niche formation, and transcription/ splicing factors known to induce EMT^[12].

Exosomes (typically, 30-150 nm in diameter) and shed microvesicles (sMV, typically 50 to 1,300 nm in diameter, also referred to as microparticles and ectosomes) are two major classes of small lipid-encapsulated extracellular vesicles (EVs) that transmit molecular messengers (functional proteins, lipids, RNA species) between cells to alter the phenotype of recipient cells^[11,13,14]. Exosomes and sMVs have distinct mechanisms of biogenesis – exosomes are of endocytic origin released by all cell types following trafficking to and fusion of multivesicular bodies with the plasma membrane, while sMVs are formed by direct budding from the plasma membrane. Within each class of EV, subtypes (subpopulations) exist that can be distinguished by their distinct protein and RNA signatures^[13]. While much is known about H-Ras-transformed MDCK cell-derived exosomal proteins following EMT^[15], the contribution of sMVs in this process is unknown. In this study we examine the biochemical and functional properties of sMVs secreted from H-Ras-transformed MDCK cells (21D1 cells). We show that expression levels of typical EMT hallmark proteins seen in parental cells mirror those observed in their cognate secreted sMVs. Protein cargos of sMVs are strikingly different to that of exosomes. These results have important implications not only for EMT biology, but also provide new insights into our understanding of different EV classes as well as cancer microenvironments.

2. Experimental Section

2.1 Isolation and purification of extracellular vesicles

MDCK cells and oncogenic H-Ras transformed MDCK cells (21D1 cells)^[12], were grown to 70% confluence in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-GIBCO, Carlsbad, CA, USA) with 10% (v/v) foetal calf serum (FCS) (Invitrogen-GIBCO) and 1% (v/v) penicillin streptomycin (P/S) (Life Technologies) at 37 °C with 5% CO₂. For large-scale EV production, 3 x 10⁷ cells were transferred to a CeLLine AD-1000 Bioreactor classic flask (Integra Biosciences) and grown in continuous culture with 1% exosome-depleted FCS for three months^[16]. Cell culture media (CM, 20 mL) was collected daily from the CeLLine bioreactor device, during each collection the culture chamber was replenished with 20 mL of fresh DMEM medium (with 1% exosome-depleted FCS, P/S supplement) and every 5 days the medium chamber was replenished with fresh 5% FCS (P/S supplement) DMEM. Cell morphology, viability and pH were monitored during continuous culture. Cell culture medium was immediately centrifuged at low speed to remove floating cells, cell debris (500 x g, 10 min; and 2,000 x g, 10 min), the supernatant stored at -20 °C. The stored culture medium (250 mL CM) was thawed at 37 °C, centrifuged at 10,000 x g, 30 min at 4 °C to obtain shed microvesicles (sMV) and rinsed three times with PBS to remove soluble factors prior to reconstituting in 150 µL for biochemical and functional studies.

2.2 Protein quantitation

Samples were solubilised in sodium dodecyl sulphate (SDS) sample buffer (4% (w/v) SDS, 20% (v/v) glycerol and 0.01% (v/v) bromophenol blue, 0.125 M Tris-Hydrochloride (Tris-HCl), pH 6.8) containing 100 mM dithiothreitol (DTT) and protein quantitation determined by 1D SDS-PAGE / SYPRO Ruby protein staining-based densitometry^[12].

2.3 Western blot analysis

Protein samples (20 µg) were electrophoresed on 4-12% Bis-Tris NuPAGE gels (Novex™, Thermo Fischer) with MES running buffer at 150 V for 1 h and then electro transferred onto nitrocellulose membranes (iBlot™ Dry blotting system from Life Technologies) at 12 V for 10 min, as described^[12]. Blots were probed with the following antibodies: mouse anti-TSG101 (BD Transduction Laboratories; 1:500), mouse anti-H-Ras (Santa Cruz Biotechnology; 1:500), rabbit anti-KRAS^{G12V} (Cell signalling, 1:500), mouse anti-E-cadherin/CDH1 (BD Transduction Laboratories™; 1:1000), mouse anti-Vimentin (BD Pharmingen™; 1:1000) and mouse anti-beta actin (Sigma; 1:1000) rabbit anti-fibroblastic/1/FN1 (Abcam; 1:1000), mouse anti-N-cadherin/CDH2 (BD Transduction 1:1000), anti-transglutaminase2/TGM2 (Abcam; 1:500), mouse anti-integrin beta1/ITGB1 (BD Transduction),

rabbit anti-ERK (Cell Signalling; 1:1000), rabbit anti-phospho MAPK (Cell signalling; 1:1000), rabbit anti-calnexin (1:1000). Secondary antibodies used were IRDye-800 goat anti-mouse IgG or IRDye-700 goat anti-rabbit IgG (1:15000, LI-COR Biosciences) and incubated for 1 h at room temperature in TTBS-Tween. Western blots were imaged using an OdysseyTM Infrared Imaging System (v3.0, LI-COR Biosciences).

2.4 Cryo-electron microscopy

sMVVs derived from MDCK and 21D1 cells were imaged by cryo-transmission electron microscopy (cryo-EM). Briefly, Aurion Protein-G gold 10 nm (ProSciTech, QLD, Australia) was mixed at a 1:3 ratio with EVs (2 μ g) in PBS and transferred onto glow-discharged C-flat holey carbon grids (ProSciTech). 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, Eindhoven, NL), in low-dose mode.

2.5 Nanoparticle tracking analysis

Particle size was determined using nanoparticle tracking analysis (NTA) (Nano Sight NS300, Malvern) fitted with a NS300 flow-cell top plate with a 405-nm laser. sMVVs samples (1 μ g/ μ L) were diluted in PBS (1:1000) and 500 μ L injected. NTA settings: detection threshold, 10; flowrate = 50 μ L/ min; temperature, 25 $^{\circ}$ C). Each analysis consisted of three 60-sec video captures. Samples were manually advanced $\sim$ 100 μ L between static captures. Video data was analysed using NTA software 3.0 (Malvern).

2.6 Lipophilic labelling of shed microvesicles

sMVVs ($\sim$ 300 μ g) resuspended in 300 μ L PBS were labelled with lipophilic fluorescent dye, DiI (Invitrogen) at 1 μ M concentration respectively for 15 min at room temperature. Fluorescently-labelled sMVVs were purified by density (iodixanol) gradient centrifugation. NIH3T3 cells were cultured (DMEM containing 1% Pen/Strep) on glass cover slips to 70% confluence and then incubated with DiI-labelled sMVVs (5 μ g) at 37 $^{\circ}$ C for 2 h. For microscopy, nuclei were stained with

Hoechst stain (10 µg/mL) for 30 min and followed by imaging using fluorescence microscopy (Zeiss Z1 Axio Observer).

2.7 Scratch wound-healing assay

MDCK cells were plated onto 10-cm culture dishes and grown to 70-80% confluency. A wound (scratch) was created with a pipette tip, followed by careful washing with PBS to remove detached cells. DMEM or DMEM supplemented with MDCK-sMV_s or 21D1-sMV_s (10 µg/ml) was added to the MDCK cells and incubated for 24 h at 37° C with 5% CO₂. Phase contrast images were obtained at 0 h and 24 h, and cell migration capability was analysed by measuring the width of the wound (scratched areas).

2.8 Transwell-Matrigel™ invasion assay

Transwell-Matrigel™ invasion assays were performed as described elsewhere^[17]. Briefly, Transwell inserts were coated with Matrigel™ (100 µL of 1 mg/mL reagent) and polymerized (4 h, 37 °C). Fibroblasts (5 x 10⁴ NIH 3T3 cells) in DMEM (1% P/S) were treated (2 h at 37 °C) with either MDCK-sMV_s or 21D1-sMV_s (30 µg/mL) or PBS alone. Treated NIH3T3 cells were harvested at 500 x g (5 min), resuspended in 100 µL DMEM medium and then carefully overlaid onto the Matrigel™-coated inserts of the 24-well plate companion plate containing DMEM (5% FCS, 1% Pen/Strep) supplemented with either sMV_s (30 µg/mL) or PBS alone. Where indicated, lower chamber medium was supplemented with sMV_s derived from MDCK and 21D1 cells. Invasion was allowed to proceed overnight at 37 °C and then the inserts were washed, cells fixed (4% (v/v) formaldehyde, 5 min), and nuclei stained with Hoechst stain (10 µg/mL) for 20 min. After non-invading cells were removed from the upper side of the inserts using a cotton swab, fibroblasts that had invaded imaged (following nuclei staining) using Zeiss Z1 Axio Observer microscope. Five fields of view/ per insert were obtained per insert and. images quantified (Image J software v1.49e).

2.9. Soft-Agar colony formation assay

Soft-Agar colony formation assays were performed as described previously^[17]. Briefly, MDCK cells or NIH3T3 fibroblasts (2×10³) in 100 µL DMEM (1% Pen/Strep) were treated with MDCK-sMV_s or 21D-sMV_s (10 µg) or 10 µL PBS vehicle for 2 h at 37 °C and then mixed with 300 µL of 0.3% agarose

(in DMEM with 10% FBS, 1% Pen/Strep) pre-warmed at 40 °C. Mixtures were overlaid onto wells in a 24-well plate pre-coated with 300 µL of 0.6% agarose (in DMEM with 10% FBS, 1% Pen/Strep). Mixtures were solidified (37 °C, 15 min) and then the wells were gently overlaid with 500 µL DMEM (5% FBS, 1% Pen/Strep) and incubated at 37 °C for 10 days with the medium replaced twice weekly. Colonies were imaged using Zeiss Z1 Axio Observer microscope under bright field.

2.10 Induction of EMT in epithelial MDCK cells

MDCK (1 x10⁵ cells) pre-incubated with MDCK-sMV or 21D1-sMV (20 µg) at 37 °C for 2 h before seeding in 6-well petri dish, and medium was replaced with new medium containing MDCK-sMV or 21D1-sMV (10 µg) cells, respectively, per well every second day. The wells maintained at 37 °C for 10 days. Western blot analysis of MDCK cells treated with 21D1-sMV or MDCK-sMV and post 10 days probed with anti-CDH1 (epithelial maker), anti-CDH2 (mesenchymal maker), anti-VIM (mesenchymal maker), anti-H-RAS, anti-RAS^{G12V}, and anti β-actin antibodies. Immuno-fluorescence microscopic analysis was performed after 10 days as follows: briefly, MDCK-sMV or 21D1-sMV treated MDCK cells were trypsinized and 2 x 10⁴ cells were seeded on 15-mm glass coverslips in 6-well plate holders (Nunc) and grown to 70% confluency at 37 °C with 5% CO₂ for 48 h. Cells were then washed three times with PBS and fixed in 4% formaldehyde for 15 min at RT, followed by PBS wash (twice) and permeabilised using 0.2% (v/v) Triton X-100 in PBS for 10 min at RT. Cells were washed with PBS (three times) and blocked with 3% (w/v) BSA (Sigma) in PBS (by 0.2% (v/v) Triton X-100) for 30 min at RT, followed by PBS wash (twice). Cells were then incubated with primary antibodies (mouse anti-CDH1 and mouse anti-VIM antibodies, 1:200) for 1 h, washed with PBS and incubated with secondary antibodies (Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), 1:200) in PBS at RT in the dark. Cells were washed again (x1) with PBS and nuclei stained with TO-PRO (10 µg/ml) for 1 min. Finally, cells were imaged using Zeiss Z1 Axio Observer microscope (Zeiss) and images captured using Zen 2011 (Blue edition, Zeiss).

2.11. Immunoprecipitation of TGM2 complex

21D1-sMV (200 µg) were lysed using 1% TX-100 HEPES lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl₂) with protease inhibitor cocktail (Roche) for 1 h on ice. The lysate was subjected to centrifugation at 10,000 x g for 30 min to remove insoluble material. To remove non-specific binding proteins, the supernatant was pre-incubated with 10 µL protein G conjugated Dynabeads at 4 °C for 1 h. Beads were removed and anti-TGM2 or isotype control anti-IgG (1-2 µg) antibodies was

added to the pre-cleared 21D1-sMV lysate and the mixture incubated overnight at 4 °C. TGM2 protein complex was isolated by adding protein G-Dynabeads (50 µl) and incubating the mixture for 3 h at 4 °C with gentle rotation. Protein G-Dynabeads were washed with PBS (three times) and TGM2 complex components were eluted using SDS sample buffer and subjected to SDS-PAGE and western blot analysis for identification of TGM2 interacting protein partners FN1 and ITGB1; total 21D1-sMV protein extract (20 µg) was used as a positive control and isotype control, and anti-IgG as a negative control.

2.12. Mass spectrometry-based proteome profiling

Proteome profiling of MDCK-sMVs and 21D1-sMVs samples were performed in biological triplicate (n=3), as previously described^[18]. Briefly, samples (20 µg protein) were electrophoresed (4-12% Bis-Tris SDS-PAGE) for 7 min at 150V and (i.e., ~ 1 cm into gel) and then visualized using Imperial Protein Stain (Thermo Fischer Scientific). Stained gel bands were excised (cut into two ~5-6 mm gel slices). Individual gel slices were destained (50 mM ammonium bicarbonate/acetonitrile), reduced with 2 mM tri(2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich, C4706) at 22 °C for 4 h with gentle rotation, alkylated by treatment with 25 mM iodoacetamide (Sigma-Aldrich) for 30 min, and then proteolytically digested (0.4 µg bovine sequencing grade trypsin ,Promega, V5111) at 37 °C for 18 h^[18,19]. Generated tryptic peptides were extracted from the gel and concentrated on reversed-phase C18 Stage Tips (Sep-Pack cartridge, Waters). Peptides were eluted from the Sep-Pack cartridge using 85% (v/v) acetonitrile (ACN) in 0.5% (v/v) formic acid (FA). Eluted peptides were lyophilized, dissolved in aqueous 0.5 (v/v) FA/ 2% (v/v) ACN and then loaded onto an Acclaim PepMap100, 5 mm × 300 µm i.d., µ-precolumn packed with 5 µm C18 beads (Thermo Fisher Scientific) and separated on a BioSphere C18 1.9 µm 120Å, 360/75 µm × 400 mm column (NanoSeparations) with a 120-min gradient from 2–100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) ACN) (2–100% 0.1% FA in acetonitrile (2–40% from 0–100 min, 40–80% from 100–110 min at a flow rate of 250 nL/min operated at 55°C. The nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to a Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Details of the operation of the mass spectrometer as previously described^[18].

The mass spectrometer was operated in data-dependent mode where the top 7 most abundant precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 60,000 with an MS/MS resolution of 15,000. Unassigned

precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 1.4 Th and selected precursors fragmented by HCD with normalized collision energies of 25 with a maximum ion injection time of 110 msec. Ion target values were set to 3e6 and 1e5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 30 sec. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). Sample and RAW data files are publicly available in ProteomeXchange (#PXD022290).

2.13 Bioinformatic analysis

MS raw data were pre-processed as described ^[20] and processed using MaxQuant ^[21] (v1.6.0.1) with Andromeda (v1.5.6), using a combined Human/Canine-only (UniProt #106294 entries) sequence database (Jan-2018) as described ^[12] with a taxonomy filter (Supplemental Table S1). This combined database search strategy was employed to overcome limitations with incomplete canine reference proteome database, issues with redundancy, and protein annotation ^[12,22]. Data was stringently searched as described ^[18] with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 6, with false discovery rate (FDR) 1% at the peptide and protein levels, tryptic digestion with up to two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation and protein N-terminal acetylation as variable modifications, and data analyzed with label-free quantitation (LFQ) ^[23]. LFQ intensity values were normalized for protein length and fold change ratios calculated. Contaminants, and reverse identification were excluded from further data analysis. Additionally, a taxonomy filter (UniProt) was applied to each protein to differentiate organism type (2,225/2797 protein identifications are canine annotated, Supplemental Table S1). Differentially expressed proteins were identified using the criteria: Fold change ratios ≥ 2.0 and $p < 0.05$, with identifications in at least two biological sample replicates. Protein-protein interactions were analysed using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) version 10.5 ^[24] with a high confidence (0.700) and active interaction sources (experiments and databases). Venn diagrams were created using “InteractiveVenn” online platform (<http://www.interactivenn.net/>). Binary protein interactions of TGM2 were obtained using the IntAct database ^[25] and visualized using Cytoscape (v.3.8.0) ^[26].

3. Results

3.1. Oncogenic H-RAS transformation of MDCK cells alters protein spectrum of secreted sMVs

To characterise the biophysical and functional properties of sMVs following H-Ras-transformation of MDCK cells (21D1-sMVs) we first generated large amounts of purified sMVs from cell culture medium using continuous-culture technology (CeLLine AD-1000 Bioreactor flask device) ^[16,17,27,28] (see Experimental Section). Separation of sMVs from exosomes was achieved using a differential centrifugation strategy (**Fig. 1A**), essentially by pelleting down larger particles before exosomes. The yields of MDCK- and 21D1- cell-derived sMVs from 2,000 mL culture medium, based upon protein content, were 3500 µg and 3850 µg, respectively. We conducted cryo-electron microscopy (cryo-EM) (**Fig. 1B**) and nanoparticle tracking analysis (Supplemental **Fig. S1A/B**) to characterise our sMV preparations. Cryo-EM revealed ellipsoid morphology for both MDCK-/ 21D1-sMVs and a particle size range 50-950 nm diameter. Our sMV preparations were further analysed by immunoblot for the expression of stereotypic epithelial and mesenchymal markers (**Fig. 1C**). In agreement with our MDCK cell model for EMT, MDCK-sMVs were positive for the epithelial marker cadherin-1 (CDH1), and 21D1-sMVs positive for the mesenchymal markers fibronectin (FN1), vimentin (VIM) and N-cadherin (CDH2). Both MDCK- and 21D1-sMVs are devoid of the stereotypic exosomal marker protein CD63, exhibit diminished expression of TSG101, and show expression of CANX (Supplemental **Fig. S1C**). A comparative analysis of proteins selectively sorted into 21D1-sMVs and 21D1-exosomes (shown in heatmap, Supplemental **Fig. S3**) show that sMVs and exosomes are biochemically distinct.

Next we examined the protein profiles of MDCK-/ 21D1-sMVs using a label-free MS approach ^[27]. Overall, 2659 and 1712 proteins were identified in MDCK-/21D1-sMVs, respectively, with 1085 proteins uniquely present in MDCK-sMVs (based on presence/ absence of peptide ion spectra) and 138 unique protein identifications in 21D1-sMVs; 1574 proteins were common to both MDCK- / 21D1 sMVs (**Fig. 2A**, see Supplemental **Table S2** for global protein identifications, and Supplemental **Table S3** for identified unique proteins). These data indicate selective trafficking of many MDCK- and 21D1-cellular proteins to their respective sMVs.

A deeper interrogation of the protein data in **Fig. 2A** revealed 222 proteins were selectively enriched ($\log_2FC > 1$) in 21D1-sMVs, relative to 2309 proteins in MDCK-sMVs. Selected examples of enriched protein identifications are given in Table 1; a full list is provided in Supplemental **Table S4**.

Examination of these specifically enriched sMV proteins revealed many cellular proteins implicated in core EMT change categories such as – (i)-cytoskeleton/ECM remodelling, (ii)-loss of apical-basal cell polarity, (iii)-cell-cell adhesion weakening, (iv)-cell-matrix adhesion remodelling, (v)-acquisition of cell motility, and (vi)-basement membrane perturbation/cell migration’ ^[3,6,29] (**Table 1**). Criteria for preferentially sorted in MDCK-sMVs or 21D1-sMVs was based on those proteins not being evident, or of very low abundance, in one, but not the other sMV type.

First, we examined proteins specifically sorted into MDCK-sMV. This analysis revealed an abundance of proteins important in maintaining epithelial cell apical-basal polarity: for example, proteins associated with tight junctions (Tight junction proteins ZO-1, -2, -3 (TJP1/2/3/), claudins 2, 3, 5, 6, 7 (CLDN 2/3/5/6/7), PATJ, crumbs cell polarity complex, par-6 family cell polarity regulator β (PARD6B) etc), adherens junctions (E-cadherin (CDH1), epithelial cell adhesion molecule (EpCAM), catenin β -1 (CTNNB1) etc), desmosomes (desmoglein 2 (DSG2), plakophilins 2/3/4 (PKP2/3/4), periplakin (PPL), desmoplakin (DSP), envoplakin ((EVPL), epiplakin (EPPK1), hemidesmosome (collagen type alpha 1 (XVII chain) (COL17A1), laminin-1 subunits alpha-3/ gamma-1 (LAMA3/LAMC1), α 6 β 4 integrins (ITGA6/ ITGB4) etc), and cell-matrix proteins involved in BM assembly (perlecan (HSPG2), agrin (AGRN). Other prominent proteins associated with cell polarity include members of the protein-tyrosine phosphatase (PTPs) family (PTPRJ, PTPRF, PTPA, PTP4A2, PTPN14, PTPN21 etc) and EMT suppressors (GSBP2, LMNB1, Rab25 etc).

Next, we examined proteins selectively sorted in 21D1-sMV critical for the EMT process (**Table 1**). Prominent amongst these include cellular modulators of EMT such as N-cadherin (CDH2), fibronectin (FN1), vimentin (VIM), β 1 β 3 integrins (ITGB1/ ITGB3), MAPK3, MAP4K4, ARAF, PGPEP1, TGFBI, TIMP1, Serpin family E member2, TGM2, AXL, GAS6, etc. Other abundant proteins selectively enriched in 21D1-sMV relative to MDCK-sMV include proteins associated with cytoskeleton/ ECM remodelling (cadherin-2, WNT5A, integrins A5/A6/ B1 etc). Interestingly, 21D1-sMV were also selectively enriched in key modulators of metastatic niche development and tumour progression (e.g., S100A9, S100A4, S100A6, receptor-type tyrosine-protein phosphatase alpha (PTPRA), cluster of differentiation 155 (CD155)/ polio virus receptor (PVR)), milk fat globule-EGF factor 8 protein (MFGE8), lactadherin (MFG-E8), glyican-4 (GPC4), syndecan-1 (SDC1), transglutaminase 2 (TGM2) etc). Further confirmation of fibronectin, MAPK/ERK1, phosphorylated MAPK, TGM2, H-Ras, vimentin -, integrin beta 1 subunit in 21D1-sMV was provided by immunoblot analysis (Fig. 2B).

To focus on the 222 selectively enriched proteins in 21D1-sMV in the context of signalling pathways implicated in EMT we performed global STRING based protein-protein interaction network analysis. This interrogation revealed prominent protein-protein interactions of the MAPK cascade (HRAS/ARAF/MAPK3), transglutaminase 2 (TGM2) ternary complex (TGM2, FN1, ITGB1), integrin signalling (ITGA5, ITGA6, FN1), and non-canonical Wnt signalling (WNT5A, GNG5, GNG10, HRAS)^[2,30-32] (**Fig. 2C**). Because of the unexpected finding of specific sorting of TGM2 and key components of the TGM2/ FN1 complex in 21D1-sMV we decided to interrogate the 1574 protein identifications common to both MDCK-/ 21D1-sMV (**Fig. 2A**) for other known TGM2 interaction partners. Known binary interacting partners of TGM2 were sourced using IntAct molecular interaction database^[25].

Apart from FN1 and ITGB1 identified in **Fig. 2C**, interrogation of 11 molecular interaction databases using IntAct platform revealed 16 additional interacting partners that we see in our 21D1-sMV proteome dataset (COL5A2, ABCA1, CDC42, CDH1, CEACAM1, COL18A1, GNAQ, HNRNPF, HSPA8, MVP, NME1, PLCD1, PSMD4, RBM25, SRC, and YWHAE) (**Fig. 2D**, Supplemental **Table S5**).

3.2. Distinct RNA-binding protein and mitochondrial protein profiles in 21D1-sMVs

Since we previously detected RNA-binding proteins (RBPs) and mitochondrial proteins in tumour-derived exosomes^[33,34] and sMVs^[19,27], we determined their selective sorting into 21D1-sMVs. Specifically, 15 RBPs (13 unique, i.e., not seen in MDCK-sMVs) and 5 mitochondrial proteins (all unique to 21D1-sMVs) were preferentially enriched in 21D1-sMVs, relative to MDCK-sMVs (**Table 2**). Interestingly, 417 RBPs are selectively enriched in MDCK-sMVs when compared to 21D1-sMVs, of which 177 are unique, not being observed in 21D1-sMVs (Supplemental Table S6). In the case of mitochondrial proteins, they are abundant in MDCK-sMVs – 170 selectively enriched, of which 76 uniquely distribute to MDCK-sMVs, relative to 21D1-sMVs Supplemental **Table S7**). The differential distribution pattern of RBPs and mitochondrial proteins in MDCK-sMVs when compared to 21D1-sMVs indicates that they are unlikely to be contaminants of the isolation; further studies are required to unravel their functional role in EMT.

3.3. 21D1-sMVs induce migration, anchorage-independent growth capability, and EMT in parental MDCK cells

Because 21D1-sMVs are enriched with factors associated with cell proliferation and pre-metastatic niche development (**Table 1**) we reasoned that they might confer migration, anchorage-independent growth capability, and induce EMT in parental MDCK cells. To address this hypothesis we first compared the functional capabilities of MDCK-sMVs and 21D1-sMVs using the scratch wound-healing assay on confluent cultures of MDCK cells treated with MDCK-sMVs or 21D1-sMVs (10 µg vesicle/mL). Within 24 h of disrupting the cell monolayers by scratching with a pipette tip, the 21D1-sMV-treated MDCK cells began to form leading edges (**Fig. 3A**, compare middle and right-hand panels).

The ability of 21D1-sMVs to induce the anchorage-independent growth (i.e., colony formation in soft agar) was also investigated (**Fig. 3B**). Previously, we showed that MDCK cells when transformed with oncogenic H-Ras gain the ability to form colonies in soft agar^[35]. As expected, MDCK cells either left untreated (no co-culture with sMVs) or treated with MDCK-sMVs for 10 days remained primarily as

single cells (**Fig. 3B**, *left and centre panels*). However, when MDCK cells were treated with 21D1-sMV they formed colonies (**Fig. 3B**, compare *right panel* with *left and middle panel*); an approximate 10-fold increase in colonies over 10 days (**Fig. 3C**). Fluorescent microscopic analysis of monolayers of MDCK-/ 21D1-sMV treated MDCK cells were examined for morphological changes (**Fig. 3D**). Interestingly, after 10 days 21D1-sMV-treated MDCK cells exhibited fibroblast-like, elongated spindle-shaped morphology, attenuated expression of adhesion junction protein CDH1 (E-cadherin) and elevated expression of the mesenchymal marker VIM, characteristic properties of EMT induction (**Fig. 3D**). Further support of EMT induction in MDCK cells following treatment with 21D1-sMV was evidenced by immunoblot analysis (for example, decreased expression of CDH1 (E-cadherin) and elevated expression of CDH2 (N-cadherin) (**Fig. 3E**).

Collectively, our findings suggest that MDCK-sMV cargo changes that occur following oncogenic H-Ras transformation are sufficient to confer cellular migration, anchorage-independent growth capabilities and induce EMT in recipient MDCK cells.

3.4. Microvesicle-associated transglutaminase-2 (TGM2) can impart invasive capability to fibroblasts

An unexpected finding was the specific enrichment of tissue transglutaminase-2 (TGM2) in 21D1-sMVs (Table 1) and components of the TGM2 ternary complex (TGM2/FN1/ITGB1)^[36] in our protein-protein interaction analysis (**Fig. 2C**). TGM2 is not sorted into MDCK-sMVs, nor MDCK-exosomes^[12], and appears to be a direct consequence of oncogenic H-Ras-induced EMT. Here, we examined whether the invasive capability of 21D1-sMVs (see **Fig. 3**) could be influenced by targeting the surface exposed ternary TGM2 complex (TGM2:FN:ITGB1). First we determined that fluorescently-labelled (using lipophilic tracer Dil) MDCK-sMVs/ 21D1-sMVs could be successfully taken up by NIH3T3 cells by using fluorescent microscopic analysis (**Fig. 4A**, Supplemental **Fig. S2**). Next, we used the Transwell cell migration and invasion assay to determine invasion capability of fluorescently-labelled 21D1-sMVs. NIH3T3 fibroblasts were treated with MDCK-sMVs or 21D1-sMVs (30 µg/ mL) for 2 h and then allowed to invade across Matrigel™ matrix for 16 h. Fluorescence microscopic analysis revealed that fibroblasts treated with 21D1-sMVs were > 60-fold more invasive than their MDCK-sMV-treated counterparts (**Fig. 4**). The ability of 21D1-sMVs to induce anchorage-independent growth (i.e., colony formation in soft agar) in NIH3T3 fibroblast was also investigated (**Fig. 4C, D**). As expected, NIH3T3 fibroblast either left untreated (no co-culture with sMVs) or treated with MDCK-sMVs for 10 days remained primarily as single cells (**Fig. 4C**, *left and centre*

panels), but when treated with 21D1-sMV formed colonies (**Fig. 4C**, compare *right panel* with *left* and *middle panel*); > 20-fold increase in colonies over 10 days, compared with MDCK-sMV treatment (**Fig. 4D**).

To confirm the presence of ternary TGM2 complex (TGM2:FN1:ITGB1) in 21D1-sMVs we immunoprecipitated TGM2 from 21D1-sMV lysate and then immunoblotted the IP using anti-FN1 and anti-ITGB1 antibodies (**Fig. 4E**). Because TGM2 and FN1 have been detected on the surface of extracellular vesicles^[37-39], and associated with metastatic niche establishment^[32], we questioned whether pre-treatment of 21D1-sMVs with neutralizing anti-TGM2 or anti-FN1 antibodies might attenuate their invasive capability on fibroblasts. For this experiment, NIH3T3 fibroblasts were pre-treated 21D1-sMVs (30 µg/ mL) that had been exposed (2 h) with anti-FN1, or anti-TGM2 antibodies or anti-IgG antibodies and then allowed to invade across the matrix for 16 h. In **Fig. 4F** it can be seen that prior treatment of 21D1-sMVs with neutralizing anti-TGM2 or anti-FN1 antibodies attenuates (2-4 fold) their invasive capability in the Transwell-Matrigel™ matrix invasion assay.

3.5. Proteins associated with suppression of *anoikis* are evident in sMVs following oncogenic H-Ras transformation

Anchorage-independent growth and oncogenic EMT are both linked to suppression of anoikis, a programmed cell death induced by disruption of epithelial cell-matrix interactions^{[40], [8], [41]}. Our finding that oncogenic H-Ras reprograms MDCK-derived sMV proteins, and that these sMV cargo changes promote anchorage independency in non-transformed cells upon 21D1-sMV uptake, led us to ask whether proteins associated with anoikis resistance might be evident in 21D1-sMVs.

Examination of the proteomic content of MDCK-sMVs and 21D1-sMVs (Table 1, and Supplemental Table S3) revealed a number of proteins involved in pathways implicated in overcoming anoikis (anoikis resistance) in 21D1-sMVs (for example, anamorsin/ cytokine induced apoptosis inhibitor 1 (CIAPIN1), WNT5A, proteins involved in constitutive activation of RAS/MAPK pathway (HRAS, ARAF), integrins ITGA5, ITGA6, and ITGB1). In contrast, proteins central to apoptosis (programmed cell death) were identified in MDCK-sMVs (**Fig. 5**).

4. Discussion

EMT is a highly organized morphogenic process whereby cellular organisation from epithelial to mesenchymal phenotypes occurs leading to functional changes in cell migration and migration^[1,2].

This evolutionary-conserved program occurs in a diverse range of physiological and pathological conditions. Over the past decade we have employed oncogenic H-Ras-transformed MDCK cells (21D1 cells) as an EMT model system to gain insights into proteomic changes that might modulate the EMT process - focussing on the contribution of soluble secreted proteins (the secretome) ^[9,10], membrane-associated proteins ^[42] and, more recently, the protein cargo of secreted extracellular vesicles (exosomes) ^[12]. There is a growing awareness that EVs comprise at least two major classes - exosomes and sMVVs (also termed microparticles/ microvesicles/ ectosomes) – and that distinct sub-populations exist for each of these classes (for reviews, see ^[11,43] and references therein). Exosomes and sMVVs differ in biochemical composition ^[27] and mode of biogenesis, but little is known about functional differences, especially in the context of EMT biology. Here, we dissected the proteome of sMVVs secreted by oncogenic H-Ras-transformed MDCK cells (21D1-sMVVs) and explored the effect of sMVVs on cell migration and invasiveness capability.

In this study we employed a differential centrifugation strategy to successfully separate sMVVs from exosomes (**Fig. 1**) in high yield. For example, ~3.6 mg purified microvesicles (based on protein content) were achieved from 2,000 mL cell culture media harvested each day over three months using continuous-culture technology (CeLLine AD-1000 Bioreactor flask device). Purified MDCK sMVVs are larger and more heterogenous in size (50-950 nm) than exosomes (50-150) and, morphologically, are more ellipsoid in shape than spherical exosomes.

Our proteomic analysis revealed selective sorting of prominent cellular modulators of EMT (WNT5A, MPAK3, HRAS, and TGM2, for example) into 21D1-sMVVs (**Table 1**, and Supplemental **Table S2-4**). Notably, AXL (member of TAM (Tyro3, Axl, Mer) family of receptor tyrosine kinases), a well-documented inducer of EMT and regulator of cancer progression and metastasis ^[44-46] selectively traffics to MDCK sMVVs following H-Ras-induced transformation. Proteins associated with cytoskeleton/ ECM remodelling (cadherin-2, WNT5A, integrins A5/A6/ B1 etc) ^[29] and key modulators of metastatic niche development and tumour progression (e.g., S100A9, S100A4, S100A6, receptor-type tyrosine-protein phosphatase alpha (PTPRA), cluster of differentiation 155 (CD155)/ polio virus receptor, PVR), milk fat globule-EGF factor 8 protein (MFGE8), lactadherin (MFG-E8), glypican-4 (GPC4), syndecan-1 (SDC1), transglutaminase 2 (TGM2) were also evident. 21D1-sMVVs and 21D1-exosomes are biochemically distinct, as shown in heatmap of comparative proteome analysis (Supplemental **Fig. S3**). For example, ECM proteins and proteins associated with EMT induction, RNA binding and mitochondrion selectively traffic to 21D1-sMVVs, membrane-associated proteins, transcriptional factors and splicing factors selectively traffic to 21D1-exosomes.

We found further differences between MDCK-sMV and 21D1-sMV cargo, especially RNA-binding proteins (RBPs) and mitochondrial proteins. For example, selective sorting (Log2FC >1) of 14 RBPs into 21D1-sMVs (**Table 2**). According to the manually-curated databases of RNA-binding specificities (RBPDB) ^{[47], [48]} 7 of the identified RBPs - Zinc finger protein 638/ NP220 (*ZNF638*), Elongation factor Ts (*TSFM*), Eukaryotic translation initiation factor 4 gamma 2 (*EIF4G2*), Ribosome-releasing factor 2, mitochondrial (*GFM2*), Calcium-regulated heat stable protein1 (*CARHSP1*), Eukaryotic translation initiation factor 3 subunit J (*EIF3J*), Nuclear RNA export factor 1 (*NXF1*), Signal recognition particle 9 kDa protein (*SRP9*), antiviral innate immune response receptor RIG1 (*DDX58*), bind specifically to mRNA species, two RBPs bind to ribosomal RNA (60S acidic ribosomal protein P2 (*RPLP2*) and Mitochondrial ribosomal protein S27 (*MRPS27*)), three bind tRNA specifically (Elongation factor beta-1 (*EEF1B2*), Elongation factor 1-gamma (*EEF1G*), Interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*), and two bind specifically to ncRNA (antiviral innate immune response receptor RIG-I (*DDX58*), signal recognition particle 9 kDa protein (*SRP9*)). Many of these RBPs are reported to induce EMT pathways ^{[49], [50], [51], [52]}, mitochondrial protein synthesis ^[53], and are expressed in several cancer types ^[54].

Importantly, our observation that several mitochondrial proteins have different enrichment distribution patterns in MDCK-sMVs/21D1-sMVs (**Table 2**) suggests they are not contaminants, but might have physiological significance. For example, the 5 mitochondrial proteins listed in **Table 2** (Reticulocalbin 1 (*RCN1*), Anamorsin (*CIAPIN1*), Ribosome-releasing factor 2, mitochondrial (*GFM2*), OCIA domain-containing protein 2 (*OCIAD2*), Mitochondrial antiviral signalling protein (*MAVS*)) are unique for 21D1-sMVs – i.e., not evident in MDCK-sMVs. Conversely, many mitochondrial proteins are selectively sorted in MDCK-sMVs compared to 21D1-sMVs (see Table 1 and Supplemental Table S7). Our finding of selective sorting of mitochondrial proteins in MDCK sMVs is consistent with previous reports from our lab ^[19,27], as well as others (for example, MVs released by LPS-treated monocytes ^{[55], [56]}, activated platelets ^{[57], [58], [59]}, mesenchymal stem cells exposed to oxidative stress ^[60], and airway myeloid-derived regulatory cells ^[61]). Intriguingly, there are emerging reports that EVs harbour intact, functional mitochondrion (for example, 300-1100 nm size range astrocyte-derived particles ^[62], mesenchymal stem cell-derived microvesicles ^[60], airway myeloid-derived regulatory cell exosomes ^[61], and activated monocyte-derived microvesicles ^[55]). Interestingly, Phinney and colleagues ^[60] do not find intact mitochondrion in mesenchymal stem cell-derived exosomes, a finding consistent with our data for colorectal cancer cell line (SW480, SW620,

LIM1863, LIM1215) and MDCK-derived exosomes. Although we do not observe intact mitochondria in our present study based on cryo-EM, we cannot exclude the possibility of mitochondrial fragments contaminating our sMV preparation. The question of how mitochondria and mitochondrial proteins in EVs might interact with extracellular signalling warrants further exploration in a variety of cells types and body fluids; elucidating the biogenesis of mitochondrion-containing EVs will be essential in determining their role(s) in cellular and organ function, especially in the context of EMT.

Our analysis also showed proteins involved in protein-protein interaction networks were selectively sorted in 21D1-sMVs. Of these, the MAPK signalling cascade (HRAS/ ARAF/ MAPK3), tissue transglutaminase (TGM2) ternary complex (TGM2/ FN1/ ITGB1), and non-canonical Wnt signalling (WNT5A/ GNG5/ GNG10/ HRAS) were most prominent^{[2], [30-32]} (**Fig. 2**). This finding suggests these interaction networks, amongst others, may influence phenotypic changes associated with oncogenic H-Ras induced EMT.

Because 21D1-sMVs are enriched with factors associated with cell proliferation and pre-metastatic niche development (**Table 1**) we explored the functionality of 21D1-sMVs on parental MDCK cells. First, we investigated whether 21D1-sMVs could influence cellular migration behaviour of confluent cultures of MDCK cells on two-dimensional (2-D) surfaces using the scratch wound-healing assay. Within 24 h of disrupting the cell monolayers by scratching, the 21D1-sMV-treated MDCK cells, in contrast to MDCK-sMV treated cells, formed leading edges (**Fig. 3**). Previously, we reported that MDCK cells when transformed with oncogenic H-Ras gain the ability to form colonies in soft agar^[35]. This led us to ask whether 21D1-sMVs alone could induce MDCK cells to undergo colony-independent growth. As expected, MDCK cells either left untreated (no co-culture with sMVs) or treated with MDCK-sMVs for 10 days remained primarily as single cells (**Fig. 3B**), while treatment with 21D1-sMVs they formed colonies. Interestingly, fluorescent microscopic analysis of monolayers of 21D1-sMV treated MDCK cells revealed fibroblast-like, elongated spindle-shaped morphology, E-cadherin expression attenuation and elevated expression of vimentin, characteristic of EMT induction (**Fig. 3D**). Further support of 21D1-sMV induced EMT in MDCK cells was provided by immunoblot analysis (for example, elevated expression of CDH2 (N-cadherin) (**Fig. 3E**).

An unexpected and novel finding was the unique trafficking of cellular TGM2 into 21D1-sMVs. We found no evidence of TGM2 in MDCK-sMVs or MDCK-exosomes^[12]. TGM2 belongs to a family of

enzymes (EC 2.3.2.13) that catalyse post-translational modifications of proteins through a calcium-dependent acyl-transfer reaction between the γ -carboxamide group of a peptide-bound glutamine residue and the ϵ -amino group of a peptide-bound lysine leading to formation of ϵ -(γ -glutamyl)lysine crosslinks, either inter- or intramolecular bonds, highly resistant to proteolysis ^[63,64]. Moreover, TGM2 has been shown to function as a GTPase, a protein disulphide isomerase and a molecular scaffold ^[65]. This key multifunctional enzyme has been associated with various physiological and pathological conditions, has pleiotropic nonenzymic functions based on its noncovalent interactions with multiple cellular proteins ^[36], is a mediator EMT and plays an essential role in tumour metastasis ^[31]. In a recent report TGM2 was shown to be present in metastatic breast cancer cell-derived EVs and promote EV-mediated metastatic niche formation ^[32]. Intriguingly, our finding that prior treatment of 21D1-sMV with neutralizing anti-TGM2 or anti-FN1 antibodies attenuates the invasive capability of fibroblasts (Fig. 4) suggests that vesicular TGM2 may have yet another important biological activity in its functionality pleiotropy. an important contributory role in the EMT process that warrants further investigation.

It is well recognized that anchorage-independent growth and EMT are both linked to suppression of anoikis, a programmed cell death induced by disruption of epithelial cell-matrix interactions ^[8,40,41]. In this context, our finding of multiple proteins implicated in overcoming anoikis (anoikis resistance) (see list of protein identifications in **Fig. 5**) in 21D1-sMV is unsurprising. Interestingly, it has been reported that the TGM2:FN1 complex ^[36], but not FN alone, could rescue TGM2-deficient mouse dermal fibroblast cells from anoikis ^[66], and Wnt5a activates RhoA to inhibit anoikis ^[67]. By contrast, proteins central to apoptosis (programmed cell death) and anoikis sensitivity were found to be selectively enriched in MDCK-sMV (see list in **Fig. 5, Table 1, Supplemental Table S4**). The tissue inhibitor of metalloproteinase (TIMP1), a well-known inhibitor of apoptosis in a variety of cell types that acts through classical PI3-kinase/ MAPK ^[68] signalling cascades is also evident in 21D1-sMV (Table 1). Our identification of cargo proteins in 21D1-sMV that are associated with pathways implicated in overcoming anoikis (anoikis resistance) further supports our hypothesis that sMV might act as vehicles to promote cellular migration and transformation in non-transformed cells.

Collectively, our findings suggest that MDCK-sMV cargo changes that occur following oncogenic H-Ras transformation are sufficient to confer cellular migration, anchorage-independent growth

capabilities and induce EMT in recipient MDCK cells. Among the important question for the future will be to better understand how the cargo of different EV classes (microvesicles and exosomes) along with the secretome act in a coordinated fashion to mediate EMT. It's also important to explore further what the impacts the sMV make on the cancer microenvironments as an enabler of cancer progression. Harnessing this knowledge will open avenues for future EV studies aimed at furthering our understanding of the role of sMVs in the EMT programme and the targeting of EVs as potential druggable molecules for therapeutic application.

Supporting Information

Supplemental Figs. S1, S2 and S3.

Supplemental Tables S1-7.

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Conflict of Interest

The authors declare no conflict of interest

Table 1. List of selected proteins identified in 21D1- and MDCK-sMV.

Category	Protein accession (UniProt)	Protein description	Gene name	Average LFQ Intensity ^a		Log2 (21D1-sMV/MDCK-sMV) ^b
				21D1-sMV	MDCK-sMV	
Receptors	F6UZY1 [#]	Adhesion G protein-coupled receptor G1	<i>ADGRG1</i>	7.08E+06	3.36E+06	1
	FIPGV4 [#]	AXL receptor tyrosine kinase	<i>AXL</i> *	2.03E+06	1.00E+00	21
	E2QWE7 [#]	G protein-coupled receptor 183	<i>GPR183</i> *	4.98E+06	1.00E+00	22
	F6XR14 [#]	Protein C receptor	<i>PROCR</i> *	3.98E+06	1.00E+00	22
	W5VNF7 [#]	Sphingosine-1-phosphate receptor 1	<i>S1PR1</i> *	2.70E+06	1.00E+00	21
Cytoskeleton	FIPCT1 [#]	BRICK1	<i>BRK1</i>	1.09E+07	2.06E+06	21
	E2R887 [#]	Formin like 3	<i>FMNL3</i> *	5.95E+06	1.00E+00	23
	FIPUI4 [#]	Serpin family E member 1	<i>SERPINE1</i>	5.51E+07	7.94E+06	3
	F6Y2H4 [#]	Serpin family E member 2	<i>SERPINE2</i> *	3.47E+07	1.00E+00	25
ECM	F1PG08 [#]	Collagen type V alpha 2 chain	<i>COL5A2</i> *	9.26E+06	1.00E+00	23
	J9P8M2 [#]	Fibronectin	<i>FN1</i> *	1.08E+08	1.00E+00	27
ECM Remodelling	F1PY05 [#]	Integrin subunit alpha 5	<i>ITGA5</i>	2.43E+07	3.01E+06	3
	J9NVU0 [#]	Integrin subunit alpha 6	<i>ITGA6</i>	3.65E+08	3.76E+07	3
	E2RFE1 [#]	Integrin beta	<i>ITGB1</i>	3.20E+08	6.14E+07	2
	F1PFZ5 [#]	Milk fat globule-EGF factor 8 protein	<i>MFG-E8</i>	2.42E+09	8.30E+08	2
EMT Inducer/Modulator	J9P5H0 [#]	Protein Wnt	<i>WNT5A</i> *	1.46E+07	1.00E+00	24
	E9PQW4	Mitogen-activated protein kinase	<i>MAPK3</i> *	5.38E+06	1.00E+00	22
	F1PEC4 [#]	Cadherin-2	<i>CDH2</i>	13239933	1	23.7
	E2RL20 [#]	A-Raf proto-oncogene, serine/threonine kinase	<i>ARAF</i> *	2.09E+07	1.00E+00	24
	P01112	GTPase HRas	<i>H-RAS</i>	4.43E+09	1.97E+08	4
	E2RB45 [#]	Phosphatidylinositol 4-kinase type 2-alpha	<i>PI4K2A</i>	1.09E+07	2.33E+06	2
	F1PMC5 [#]	Phosphatidylinositol 4-kinase alpha	<i>PI4KA</i>	1.18E+08	3.90E+07	2
	FIPUQ4 [#]	RPTOR independence companion of MTOR complex 2	<i>RICTOR</i> *	1.98E+07	1.00E+00	24
	F1Q435 [#]	Transglutaminase 2	<i>TGM2</i> *	1.04E+06	1.00E+00	20
	F1PQS2 [#]	Metalloproteinase inhibitor 1	<i>TIMP1</i> *	3.34E+08	1.00E+00	28

	E2RGN0 [#]	Plasminogen activator, urokinase receptor	<i>PLAUR*</i>	5.66E+06	1.00E+00	22
	F1PWE1 [#]	Plasminogen activator	<i>PLAT</i>	6.17E+07	7.21E+06	3.1
	F6XVD7 [#]	Ubiquitin carboxyl-terminal hydrolase	<i>UCHL1*</i>	4.65E+07	1.00E+00	25
	F1PW10 [#]	Transforming growth factor beta induced	<i>TGFB1</i>	1.01E+08	1.71E+07	3
Pre-metastatic niche factors	Q9TV56 [#]	S100 calcium-binding protein A4	<i>S100A4</i>	6.23E+08	4.41E+07	4
	P06702	S100 calcium-binding protein A9	<i>S100A9*</i>	7.35E+05	1.00E+00	19
	E2RFE1 [#]	Integrin beta 1	<i>ITGB1</i>	3.20E+08	6.14E+07	2
	F1PY05 [#]	Integrin subunit alpha 5	<i>ITGA5</i>	2.43E+07	3.01E+06	3
	J9NVU0 [#]	Integrin subunit alpha 6	<i>ITGA6</i>	3.65E+08	3.76E+07	3
	J9P8M2 [#]	Fibronectin	<i>FN1*</i>	1.08E+08	1.00E+00	27
	F1PQS2 [#]	Metalloproteinase inhibitor 1	<i>TIMP1*</i>	3.34E+08	1.00E+00	28
	J9P423 [#]	CD44 antigen	<i>CD44</i>	7.03E+08	2.52E+08	1
Drug-resistance	E2QUX2 [#]	ATP binding cassette subfamily A member 1	<i>ABCA1</i>	8.91E+06	1.00E+00	23
	F1PH11 [#]	ATP binding cassette subfamily B member 4	<i>ABCB4</i>	2.06E+07	7.78E+06	1
Cell Invasion	J9P8M2 [#]	Fibronectin	<i>FN1*</i>	1.08E+08	1.00E+00	27
	F1Q435 [#]	Transglutaminase 2	<i>TGM2*</i>	1.04E+06	1.00E+00	20
	E2RJI1 [#]	Galectin-1	<i>LGALS1</i>	3.57E+08	5.76E+06	6
	E2RT70 [#]	Syndecan-1	<i>SDC1*</i>	3.30E+06	1.00E+00	21.7
	F1PFZ5 [#]	Milk fat globule-EGF factor 8 protein	<i>MFGE8</i>	2.42E+09	8.30E+08	1.5
	F1PCT1 [#]	BRICK1	<i>BRK1</i>	1.09E+07	2.06E+06	21
	F1PM35 [#]	Glypican-4	<i>GPC4*</i>	7.56E+06	1.00E+00	22.8
Cell migration	P98172	Ephrin-B1	<i>EFNB1</i>	1.49E+07	1.92E+06	3
	F1PM35 [#]	Glypican-4	<i>GPC4*</i>	7.56E+06	1.00E+00	23
	J9NX46 [#]	GLI pathogenesis related 2	<i>GLIPR2</i>	1.01E+08	3.23E+06	5
Anchorage-independent growth	E2R1X9 [#]	Growth associated protein 43	<i>GAP43*</i>	4.70E+07	1.00E+00	25.5
	E2RRR8 [#]	Armadillo repeat gene deleted in velocardiiofacial syndrome	<i>ARVCF</i>	1.30E+08	1.51E+07	3.1
	T2AX92 [#]	High mobility group AT-hook 2	<i>HMGA2*</i>	7.94E+06	1.00E+00	22.9
	P01112	GTPase HRas	<i>H-RAS</i>	4.43E+09	1.97E+08	4

Anoikis resistant	E2RL20 [#]	A-Raf proto-oncogene, serine/threonine kinase	<i>ARAF</i>	2.00E+07	1.00E+00	24.3
	J9P423 [#]	CD44 antigen	<i>CD44</i>	7.03E+08	2.52E+08	1
	F1PY05 [#]	Integrin subunit alpha 5	<i>ITGA5</i>	2.00E+07	3.00E+06	2.7
	J9NVU0 [#]	Integrin subunit alpha 6	<i>ITGA6</i>	4.00E+08	4.00E+07	3.3
	E2RFE1 [#]	Integrin beta	<i>ITGB1</i>	3.00E+08	6.00E+07	2.3
	F1P8D5 [#]	EGF like repeats and discoidin domains 3	<i>EDIL3</i>	6.00E+07	4.00E+06	4
	J9P5H0 [#]	Protein Wnt	<i>WNT5A</i> *	1.46E+07	1.00E+00	24
	E2QUR [#] 0	Delta-like protein	<i>JAG1</i>	2.00E+07	1.00E+07	1
	E9PQW4	Mitogen-activated protein kinase	<i>MAPK3</i>	5.00E+06	1.00E+00	22.3
	F1PUQ4 [#]	RPTOR independent companion of MTOR complex 2	<i>RICTOR</i>	2.00E+07	1.00E+00	24.3
	E2RGN0 [#]	Plasminogen activator, urokinase receptor	<i>PLAUR</i>	5.66E+06	1.00E+00	22
	J9P8M2 [#]	Fibronectin	<i>FN1</i> *	1.08E+08	1.00E+00	27
	F1Q435 [#]	Transglutaminase 2	<i>TGM2</i> *	1.04E+06	1.00E+00	20
	F1PQS2 [#]	Metalloproteinase inhibitor 1	<i>TIMP1</i>	3.00E+08	1.00E+00	28.2
	Q9TV56 [#]	S100 calcium-binding protein A4	<i>S100A4</i>	6.00E+08	4.00E+07	4
Proliferation	F1PVS7 [#]	Poliovirus receptor (CD155)	<i>PVR</i>	1.14E+08	3.02E+07	1.9
	E2R5J7 [#]	Receptor-type tyrosine-protein phosphatase	<i>PTPRA</i>	1.23E+07	5.22E+06	1.2
	J9P211 [#]	Hippocalcin like	<i>HPCAL1</i> *	3.20E+06	1.00E+00	21.6
Metabolic reprogramming	F1PGJ3 [#]	Glutamine-fructose-6-phosphate transaminase 2	<i>GFPT2</i> *	1.32E+09	1.00E+00	30.3
	F6XVD7 [#]	Ubiquitin carboxyl-terminal hydrolase-L1	<i>UCHL1</i> *	4.65E+07	1.00E+00	25.5
	E2R311 [#]	UDP-glucose 6-dehydrogenase	<i>UGDH</i>	1.52E+08	7.05E+07	1.1
Angiogenesis	F1PYE3 [#]	Heat shock protein 27 kDa beta-1	<i>HSPB1</i>	3.82E+08	1.88E+08	1
	J9P5H0 [#]	Protein Wnt	<i>WNT5A</i> *	1.46E+07	1.00E+00	24
	E2QUR0 [#]	Delta-like protein	<i>JAG1</i>	2.43E+07	1.09E+07	1
	P21359	Neurofibromin	<i>NF1</i>	1.39E+07	4.73E+06	2
	E2RGN0 [#]	Plasminogen activator, urokinase receptor	<i>PLAUR</i> *	5.66E+06	1.00E+00	22
	E2RFE1 [#]	Integrin beta 1	<i>ITGB1</i>	3.20E+08	6.14E+07	2

Tight junction	O62683 [#]	Tight junction protein ZO-3 (Zona occludens protein 3)	<i>TJP3</i>	1.00E+00	2.32E+06	-21.1
	J9P749 [#]	Tight junction protein ZO-2 (Zona occludens protein 2)	<i>TJP2</i>	2.66E+07	1.16E+08	-2.1
	O97758 [#]	Tight junction protein ZO-1 (Zona occludens protein 1)	<i>TJP1</i>	1.16E+07	4.63E+07	-2
	E2R3X4 [#]	Occludin	<i>OCLN</i>	1.00E+00	2.97E+06	-21.5
	Q95KM6 [#]	Claudin-2	<i>CLDN2</i>	1.00E+00	1.89E+07	-24.2
	Q95KM5 [#]	Claudin-3	<i>CLDN3</i>	4.80E+06	6.14E+07	-3.7
	E2RNC2 [#]	Claudin-4	<i>CLDN4</i>	1.00E+00	3.72E+07	-25.1
	L7N0I6 [#]	Claudin-6	<i>CLDN6</i>	1.00E+00	1.36E+07	-23.7
	E2R5S3 [#]	Claudin-7	<i>CLDN7</i>	1.00E+00	6.74E+06	-22.7
	J9JHV1 [#]	PATJ, crumbs cell polarity complex component	<i>PATJ</i>	1.00E+00	3.88E+07	-25.2
	Q9BYG5	Partitioning defective 6 homolog beta	<i>PARD6B</i>	1.43E+06	5.13E+07	-5.2
	E2RM32 [#]	LLGL2, scribble cell polarity complex component	<i>LLGL2</i>	9.02E+06	1.42E+08	-4
Adhesion Junction	F1PAA9 [#]	Cadherin-1 (Epithelial cadherin)	<i>CDH1</i>	7.78E+06	1.42E+08	-4.2
	P35222	Catenin beta-1 (Beta-catenin)	<i>CTNNB1</i>	1.79E+08	8.57E+08	-2.3
	E2R9S7 [#]	Catenin alpha 1	<i>CTNNA1</i>	4.37E+08	1.34E+09	-1.6
	F1Q1M9 [#]	Epithelial cell adhesion molecule	<i>EPCAM</i>	5.91E+06	9.79E+08	-7.4
	J9P8W6 [#]	Tetraspanin	<i>TSPAN4</i>	1.00E+00	6.05E+06	-22.5
Desmosome	F1PGX2 [#]	Desmoglein 2	<i>DSG2</i>	1.70E+06	1.95E+08	-6.8
	E2RHY0 [#]	Plakophilin 2	<i>PKP2</i>	2.75E+06	7.23E+07	-4.7
	F1PY11 [#]	Plakophilin 3	<i>PKP3</i>	1.00E+00	4.19E+07	-25.3
	J9NU50 [#]	Plakophilin 4	<i>PKP4</i>	1.00E+00	9.55E+06	-23.2
	A0A140T8E6 [#]	Junction plakoglobin	<i>JUP</i>	4.20E+08	1.28E+09	-1.6
	J9P8T8 [#]	Desmoplakin	<i>DSP</i>	1.00E+00	8.57E+07	-26.4
	J9JH91 [#]	Envoplakin	<i>EVPL</i>	1.00E+00	6.01E+06	-22.5
	A0A075B730	Epiplakin	<i>EPPK1</i>	1.00E+00	1.00E+08	-26.6
	F1Q1I3 [#]	Periplakin	<i>PPL</i>	1.00E+00	4.20E+07	-25.3
Hemidesmosome	Q15149	Plectin	<i>PLEC</i>	5.96E+06	1.05E+08	-4.1
	Q28271 [#]	Collagen type IV alpha 1 chain	<i>COL4A1</i>	1.00E+00	4.19E+08	-28.6

	F1PFM5 [#]	Laminin subunit beta 3	LAMB3	1.00E+00	1.82E+08	-27.4
	Q867A2 [#]	Laminin-5 gamma 2	LAMC2	1.00E+00	1.20E+08	-26.8
	Q867A1 [#]	Laminin alpha 3	LAMA3	1.00E+00	5.43E+06	-22.4
Tyrosine Phosphatases	E2R341 [#]	Protein tyrosine phosphatase type IVA, member 2	PTP4A2	1.00E+00	1.26E+07	-23.6
	E2RLF5 [#]	Tyrosine-protein phosphatase non-receptor type	PTPN21	1.00E+00	7.04E+06	-22.7
	F1PD37 [#]	Serine/threonine-protein phosphatase 2A activator	PTPA	1.00E+00	5.79E+06	-22.5
	F1PMK1 [#]	Protein tyrosine phosphatase, non-receptor type 13	PTPN13	1.00E+00	2.52E+06	-21.3
	F1P983 [#]	Protein tyrosine phosphatase, receptor type J	PTPRJ	1.86E+06	8.82E+07	-5.6
	F1PU81 [#]	Tyrosine-protein phosphatase non-receptor type	PTPN14	1.83E+06	1.99E+07	-3.4
	E2RE80 [#]	Protein tyrosine phosphatase, receptor type F	PTPRF	9.76E+06	5.43E+07	-2.5
EMT suppressors	F1PBK4 [#]	Lamin B1	LMNB1	1.00E+00	8.17E+07	-26.3
	E2RQ15 [#]	Ras-related protein Rab-25	RAB25	1.00E+00	4.46E+07	-25.4
	Q9UN86	Ras GTPase-activating protein-binding protein 2	G3BP2	1.00E+00	4.44E+07	-25.4
	E2QWV0 [#]	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	SMARCA5	1.00E+00	6.69E+07	-26
Cell-matrix contacts	F1PCD8 [#]	Laminin subunit beta 1	LAMB1	1.00E+00	2.08E+07	-24.3
	F1PHK9 [#]	Laminin subunit gamma 1	LAMC1	1.70E+06	1.73E+07	-3.3
	F1Q2Z6 [#]	Agrin	AGRN	4.58E+07	6.87E+08	-3.9
	F6XBP8 [#]	Collagen type XVII alpha 1 chain (Bullous pemphigoid antigen 2)	COL17A1	4.41E+06	2.70E+07	-2.6
	F1Q129 [#]	Collagen type IV alpha 2 chain	COL4A2	4.56E+05	3.41E+08	-9.5

a) LFQ (label free precursor intensity) for proteins identified in each biological replicate in MDCK-sMV, 21D1-sMV. Taxonomy (organism) filter based on UniProt, indicating canine (#)

b) Log2 value of LFQ protein abundance ratio between 21D1-sMV and MDCK-sMV (refer Supplemental Table S2), log2 (21D1-sMV/Exos) > 1 (Fold Change (FC) > 1), which represents proteins selectively sorted in 21D1-sMV compared with MDCK-sMV were selected.

* Proteins uniquely sorted into 21D1-sMV, but not in MDCK-sMV

Table 2. RNA-binding proteins and mitochondrial proteins selectively sorted into 21D1-MVs, relative to MDCK-sMVs.

A. RNA-binding proteins^a

Category	Protein accession (UniProt)	Protein description	Gene name	Average LFQ Intensity ^b		Log2 (21D1-sMVs/MDCK-sMVs) ^c	Tauro MCP 2013 ^d
				21D1-sMVs	MDCK-sMVs		
mRNA	Q14966	Zinc finger protein 638(Nuclear protein 220/NP220)	<i>ZNF638*</i>	1.90E+09	1.00E+00	30.8	N
	A0A0E3ZR37 [#]	Elongation factor Ts	<i>TSFM*</i>	5.71E+08	1.00E+00	29.1	N
	E9PKF8	Eukaryotic translation initiation factor 4 gamma 2(Death-associated protein 5/DAP5)	<i>EIF4G2*</i>	2.89E+08	1.00E+00	28.1	N
	D6RAL1	Ribosome-releasing factor 2, mitochondrial	<i>GFM2*</i>	5.39E+07	1.00E+00	25.7	N
	Q9Y2V2	Calcium-regulated heat-stable protein 1	<i>CARHSP1*</i>	6.06E+06	1.00E+00	22.5	N
	E2QUU1 [#]	Eukaryotic translation initiation factor 3 subunit J	<i>EIF3J*</i>	1.46E+06	1.00E+00	20.5	N
	J9NV67 [#]	Nuclear RNA export factor 1	<i>NXF1*</i>	3.62E+05	1.00E+00	18.5	N
ncRNA	F1Q0Z5 [#]	Signal recognition particle 9 kDa protein	<i>SRP9*</i>	8.06E+06	1.00E+00	22.9	N
	E2RMV6 [#]	Antiviral innate immune response receptor RIG-I(DEXD/H-box helicase 58)	<i>DDX58</i>	2.27E+08	3.70E+07	2.6	N
ribosome	E2R9Y9 [#]	60S acidic ribosomal protein P2	<i>RPLP2*</i>	4.41E+06	1.00E+00	22.1	Y
	E2R5P9 [#]	Mitochondrial ribosomal protein S27	<i>MRPS27*</i>	3.64E+05	1.00E+00	18.5	N
tRNA	P26641	Elongation factor 1-gamma	<i>EEF1G*</i>	2.42E+07	1.00E+00	24.5	Y
	J9NXC5 [#]	Eukaryotic translation elongation factor 1 beta 2	<i>EEF1B2*</i>	5.12E+06	1.00E+00	22.3	N
	F1PWG0 [#]	Interferon-induced protein with tetratricopeptide repeats 5	<i>IFIT5*</i>	2.40E+06	1.00E+00	21.2	N

B. Mitochondrial proteins^f

Mitochondrial proteins	E2RTJ0 [#]	Anamorsin (Cytokine-induced apoptosis inhibitor1)	<i>CIAPIN1*</i>	8.10E+05	1.00E+00	19.6	N
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	D6RAL1	Ribosome-releasing factor 2, mitochondrial	GFM2*	7.74E+05	1.00E+00	25.7	N
	J9P0Y1 [#]	OCIA domain containing 2	OCIAD2*	3.64E+05	1.00E+00	20.1	N
	E2R3V7 [#]	Mitochondrial antiviral signaling protein	MAVS*	1.16E+06	1.00E+00	19.6	N
	E2R5P9 [#]	Mitochondrial ribosomal protein S27	MRPS27*	5.39E+07	1.00E+00	18.5	N

a), According to Gerstberger et al., 2014^[48] and RBPDB; a database of RNA-binding specificities^[47]. Taxonomy (organism) filter based on UniProt, indicating canine (#)

b) LFQ (label free precursor intensity) for proteins identified in each biological replicate in MDCK-sMV, 21D1-sMV.

c) Log2 value of LFQ protein abundance ratio between 21D1-sMV and MDCK-sMV (refer Supplemental Table S2), log2 (21D1-sMV/Exos) > 1 (Fold Change (FC) > 1), which represents proteins enriched in 21D1-sMV compared with MDCK-sMV were selected.

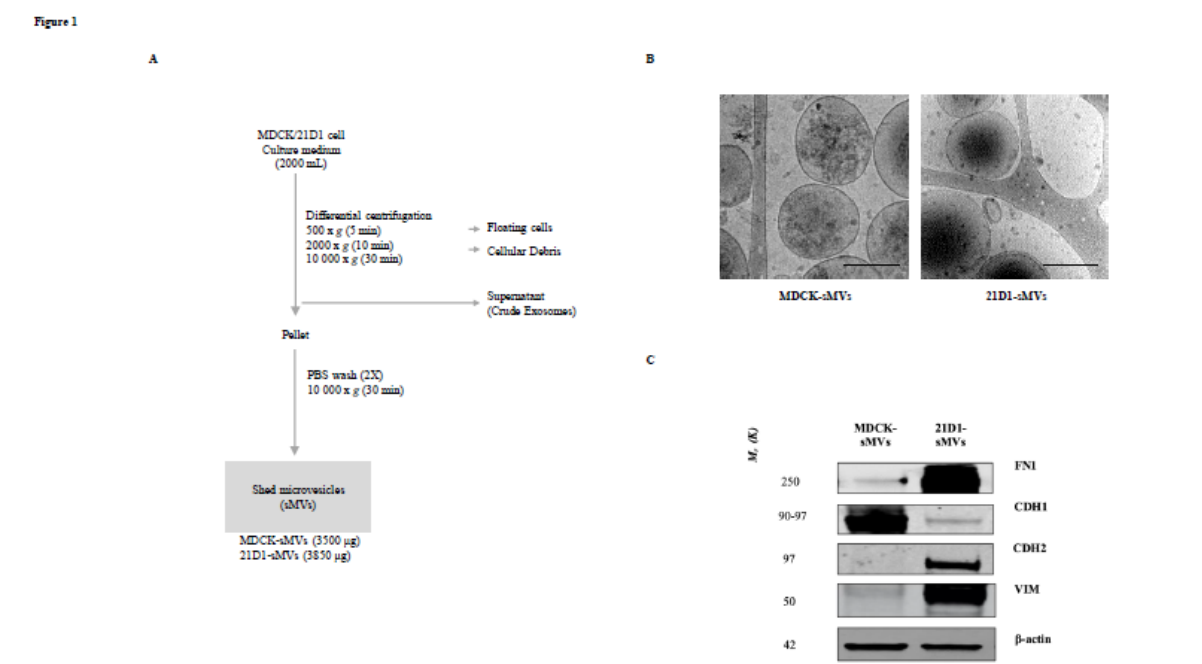
d) According to Tauro et al., 2013^[12]

f), Human protein atlas <https://www.proteinatlas.org>, Uhlen et al., 2015,^[69]

* Proteins uniquely sorted into sMV (i.e., present in 21D1-sMV, but not MDCK-sMV)

Figure Legends

Figure 1. Isolation and characterisation of MDCK- and 21D1-sMV. (A) Experimental workflow used for isolation of shed microvesicles (sMVs) from conditioned media of MDCK or 21D1 cells using differential centrifugation. Conditioned media was centrifuged to pellet floating cells (500 x g) and cellular debris (2,000 x g). The supernatant was then subjected to centrifugation at 10,000 x g to obtain purified sMVs. (B) Cryo-electron microscopic analysis of MDCK-sMVs and 21D1-sMVs. Scale bar, 200 nm. (C) Western blot analysis of purified sMVs using antibodies to stereotypic epithelial and mesenchymal markers (20 µg protein loaded per lane), n=5.



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Figure 2. Proteins preferentially sorted into MDCK- and 21D1-sMVs. (A) Two-way Venn diagram represents 1574 proteins commonly identified in MDCK- and 21D1-sMVs, and 1085 and 138 proteins uniquely sorted into MDCK-sMVs and 21D1-sMVs, respectively. Selective enrichment analysis revealed 222 proteins were sorted into 21D1-sMVs ($\log_2(\text{FC})$ 21D1-sMVs/ MDCK-sMVs value ≥ 1 , see Supplemental **Table S4**, and 2309 proteins sorted into MDCK-sMVs ($\log_2(\text{FC})$ (21D1-sMVs/ MDCK-sMVs value ≤ -1 (Supplemental **Table S4**). **(B)** Protein-protein interaction network analysis of 222 proteins sorted into 21D1-sMVs compared to MDCK-sMVs using STRING database analysis (Search Tool for the Retrieval of Interacting Genes/Proteins ^[24], version 10.5, high confidence (0.700), active interaction sources as ‘experiments’ and ‘databases’). **(C)** Western blot analysis of purified MDCK- / 21D1-sMVs (20 μg protein per lane) using anti-H-Ras, anti-p-MAPK, anti-FN1, anti-TGM2, anti-ITGB1, and anti- β -actin antibodies. **(D)** TGM2 binary protein interactions identified using the IntAct database, and visualized by Cytoscape. FN1, COL5A2 and ABCA1 (Red) were experimentally identified in list of specifically sorted proteins in 21D1-sMVs (Table 1), CDC42, CDH1, CEACAM1, COL18A1, GNAQ, HNRNPF, HSPA8, MVP, NME1, PLCD1, PSMD4, RBM25, SRC, and YWHAE (Green) were identified in list of proteins common to both MDCK-sMVs and 21D1-sMVs (Supplemental **Table S5**).

Figure 2

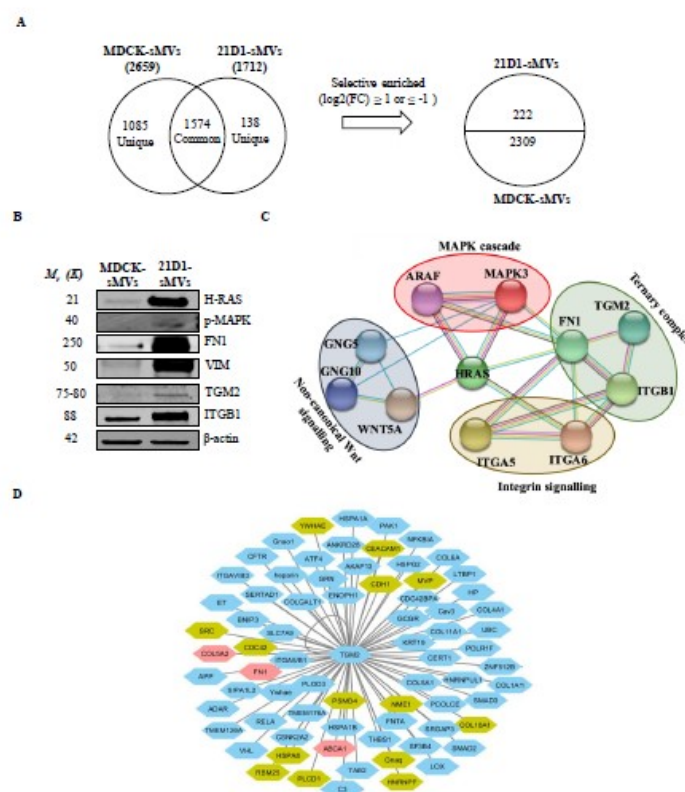
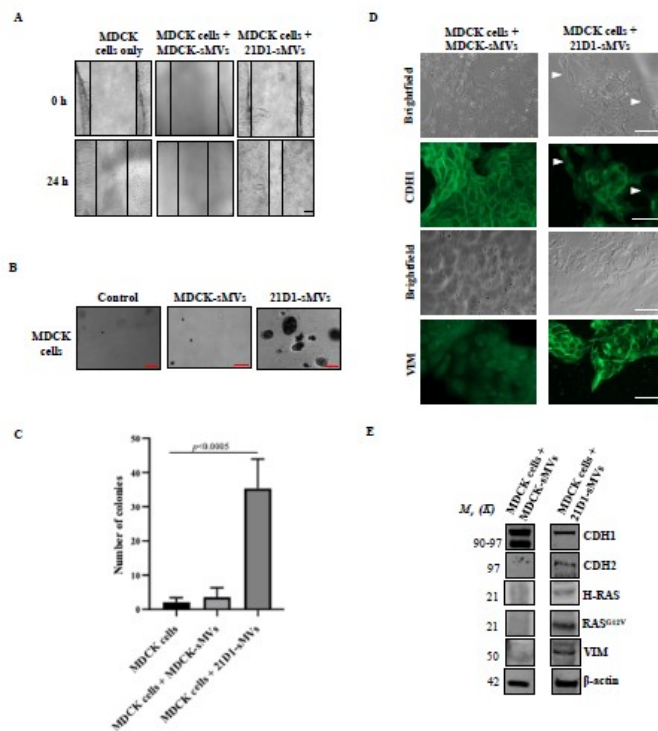


Figure 3. 21D1-sMVVs induce EMT in parental MDCK cells.

(A) Scratch wound-healing assay was performed on MDCK cells treated without (*Untreated*, MDCK cells only) or with MDCK-sMVVs / 21D1-sMVVs (10 μ g vesicle/mL). Twenty four hours after scratching the wounds, the cells were fixed and visualized by light microscopy to determine the extent of wound closure. One untreated plate of cells was fixed immediately after scratching the wound (0 h - *Untreated*) to show the size of the wounds at the start of the assay. Each panel represents a single image that shows both edges of a wound that was scratched in a monolayer of MDCK exposed to the indicated culturing condition (see Experimental section). The widths of the initially scratched wounds are indicated by *solid lines*. **(B and C)** Soft agar assays were performed on control MDCK cells (*Untreated*) and MDCK cells treated with MDCK-sMVVs or 21D1-sMVVs (10 μ g vesicle/mL) for 10 days with medium changed twice weekly; biological replicates (n=3), scale bar, 1000 μ m. Representative bright field images of the assays are shown in **B**, and **C** shows the number of colonies that formed for each condition. (data represent mean $\pm$ S.E). **(D)** Fluorescent microscopic analysis of monolayers of MDCK cells co-cultured for 10 days with 21D1-sMVVs or MDCK-sMVVs (10-20 μ g/mL). Culture media was changed every 3 days. At day 10 cells were washed, fixed and stained with anti-CDH1/E-cadherin or anti-VIM/vimentin antibodies (Green color). *White arrows* in bright field image and fluorescent image indicate 21D1-sMV-treated MDCK cells exhibit fibroblast-like, elongated spindle-shaped morphology. Fluorescent images show downregulated expression of adhesion junction protein E-cadherin (CDH1) and upregulated expression of mesenchymal marker VIM (n=3) – see Supplemental Fig. S2 for other fluorescent images, scale bar 100 μ m. **(E)** Western blot analysis of 21D1-sMVVs treated MDCK cells, cultured as in **D**, using anti-E-cadherin (CDH1), anti-N-cadherin (CDH2), anti-H-Ras, anti-RasG12V, anti-VIM, and anti- β -actin antibodies.

Figure 3

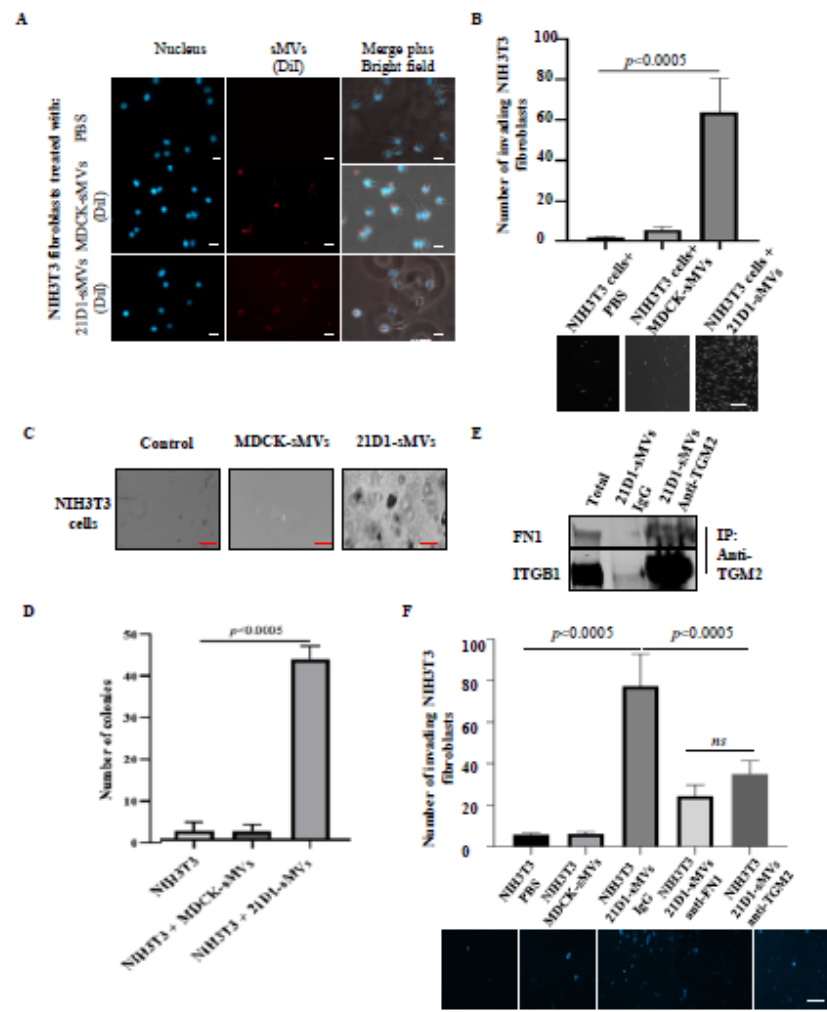


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Figure 4. TGM2 can impart invasive capability in fibroblasts.

(A) Fluorescence microscopic analysis of NIH3T3 cells incubated with MDCK- or 21D1-sMV (labelled with lipophilic dye Dil (Red)). Nuclei are stained with Hoechst stain (Blue). Scale bar, 10 μ m. **(B)** Transwell-Matrigel™ matrix invasion assay. NIH3T3 fibroblasts were treated with PBS (Untreated control), MDCK-sMV or 21D1-sMV for 2 h and allowed to invade across matrix for 16 h. Invading cells were stained for their nuclei (Hoechst stain, white) and imaged using fluorescence microscopy (*lower panels*). Scale bar, 50 μ m. Number of invading NIH3T3 fibroblasts are shown in *upper panel* (mean $\pm$ standard error of mean (s.e.m.)). **(C and D)** Soft agar assays were performed on control NIH 3T3 cells (*Untreated control*) and NIH 3T3 cells treated with MDCK-sMV or 21D1-sMV (10 μ g vesicle/mL) for 10 days with medium changed twice weekly; biological replicates (n=3), scale bar, 1000 μ m. Representative bright field images of the assays are shown in **C**, and **D** shows the number of colonies that formed for each condition. (data represent mean $\pm$ S.E). **(E)** TGM2 immunoprecipitation (IP) from 200 μ g 21D1-sMV lysate (1% TX-100/HEPES lysis buffer containing 25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl₂). Western blotting analysis of IP (20 μ g protein loaded) using anti-FN1 and anti-ITGB1 antibodies. *Left panel*, 21D1-sMV lysate (*Positive control, Total*); *Middle panel*, *Negative control* (21D1-sMV IP treated with anti-IgG isotype antibody), *Right panel* (21D1-sMV IP). **(F)** Transwell-Matrigel™ matrix invasion assay. NIH3T3 cells were treated with PBS (Untreated), MDCK-sMV, or 21D1-sMV with either anti-IgG, anti-FN1, or anti-TGM2 antibodies for 2 h and then allowed to invade across matrix for 16 h. Invading cells were stained for their nuclei (Hoechst stain, white) and imaged using fluorescence microscopy (*Lower panel*). Scale bar, 50 μ m. Number of invading NIH3T3 cells are shown (*Upper panel*), mean $\pm$ standard error of mean.

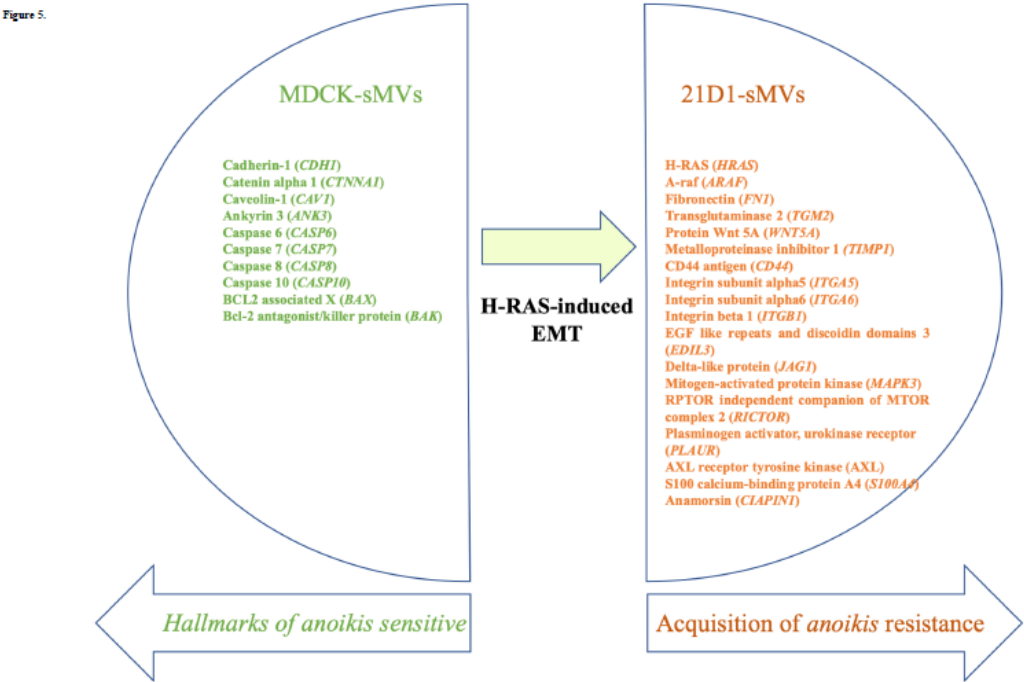
Figure 4.



Author

Figure 5. 21D1-sMV and MDCK-sMV anoikis-associated.

Schematic showing proteins identified in MDCK-sMVs that are central to apoptosis (programmed cell death) and proteins identified in 21D1-sMVs that are implicated in overcoming anoikis (anoikis resistance).



Supplemental Figure Legends

Supplemental Figure S1. Isolation and characterisation of MDCK- and 21D1-sMV.

sMVs were purified from culture media of MDCK or 21D1 cells using differential centrifugation. Culture media was centrifuged to pellet floating cells (500 x *g*) and cellular debris (2,000 x *g*). The supernatant was then subjected to centrifugation at 10,000 x *g* to obtain purified sMVs. **(A and B)** Nanoparticle tracking analysis (NTA) of MDCK-sMVs and 21D1-sMVs. **(C)** Western blot analysis of MDCK cell lysate, purified sMVs, and exosomes using antibodies to stereotypic exosomal and microvesicle markers (20 µg protein loaded per lane), n=3.

Supplemental Figure S2. 21D1-sMVs induce EMT in parental MDCK cells.

Fluorescent microscopic analysis of monolayers of MDCK cells co-cultured for 10 days with 21D1-sMVs or MDCK-sMVs (10-20 µg/mL). Culture media was changed every 2 days. At day 10 cells were washed, fixed and stained with anti-CDH1/E-cadherin or anti-VIM/vimentin antibodies (Green color). *White arrows* in bright field image and fluorescent image indicate 21D1-sMV-treated MDCK cells exhibit fibroblast-like, elongated spindle-shaped morphology. Fluorescent images show downregulated expression of adhesion junction protein E-cadherin (CDH1) and upregulated expression of mesenchymal marker VIM (n=3).

Supplemental Figure S3. Heat map illustration of proteins specifically associated with 21D1-/MDCK-sMVs and Exos. Proteins present in high abundance in 21D1-sMVs include ECM proteins, EMT inducers, RBPs and mitochondrial proteins. Proteins present in high abundance in 21D1-Exos include membrane-associated proteins, transcriptional factors and splicing factors. * Denotes proteins uniquely identified; ^ denotes proteins selectively enriched (fold change >2). Scale shown, Z-score = (individual value-mean)/ SD. MDCK-/21D1-Exos. Exos datasets can be accessed through Tauro et al. 2013^[12].

References

- [1] Nieto, M. A., Huang, R. Y., Jackson, R. A., & Thiery, J. P. (2016). EMT: 2016. *Cell*, 166(1), 21-45. doi: 10.1016/j.cell.2016.06.028
- [2] Dongre, A., & Weinberg, R. A. (2019). New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol*, 20(2), 69-84. doi: 10.1038/s41580-018-0080-4
- [3] Brabletz, T., Kalluri, R., Nieto, M. A., & Weinberg, R. A. (2018). EMT in cancer. *Nat Rev Cancer*, 18(2), 128-134. doi: 10.1038/nrc.2017.118
- [4] Owusu-Akyaw, A., Krishnamoorthy, K., Goldsmith, L. T., & Morelli, S. S. (2019). The role of mesenchymal-epithelial transition in endometrial function. *Hum Reprod Update*, 25(1), 114-133. doi: 10.1093/humupd/dmy035
- [5] Bartsch, D., Mises, N., Mahida, R. Y., Eickelberg, O., & Thickett, D. R. (2014). Epithelial-mesenchymal transition in lung development and disease: does it exist and is it important? *Thorax*, 69(8), 760-765. doi: 10.1136/thoraxjnl-2013-204608
- [6] Thiery, J. P., & Sleeman, J. P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol*, 7(2), 131-142. doi: 10.1038/nrm1835
- [7] Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*, 15(3), 178-196. doi: 10.1038/nrm3758
- [8] Frisch, S. M., Schaller, M., & Cieply, B. (2013). Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *J Cell Sci*, 126(Pt 1), 21-29. doi: 10.1242/jcs.120907
- [9] Mathias, R. A., Chen, Y. S., Wang, B., Ji, H., Kapp, E. A., Moritz, R. L., . . . Simpson, R. J. (2010). Extracellular remodelling during oncogenic Ras-induced epithelial-mesenchymal transition facilitates MDCK cell migration. *J Proteome Res*, 9(2), 1007-1019. doi: 10.1021/pr900907g
- [10] Mathias, R. A., Wang, B., Ji, H., Kapp, E. A., Moritz, R. L., Zhu, H. J., & Simpson, R. J. (2009). Secretome-based proteomic profiling of Ras-transformed MDCK cells reveals extracellular modulators of epithelial-mesenchymal transition. *J Proteome Res*, 8(6), 2827-2837. doi: 10.1021/pr8010974
- [11] Xu, R., Rai, A., Chen, M., Suwakulsiri, W., Greening, D. W., & Simpson, R. J. (2018). Extracellular vesicles in cancer - implications for future improvements in cancer care. *Nat Rev Clin Oncol*, 15(10), 617-638. doi: 10.1038/s41571-018-0036-9
- [12] Tauro, B. J., Mathias, R. A., Greening, D. W., Gopal, S. K., Ji, H., Kapp, E. A., . . . Simpson, R. J. (2013). Oncogenic H-ras reprograms Madin-Darby canine kidney (MDCK) cell-derived exosomal proteins following epithelial-mesenchymal transition. *Mol Cell Proteomics*, 12(8), 2148-2159. doi: 10.1074/mcp.M112.027086
- [13] Greening, D. W., & Simpson, R. J. (2018). Understanding extracellular vesicle diversity - current status. *Expert Rev Proteomics*, 15(11), 887-910. doi: 10.1080/14789450.2018.1537788
- [14] Phaner, C. J., Liu, S., Ji, H., Simpson, R. J., & Reid, G. E. (2012). Comprehensive lipidome profiling of isogenic primary and metastatic colon adenocarcinoma cell lines. *Anal Chem*, 84(21), 8917-8926. doi: 10.1021/ac302154g
- [15] Gopal, S. K., Greening, D. W., Rai, A., Chen, M., Xu, R., Shafiq, A., . . . Simpson, R. J. (2017). Extracellular vesicles: their role in cancer biology and epithelial-mesenchymal transition. *Biochem J*, 474(1), 21-45. doi: 10.1042/BCJ20160006
- [16] Ji, H., Greening, D. W., Barnes, T. W., Lim, J. W., Tauro, B. J., Rai, A., . . . Simpson, R. J. (2013). Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *Proteomics*, 13(10-11), 1672-1686. doi: 10.1002/pmic.201200562
- [17] Rai, A., Greening, D. W., Chen, M., Xu, R., Ji, H., & Simpson, R. J. (2019). Exosomes Derived from Human Primary and Metastatic Colorectal Cancer Cells Contribute to Functional

- Heterogeneity of Activated Fibroblasts by Reprogramming Their Proteome. *Proteomics*, 19(8), e1800148. doi: 10.1002/pmic.201800148
- [18] Greening, D. W., Nguyen, H. P., Elgass, K., Simpson, R. J., & Salamonsen, L. A. (2016). Human Endometrial Exosomes Contain Hormone-Specific Cargo Modulating Trophoblast Adhesive Capacity: Insights into Endometrial-Embryo Interactions. *Biol Reprod*, 94(2), 38. doi: 10.1095/biolreprod.115.134890
- [19] Xu, R., Greening, D. W., Rai, A., Ji, H., & Simpson, R. J. (2015). Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct. *Methods*, 87, 11-25. doi: 10.1016/j.ymeth.2015.04.008
- [20] Gorshkov, V., Verano-Braga, T., & Kjeldsen, F. (2015). SuperQuant: A Data Processing Approach to Increase Quantitative Proteome Coverage. *Anal Chem*, 87(12), 6319-6327. doi: 10.1021/acs.analchem.5b01166
- [21] Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*, 26(12), 1367-1372. doi: 10.1038/nbt.1511
- [22] Heck, M., & Neely, B. A. (2020). Proteomics in Non-model Organisms: A New Analytical Frontier. *J Proteome Res*, 19(9), 3595-3606. doi: 10.1021/acs.jproteome.0c00448
- [23] Lubner, C. A., Cox, J., Lauterbach, H., Fancke, B., Selbach, M., Tschopp, J., . . . Mann, M. (2010). Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity*, 32(2), 279-289. doi: 10.1016/j.immuni.2010.01.013
- [24] Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., . . . von Mering, C. (2017). The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res*, 45(D1), D362-D368. doi: 10.1093/nar/gkw937
- [25] Orchard, S., Ammari, M., Aranda, B., Breuza, L., Briganti, L., Broackes-Carter, F., . . . Hermjakob, H. (2014). The MIntAct project--IntAct as a common curation platform for 11 molecular interaction databases. *Nucleic Acids Res*, 42(Database issue), D358-363. doi: 10.1093/nar/gkt1115
- [26] Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., . . . Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*, 13(11), 2498-2504. doi: 10.1101/gr.1239303
- [27] Suwakulsiri, W., Rai, A., Xu, R., Chen, M., Greening, D. W., & Simpson, R. J. (2019). Proteomic profiling reveals key cancer progression modulators in shed microvesicles released from isogenic human primary and metastatic colorectal cancer cell lines. *Biochim Biophys Acta Proteins Proteom*, 1867(12), 140171. doi: 10.1016/j.bbapap.2018.11.008
- [28] Xu, R., Greening, D. W., Chen, M., Rai, A., Ji, H., Takahashi, N., & Simpson, R. J. (2019). Surfaceome of Exosomes Secreted from the Colorectal Cancer Cell Line SW480: Peripheral and Integral Membrane Proteins Analyzed by Proteolysis and TX114. *Proteomics*, 19(8), e1700453. doi: 10.1002/pmic.201700453
- [29] Yang, J., Antin, P., Berx, G., Blanpain, C., Brabletz, T., Bronner, M., . . . Association, E. M. T. I. (2020). Guidelines and definitions for research on epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*, 21(6), 341-352. doi: 10.1038/s41580-020-0237-9
- [30] Sima, L. E., Yakubov, B., Zhang, S., Condello, S., Grigorescu, A. A., Nwani, N. G., . . . Matei, D. (2019). Small Molecules Target the Interaction between Tissue Transglutaminase and Fibronectin. *Mol Cancer Ther*, 18(6), 1057-1068. doi: 10.1158/1535-7163.MCT-18-1148
- [31] Ayinde, O., Wang, Z., & Griffin, M. (2017). Tissue transglutaminase induces Epithelial-Mesenchymal-Transition and the acquisition of stem cell like characteristics in colorectal cancer cells. *Oncotarget*, 8(12), 20025-20041. doi: 10.18632/oncotarget.15370

- [32] Shinde, A., Paez, J. S., Libring, S., Hopkins, K., Solorio, L., & Wendt, M. K. (2020). Transglutaminase-2 facilitates extracellular vesicle-mediated establishment of the metastatic niche. *Oncogenesis*, 9(2), 16. doi: 10.1038/s41389-020-0204-5
- [33] Tauro, B. J., Greening, D. W., Mathias, R. A., Mathivanan, S., Ji, H., & Simpson, R. J. (2013). Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. *Mol Cell Proteomics*, 12(3), 587-598. doi: 10.1074/mcp.M112.021303
- [34] Chen, M., Xu, R., Ji, H., Greening, D. W., Rai, A., Izumikawa, K., . . . Simpson, R. J. (2016). Transcriptome and long noncoding RNA sequencing of three extracellular vesicle subtypes released from the human colon cancer LIM1863 cell line. *Sci Rep*, 6, 38397. doi: 10.1038/srep38397
- [35] Gopal, S. K., Greening, D. W., Zhu, H. J., Simpson, R. J., & Mathias, R. A. (2016). Transformed MDCK cells secrete elevated MMP1 that generates LAMA5 fragments promoting endothelial cell angiogenesis. *Sci Rep*, 6, 28321. doi: 10.1038/srep28321
- [36] Nurminkaya, M. V., & Belkin, A. M. (2012). Cellular functions of tissue transglutaminase. *Int Rev Cell Mol Biol*, 294, 1-97. doi: 10.1016/B978-0-12-394305-7.00001-X
- [37] Diaz-Hidalgo, L., Altuntas, S., Rossin, F., D'Eletto, M., Marsella, C., Farrace, M. G., . . . Piacentini, M. (2016). Transglutaminase type 2-dependent selective recruitment of proteins into exosomes under stressful cellular conditions. *Biochim Biophys Acta*, 1863(8), 2084-2092. doi: 10.1016/j.bbamcr.2016.05.005
- [38] Purushothaman, A., Bandari, S. K., Liu, J., Mobley, J. A., Brown, E. E., & Sanderson, R. D. (2016). Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions. *J Biol Chem*, 291(4), 1652-1663. doi: 10.1074/jbc.M115.686295
- [39] Antonyak, M. A., Li, B., Boroughs, L. K., Johnson, J. L., Druso, J. E., Bryant, K. L., . . . Cerione, R. A. (2011). Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc Natl Acad Sci U S A*, 108(12), 4852-4857. doi: 10.1073/pnas.1017667108
- [40] Paoli, P., Giannoni, E., & Chiarugi, P. (2013). Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta*, 1833(12), 3481-3498. doi: 10.1016/j.bbamcr.2013.06.026
- [41] Guadamillas, M. C., Cerezo, A., & Del Pozo, M. A. (2011). Overcoming anoikis--pathways to anchorage-independent growth in cancer. *J Cell Sci*, 124(Pt 19), 3189-3197. doi: 10.1242/jcs.072165
- [42] Chen, Y. S., Mathias, R. A., Mathivanan, S., Kapp, E. A., Moritz, R. L., Zhu, H. J., & Simpson, R. J. (2011). Proteomics profiling of Madin-Darby canine kidney plasma membranes reveals Wnt-5a involvement during oncogenic H-Ras/TGF-beta-mediated epithelial-mesenchymal transition. *Mol Cell Proteomics*, 10(2), M110 001131. doi: 10.1074/mcp.M110.001131
- [43] Xu, R., Greening, D. W., Zhu, H. J., Takahashi, N., & Simpson, R. J. (2016). Extracellular vesicle isolation and characterization: toward clinical application. *J Clin Invest*, 126(4), 1152-1162. doi: 10.1172/JCI81129
- [44] Gjerdrum, C., Tiron, C., Hoiby, T., Stefansson, I., Haugen, H., Sandal, T., . . . Lorens, J. B. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A*, 107(3), 1124-1129. doi: 10.1073/pnas.0909333107
- [45] Asiedu, M. K., Beauchamp-Perez, F. D., Ingle, J. N., Behrens, M. D., Radisky, D. C., & Knutson, K. L. (2014). AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene*, 33(10), 1316-1324. doi: 10.1038/onc.2013.57
- [46] Goyette, M. A., Duhamel, S., Aubert, L., Pelletier, A., Savage, P., Thibault, M. P., . . . Cote, J. F. (2018). The Receptor Tyrosine Kinase AXL Is Required at Multiple Steps of the Metastatic Cascade during HER2-Positive Breast Cancer Progression. *Cell Rep*, 23(5), 1476-1490. doi: 10.1016/j.celrep.2018.04.019

- [47] Cook, K. B., Kazan, H., Zuberi, K., Morris, Q., & Hughes, T. R. (2011). RBPDB: a database of RNA-binding specificities. *Nucleic Acids Res*, 39(Database issue), D301-308. doi: 10.1093/nar/gkq1069
- [48] Gerstberger, S., Hafner, M., & Tuschl, T. (2014). A census of human RNA-binding proteins. *Nat Rev Genet*, 15(12), 829-845. doi: 10.1038/nrg3813
- [49] Zhu, Y., Wang, G. Z., Cingoz, O., & Goff, S. P. (2018). NP220 mediates silencing of unintegrated retroviral DNA. *Nature*, 564(7735), 278-282. doi: 10.1038/s41586-018-0750-6
- [50] Batham, J., Lim, P. S., & Rao, S. (2019). SETDB-1: A Potential Epigenetic Regulator in Breast Cancer Metastasis. *Cancers (Basel)*, 11(8). doi: 10.3390/cancers11081143
- [51] Harada, K., & Nakanuma, Y. (2010). Biliary innate immunity in the pathogenesis of biliary diseases. *Inflamm Allergy Drug Targets*, 9(2), 83-90. doi: 10.2174/187152810791292809
- [52] Lo, U. G., Pong, R. C., Yang, D., Gandee, L., Hernandez, E., Dang, A., . . . Hsieh, J. T. (2019). IFN γ -Induced IFIT5 Promotes Epithelial-to-Mesenchymal Transition in Prostate Cancer via miRNA Processing. *Cancer Res*, 79(6), 1098-1112. doi: 10.1158/0008-5472.CAN-18-2207
- [53] Suomalainen, A., & Battersby, B. J. (2018). Mitochondrial diseases: the contribution of organelle stress responses to pathology. *Nat Rev Mol Cell Biol*, 19(2), 77-92. doi: 10.1038/nrm.2017.66
- [54] Hassan, M. K., Kumar, D., Naik, M., & Dixit, M. (2018). The expression profile and prognostic significance of eukaryotic translation elongation factors in different cancers. *PLoS One*, 13(1), e0191377. doi: 10.1371/journal.pone.0191377
- [55] Puhm, F., Afonyushkin, T., Resch, U., Obermayer, G., Rohde, M., Penz, T., . . . Binder, C. J. (2019). Mitochondria Are a Subset of Extracellular Vesicles Released by Activated Monocytes and Induce Type I IFN and TNF Responses in Endothelial Cells. *Circ Res*, 125(1), 43-52. doi: 10.1161/CIRCRESAHA.118.314601
- [56] Bernimoulin, M., Waters, E. K., Foy, M., Steele, B. M., Sullivan, M., Falet, H., . . . Wagner, D. D. (2009). Differential stimulation of monocytic cells results in distinct populations of microparticles. *J Thromb Haemost*, 7(6), 1019-1028. doi: 10.1111/j.1538-7836.2009.03434.x
- [57] Dean, W. L., Lee, M. J., Cummins, T. D., Schultz, D. J., & Powell, D. W. (2009). Proteomic and functional characterisation of platelet microparticle size classes. *Thromb Haemost*, 102(4), 711-718. doi: 10.1160/TH09-04-243
- [58] Boudreau, L. H., Duchez, A. C., Cloutier, N., Soulet, D., Martin, N., Bollinger, J., . . . Boilard, E. (2014). Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood*, 124(14), 2173-2183. doi: 10.1182/blood-2014-05-573543
- [59] Duchez, A. C., Boudreau, L. H., Naika, G. S., Bollinger, J., Belleanne, C., Cloutier, N., . . . Boilard, E. (2015). Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A2-IIA. *Proc Natl Acad Sci U S A*, 112(27), E3564-3573. doi: 10.1073/pnas.1507905112
- [60] Phinney, D. G., Di Giuseppe, M., Njah, J., Sala, E., Shiva, S., St Croix, C. M., . . . Ortiz, L. A. (2015). Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. *Nat Commun*, 6, 8472. doi: 10.1038/ncomms9472
- [61] Hough, K. P., Trevor, J. L., Strenkowski, J. G., Wang, Y., Chacko, B. K., Tousif, S., . . . Deshane, J. S. (2018). Exosomal transfer of mitochondria from airway myeloid-derived regulatory cells to T cells. *Redox Biol*, 18, 54-64. doi: 10.1016/j.redox.2018.06.009
- [62] Hayakawa, K., Esposito, E., Wang, X., Terasaki, Y., Liu, Y., Xing, C., . . . Lo, E. H. (2016). Transfer of mitochondria from astrocytes to neurons after stroke. *Nature*, 535(7613), 551-555. doi: 10.1038/nature18928
- [63] Eckert, R. L. (2019). Transglutaminase 2 takes center stage as a cancer cell survival factor and therapy target. *Mol Carcinog*, 58(6), 837-853. doi: 10.1002/mc.22986
- [64] Katt, W. P., Antonyak, M. A., & Cerione, R. A. (2018). The diamond anniversary of tissue transglutaminase: a protein of many talents. *Drug Discov Today*, 23(3), 575-591. doi: 10.1016/j.drudis.2018.01.037

- [65] Gundemir, S., Colak, G., Tucholski, J., & Johnson, G. V. (2012). Transglutaminase 2: a molecular Swiss army knife. *Biochim Biophys Acta*, 1823(2), 406-419. doi: 10.1016/j.bbamcr.2011.09.012
- [66] Verderio, E. A., Telci, D., Okoye, A., Melino, G., & Griffin, M. (2003). A novel RGD-independent cell adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis. *J Biol Chem*, 278(43), 42604-42614. doi: 10.1074/jbc.M303303200
- [67] Hayakawa, Y., Ariyama, H., Stancikova, J., Sakitani, K., Asfaha, S., Renz, B. W., . . . Wang, T. C. (2015). Mist1 Expressing Gastric Stem Cells Maintain the Normal and Neoplastic Gastric Epithelium and Are Supported by a Perivascular Stem Cell Niche. *Cancer Cell*, 28(6), 800-814. doi: 10.1016/j.ccell.2015.10.003
- [68] Toricelli, M., Melo, F. H., Peres, G. B., Silva, D. C., & Jasiulionis, M. G. (2013). Timp1 interacts with beta-1 integrin and CD63 along melanoma genesis and confers anoikis resistance by activating PI3-K signaling pathway independently of Akt phosphorylation. *Mol Cancer*, 12, 22. doi: 10.1186/1476-4598-12-22
- [69] Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., . . . Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science*, 347(6220), 1260419. doi: 10.1126/science.1260419