

Exosomes derived from human primary and metastatic colorectal cancer cells contribute to functional heterogeneity of activated fibroblasts by reprogramming their proteome

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Abbreviations: CAFs, cancer-associated fibroblasts; **CRC**, colorectal cancer; **CM**, culture media; **ECM**, extracellular matrix; **EM**, electron microscopy; **Exos**, exosome; **TME**, tumour microenvironment;

Abstract

Cancer-associated fibroblasts (CAFs) are a heterogeneous population of activated fibroblasts that constitute a dominant cellular component of the tumour microenvironment (TME) performing distinct functions. Here, we investigated the role of tumour-derived exosomes in activating quiescent fibroblasts into distinct functional subtypes. Proteomic profiling and functional dissection revealed that early (SW480) and late-stage (SW620) colorectal cancer (CRC) cell-derived exosomes both activated normal quiescent fibroblasts (α -SMA⁻, CAV⁺, FAP⁺, VIM⁺) into CAF-like fibroblasts (α -SMA⁺, CAV⁻ FAP⁺, VIM⁺). Fibroblasts activated by early stage cancer-exosomes (SW480-Exos) were highly pro-proliferative and pro-angiogenic and displayed elevated expression of pro-angiogenic (IL8, RAB10, NDRG1) and pro-proliferative (SA1008, FFPS) proteins. In contrast, fibroblasts activated by late stage cancer-exosomes (SW620-Exos) displayed a striking ability to invade through extracellular matrix through upregulation of pro-invasive regulators of membrane protrusion (PDLIM1, MYO1B) and elevated secretion of matrix-remodelling proteins (MMP11, EMMPRIN, ADAM10). Conserved features of exosome-mediated fibroblast activation include enhanced ECM secretion (type I collagen, Tenascin C/X), oncogenic transformation and metabolic reprogramming (e.g., downregulation of metabolic switch CAV-1, upregulation of glycogen metabolism (GAA), amino acid biosynthesis (SHMT2, IDH2) and membrane transporters of glucose (GLUT-1), lactate (MCT4) and amino acids (SLC1A5/3A5)). This study highlights the role of primary and metastatic CRC tumour-derived exosomes in generating phenotypically and functionally distinct subsets of CAFs that may facilitate tumour progression.

1 Introduction

Cancer-associated fibroblasts (CAFs) are activated fibroblasts present in solid malignancies during all stages of cancer^[1]. As a major component of the TME, CAFs support tumour growth, angiogenesis, immune suppression, metabolic reprogramming, and metastasis^[1], and represent attractive targets for therapeutic intervention. In the last 10 years, it has become increasingly evident that multiple subsets of CAFs exist within the same tumour that can be distinguished by differential expression of conventional markers (α SMA, PDGFR β , FAP, FSP1, CAV1 and CD29)^[2-5] and specific cell-surface proteins (CD10 and GPR77^[6], CD146^[7]), and their distinct transcriptome^[4, 5], and secretome profiles^[4, 5].

More recently, these subsets of CAFs were shown to perform distinct functions. In breast cancer tissues from estrogen receptor positive breast cancer patients, two CAF subtypes defined by CD146 expression were identified^[7]. CD146⁻ CAFs suppress ER expression in ER+ breast cancer cells, decrease tumour cell sensitivity to estrogen, and increase tumour cell resistance to tamoxifen therapy. Conversely, the presence of CD146⁺ CAFs maintain ER expression in ER+ breast cancer cells and sustain estrogen-dependent proliferation and sensitivity to tamoxifen. In another study, in breast cancer tissues, two CAFs subsets could also be identified based on cell surface markers CD10 and GPR77^[6]. A subset of CD10⁺GPR77⁺ CAFs were shown to promote tumour formation and chemoresistance by sustaining cancer stemness^[6]. Targeting these CAFs with a neutralizing anti-GPR77 antibody successfully abrogated tumour growth and restored chemosensitivity^[6]. Yet in another study, based on expression of 6 different markers (α SMA, PDGFR β , FAP, FSP1, CAV1 and CD29), up to four CAF subsets with distinct properties and levels of activation were identified in breast cancer tissues^[5]. In this study, a subset of CAFs was shown to promote immunosuppression by recruiting T cells through CXCL12 secretion, triggering their cell differentiation into suppressor FOXP3⁺ T cells (also called regulatory T cells) that displayed enhanced capacity to suppress T effector proliferation^[5]. Moreover, using the same set of 6 CAF markers, four CAF subtypes were reported in ovarian cancer tissues^[4]. In this study, the same subset of CAFs (based on similar expression levels of marker proteins) were shown to perform immunosuppressive function^[4], also through elevated secretion of CXCL12. More recently, at least two subtypes of CAFs (α -SMA⁺ and α -SMA⁻) were reported in pancreatic ductal adenocarcinoma^[3]. Whereas α -SMA⁺ CAFs displayed contractile phenotype, α -SMA⁻ CAFs displayed cytokine-secreting properties (such as interleukin 6 that promoted STAT signalling in cancer cells). Collectively, these studies highlight the importance of functional heterogeneity

of CAFs in tumours. Hence, defining functions, regulation, origins, and plasticity of these CAF subtypes will have direct implication in prognostication and predicting therapeutic outcomes^[1, 4-6].

The source of heterogeneous populations of CAFs remains poorly understood. Although CAFs heterogeneity could be partly attributed to their different cellular origins^[8], it has been speculated that local quiescent fibroblasts can differentiate into different subsets of functional CAFs in response to tumour-derived signals^[1, 8]. Recently, a direct juxtacrine interaction of fibroblasts with cancer cells or fibroblasts conditioning with secreted factors from cancer cells were shown to support distinct subset of CAFs with distinct expression of protein markers, secretory profile and function^[3].

The recruitment of activated fibroblasts in many cancers is also dependent on tumour-derived signals such as TGF β and PDGF^[1] which has been shown to be delivered by tumour-derived exosomes to resting fibroblasts triggering their differentiation^[9, 10]. It is now well known that tumour-derived exosomes contain oncoproteins^[11], oncomiRs, fusion gene mRNAs^[12], oncogenic lncRNAs^[13], mutated DNA fragments^[14] and a multitude of cell-signalling molecules^[15] which they can transfer to non-cancer cells to elicit diverse functional response^[16], including reprogramming of fibroblasts^[9, 10], endothelial cells^[17], bone marrow-derived cells^[15], alveolar epithelial cells and Kupffer cells^[18]. These transformed cells in turn enhance tumour growth, support construction of pre-metastatic niche and increase metastasis^[15, 18, 19, 20]. Moreover, functional reprogramming of stromal cells by tumour-derived exosomes also appears to be context-dependant where the same quiescent fibroblasts can be activated to carry out different functions depending on the changing TME^[21]. We previously reported that early (SW480, primary adenocarcinoma) and late stage (SW620, lymph-node metastasis) colorectal cancer-derived exosomes carry distinct signal transduction and cancer-related proteins^[22]. We therefore hypothesized that these exosomes might be able to activate quiescent fibroblasts into distinct subsets of activated fibroblasts that possibly carry out cancer stage-specific functions. Using functional assays and mass spectrometry-based proteomics^[23], we show in this study that exosomes released from different pathological stage cancer cells activate normal quiescent fibroblasts into activated fibroblasts that perform specialised functions in a context-dependent manner to facilitate tumour progression. This study highlights the contribution of cancer exosomes towards functional and phenotypic heterogeneity of CAFs.

Significant statement

Defining functions, regulation, origins, and plasticity of CAF subtypes will have direct implication in prognostication and predicting therapeutic outcomes in cancer. The results presented here provide an insight into mechanisms whereby cancer exosomes regulate CAF activation and heterogeneity. By combining functional and protein dissection of normal fibroblasts activated with highly purified exosomes from early (primary) and late (metastatic) stage colorectal cancer cells, we show a switch in fibroblasts regulation from pro-tumorigenic to pro-invasive phenotype. Our study also provides molecular insights into metabolic reprogramming of fibroblasts by cancer exosomes. These findings will enable future studies seeking to characterize the underlying mechanism regulating fibroblast heterogeneity in tumours, and have implications in defining and therapeutically targeting those subsets of CAFs that are pro-tumorigenic, and potentially avoiding CAFs subtypes, which are anti-tumorigenic.

2 Materials and methods

2.1 Cell culture

The human CRC cell lines SW480 (CCL-228) and SW620 (CCL-227) were obtained from American Type Culture Collection. Human neonatal foreskin fibroblast cell line (neoHFF) was obtained as a kind gift from Dr Pritinder Kaur (Monash University, Victoria, Australia). Human CRC cell lines were cultured in RPMI-1640 media (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 1% (v/v) Penicillin Streptomycin (Pen/Strep, Life Technologies) and maintained at 37 °C with 10% CO₂. Fibroblast cell lines were routinely cultured in DMEM with high-glucose (Invitrogen) at 37 °C with 10% CO₂. Human Umbilical Vein Endothelial cells (HUVECs, Lonza) were cultured in Endothelial Basal Medium 2 (EBM, Lonza) supplemented with SingleQuot Kit Suppl. & Growth Factors (Lonza). Cells were passaged using Trypsin-EDTA (Gibco).

2.2 Exosome isolation

Cells were cultured in CELLline AD-1000 Bioreactor classic flasks (Integra Biosciences) as described^[24]. SW620 or SW480 cells (3×10^7) in 15 mL of RPMI medium supplemented with 0.5% (v/v) insulin transferrin selenium (Invitrogen) and 1% Pen/Strep was added to the lower cell-cultivation chamber. The upper nutrient supply chamber contained 500 mL RPMI (10% FBS, 1% Pen/Strep) that was replaced every 4 days. Cells were cultured at 37 °C with 10% CO₂. Cells in the lower cell cultivation chamber was allowed to adhere for 48 h. Medium in the cell cultivation chamber was replaced every day. CM harvested from cell cultivation chamber was centrifuged at 500 x g for 5 min (4 °C) and 2000 x g for 10 min (4 °C). The supernatant was then centrifuged at 10 000 x g for 30 min to pellet sMV. The supernatant was centrifuged at 100 000 x g for 1 h to pellet crude exosomes. This crude exosome pellet was subjected to isopycnic (iodixanol-density) ultracentrifugation^[22, 25] as previously described^[22, 26]. Briefly, a discontinuous gradient of OptiPrepTM (iodixanol solution) was prepared by layering 3 mL volumes of 40, 20, and 10% of iodixanol solution to a 14 × 89 mm polyallomer tube (Beckman Coulter). Dilutions were made in 0.25 M sucrose / 10 mM Tris (pH 7.5) solution. This column was topped with layer of 2.5 mL of 5% iodixanol solution. Crude exosome pellet was resuspended in 500 µL of 0.25 M sucrose / 10 mM and overlaid onto to the column and centrifuged at 100 000 x g for 18 h at 4 °C. Twelve 1 mL fractions were obtained, diluted in 2 mL PBS and centrifuged at 100 000 x g for 1 h. Pellets were washed in 500 µL PBS with final resuspension in 100 µL of PBS. Exos containing fractions were

determined based on western blot analysis of Exos markers ALIX and TSG101. Both sMV and Exos were stored at -80 °C until further use.

2.3 Protein quantification and western blotting

Cells or exosomes 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) with 100 mM Dithiothreitol (DTT). Protein amount was estimated by 1D SDS-PAGE / SYPRO Ruby protein staining-based densitometry, as previously described^[27]. For immunoblotting, cellular proteins (10-20 µg) or Exos (10-20 µg) were resolved on Norvex 4-12% Bis-Tris NuPAGE gels with MES running buffer at 150 V for 1 h. Proteins on the gel were electro transferred onto nitrocellulose membranes using iBlotTM Dry blotting system (Life Technologies) at 12 V for 8 min. The membranes were blocked with 5% (w/v) skim milk powder in TTBS-Tween (50 mM Tris, 150 mM NaCl, 0.05% (w/v) Tween 20) for 30 min (room temperature, RT). The membranes were washed and probed with primary antibodies (1:1000 dilution) for ~24 h at 4 °C in TTBS-Tween. Primary antibodies used were; rabbit polyclonal against Met (Santa Cruz Biotechnology), CD44 (Cell Signalling) GAPDH (Cell Signalling), β -actin (Abcam), and mouse monoclonal against Alix (Cell Signalling), Tsg101 (BD Biosciences), α -SMA (Abcam), and Vimentin (Cell Signalling). The membranes were washed thrice in TTBS-Tween for 45 min at room temperature. This was followed by incubation with corresponding secondary antibodies; IRDye 800 goat anti-mouse IgG or IRDye 700 goat anti-rabbit IgG (1:15000, LI-COR Biosciences), for 1 h at room temperature in TTBS-Tween. The membranes were then washed thrice in TTBS-Tween for 15 min at room temperature. Blots were then imaged using OdysseyTM Infrared Imaging System (v3.0, LI-COR Biosciences).

2.4 Electron microscopy

Vesicle morphology was analysed using cryo-electron microscopy (cryo-EM) (Tecnai G2 F30)^[28], as previously described. Briefly, fresh non-frozen Exos or sMVs (2 µg) preparations were applied to flow-discharged C-flat holey carbon grids (ProSciTech). Excess fluid was drained by blotting. Carbon grids were snap-frozen in liquid ethane. Grids were mounted onto Gatan cryoholder (Gatan Inc) in liquid nitrogen and imaged using Tecnai G2 F30 (FEI, Eindhoven) at 300 kV.

2.5 Nanoparticle tracking analysis

Vesicle particle size was determined using NanoSight NS300, Nanoparticle tracking analysis (NTA) (Malvern) fitted with a NS300 flow-cell top plate with a 405 nm laser. Exos sample (1 $\mu\text{g}/\mu\text{L}$ in PBS (1:10,000 dilution) were injected with 1 mL syringes (BD) (detection threshold = 10, flowrate = 100, temperature = 25 °C). Each analysis consisted of 60 s video captures. Data was analysed using NTA software 3.0 (ATA Scientific).

2.6 Lipophilic labelling of exosomes and uptake assay

Exos (~300 μg) in 300 μL PBS were labelled with fluorescent dye, DiI (Invitrogen) at 1 μM concentration for 15 min at room temperature, as previously described^[29]. Exos were collected at 100 000 $\times g$ (1 h) and subjected to isopycnic (iodixnol-density) ultracentrifugation. Cells were grown on glass cover-slips to 70% confluency in wells of 24-well plate. Cells were incubated with DiI-labelled Exos (5 μg) at 37 °C for 2 h. Cells were then washed with PBS and analysed by fluorescent microscopy. Nuclei were stained with Hoechst stain (10 $\mu\text{g}/\text{mL}$) for 30 min prior to imaging by Zeiss AxioObserver Z1 (Zeiss).

2.7 Immunofluorescence assay

Cells were cultured on glass coverslip to 70% confluency. Immunofluorescence was performed as previously described^[22] with modifications. Briefly, cells were fixed in 4% formaldehyde for 10 min at room temperature, washed in PBS and permeabilized using 0.2% (v/v) Triton X-100 in TTBS for 5 min at room temperature. Cells were then washed in TTBS and blocked with 3% (w/v) bovine serum albumin (BSA, Sigma) in TTBS (0.2% (v/v) Triton X-100) (blocking solution) for 30 min at room temperature. Cells were then incubated with primary antibodies (1:200) (mouse anti- α -SMA (Abcam), mouse anti-IL8 (BD Biosciences) and rabbit anti-CD31(Abcam)) in blocking solution for 1 h at room temperature, washed with TTBS and incubated with secondary antibodies (1:200) in blocking solution for 20 min at room temperature in the dark. Secondary antibodies used were Alexa Fluor 546-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Where indicated, cells were washed and nuclei stained with Hoechst stain (10 $\mu\text{g}/\text{mL}$) for 1 min. Cells were imaged using Zeiss AxioObserver Z1 microscope and images were analysed using Zen 2011 (Blue edition, Zeiss).

2.8 Fibroblast activation assay for α -SMA

Activation of fibroblasts with Exos was performed as previously described^[10] with modifications. Briefly, neoHFF (80% confluent) monolayers in wells of 96-well plate were maintained in 100 μ L of serum-free DMEM (1% Pen/Strep) for 72 h at 37 °C. Cells were treated with SW620-Exos or SW480-Exos (150 μ g/mL) or PBS vehicle alone for further 72 h. Cells were then analysed for α -SMA expression using immunofluorescence assay or western blotting.

2.9 Soft-agar colony formation assay

Fibroblasts (2×10^3) in 100 μ L DMEM (1% Pen/Strep) were stimulated with 20 μ g of exosomes or 20 μ L PBS vehicle for 2 h at 37 °C. Fibroblasts were then mixed with 300 μ L of 0.3% agarose (in DMEM with 10% FBS, 1% Pen/Strep) that was pre-warmed to 40 °C using water bath. The mixture was overlaid onto well of a 24-well plate that was pre-coated with 300 μ L of 0.6% agarose (in DMEM with 10% FBS, 1% Pen/Strep). The mixture was allowed to solidify at 37 °C for 15 min. The well was then gently overlaid with 500 μ L DMEM (5% FBS, 1% Pen/Strep) and maintained at 37 °C for 10 days. Culture medium was replaced every 2 days. Colonies were imaged using Zeiss AxioObserver Z1 microscope under bright-field.

2.10 Proliferation assay

In wells of 96 well plate, neoHFFs monolayers were treated with exosomes or PBS for 72 h as described above. Fibroblast monolayers were washed and overlaid with SW480-GFP GFP cells (400 cells) (Xu, R., et al Proteomics 2018, submitted) in 100 μ L DMEM (1% Pen/Strep). Cells were maintained at 37 °C for 6 days. Culture medium was replaced every 2 days. Cells were imaged using Zeiss AxioObserver Z1 microscope.

2.11 Transwell-Matrigel™ invasion assay

Transwell-Matrigel™ invasion assays were performed using growth factor-reduced Matrigel™ matrix (Corning), as previously described^[30] with modifications. Transwell inserts (8 μ m pore size, Corning) were coated with 100 μ L of 1 mg/mL Matrigel™ and allowed to polymerize for 4 h at 37 °C. Fibroblasts (5×10^4) in DMEM (1% Pen/Strep) were treated with either exosomes (30 μ g/mL) or PBS alone for 2 h at 37 °C. Treated fibroblasts were centrifuged at 500 x g (5 min) and resuspended in 100 μ L DMEM medium. Cells were carefully overlaid onto

MatrigelTM-coated inserts. The inserts were placed into wells of 24-well plate companion plate (Corning) that contained DMEM (5% FCS, 1% Pen/Strep) supplemented with either Exos (30 µg/mL) or PBS alone. Invasion chambers were incubated overnight (~16 h) at 37 °C to facilitate invasion. Inserts were washed, cells fixed (4% (v/v) formaldehyde, 5 min), and nuclei stained with Hoechst (10 µg/mL) for 20 min. Non-invading cells were removed from the upper side of the inserts using cotton swab. Nuclei of fibroblasts that invaded to lower side of the insert were imaged using Zeiss AxioObserver Z1 microscope. Five fields of view were obtained per insert. Images were quantified using Image J software v1.49e.

2.12 Angiogenesis antibody array

Exos-activated neoHFFs or control neoHFFs (180 µg) were subjected to Proteome ProfilerTM Human Angiogenesis Antibody Array (R&D Systems) analysis as previously described^[31]. Briefly, neoHFFs cells cultured in wells of 24-well plate were treated with Exos (60 µg in 300 µL DMEM) or PBS for 72 h. To prevent cytokine secretion, cells were incubated with Golgi-StopTM and Golgi-PlugTM (RnD systems) for 18 h before lysis at 72 h time-point. Cells were washed and lysed in 1% TX-100 in PBS with cOmpleteTM protease Inhibitor cocktail (Lifescience) for 30 min on ice. The cell lysate was centrifuged at 14,000 x g for 5 min. The supernatant was subjected to total protein quantitation. From 3 wells, the yield of cell lysate was ~150 µg. For each membrane, ~130 µg of the lysate was used.

2.13 Mass Spectrometry

Proteomic experiments were performed in biological duplicate (with technical duplicate) using GeLC-MS/MS as previously described^[32]. Briefly, protein samples (10 µg) were lysed in SDS sample buffer and proteins denatured at 95 °C (5 min). Proteins were separated by SDS-PAGE (150 V, 15 min) and visualized by ImperialTM Protein Stain (Thermo Fisher Scientific). Each lane was excised into two gel pieces, destained (50 mM ammonium bicarbonate/acetonitrile), reduced (10 mM dithiothreitol (Calbiochem) for 30 min at 37 °C), alkylated (50 mM iodoacetamide (Fluka) for 30 min at 37 °C) and trypsinized (0.2 µg trypsin (Promega Sequencing Grade) for 16 h at 37 °C). Tryptic peptides were extracted and subjected to nLC-MS/MS. A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled on-line to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Extracted peptides were loaded onto pre-column (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher

Scientific) and separated (PepMapRSLC C18, 50 cm length, 75 µm inner diameter, 2 µm beads with 100 Å pore-size) (Thermo Fisher Scientific) with a 120 min linear gradient from 0 to 100% (v/v) phase B (0.1% (v/v) FA in 80% (v/v) acetonitrile) at a flow rate of 250 nL/min. The MS was operated in a data dependant mode where top 20 most abundant precursor ions in the survey scan (300-2500 Th; scans were acquired at a resolution of 120,000 at m/z 400) were selected for MS/MS fragmentation. Unassigned precursor ions charge states and slightly charged species were rejected and peptide match disabled. The isolation window was set to 3 Th and fragmented with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 ms and 60 ms, respectively. Selected sequenced ions were dynamically excluded for 90 s.

2.14 Database searching and protein identification

Raw data was processed using Proteome Discoverer (v1.4.0.288, Thermo Fischer Scientific) and searched with Mascot (Matrix Science, London, UK; v 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, v 1.4.0.288), and X! Tandem (v2010.12.01.1) against the UniProt Human database comprising 125,803 entries. Data was searched with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 7, with FDR 1% at the peptide and protein levels. Peptide spectral matches were validated using Percolator based on q-values at a 1% false discovery rate (FDR)^[33]. Scaffold (Proteome Software Inc., Portland, OR, v 4.3.4) was employed to validate MS/MS-based peptide and protein identifications from database searching^[34]. Initial peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm^[34]. Protein identifications were accepted, if they reached greater than 99% probability and contained at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm^[35].

2.15 Semi-quantitative label-free spectral counting

Differential expression of proteins between datasets was examined using label-free quantitation (LFQ)^[36]. The relative abundance of a protein within a sample was determined using normalised spectral count (Nsc) as previously described^[22, 28, 37].

Significant peptide MS/MS spectra were summated for each individual protein, and were normalized by the total number of significant MS/MS spectra identified in the sample using equation; $N_{sc} = (n + f) / (t - n + f)$, where n = number of significant peptide spectral counts for

each protein in the sample, t = total number of significant MS/MS spectral counts identified in the sample and f = correction factor set to 1.25].

To compare relative protein abundance between samples X and Y, the ratio of normalized spectral counts (Rsc) was estimated using equation; $R_{SC} = [(n_Y + f)(t_X - n_X + f)] / [(n_X + f)(t_Y - n_Y + f)]$, where n = significant protein spectral count, t = total number of significant MS/MS spectra in the sample, f = correction factor set to 1.25.

2.16 Bioinformatics

For GO annotation protein accession IDs were submitted to DAVID Bioinformatics Resources (<https://david.ncifcrf.gov/>)^[38]. Heatmaps were generated using R-package software.

3 Results and discussion

In this study, we questioned whether tumour-derived exosomes can reprogram quiescent fibroblasts into distinct subsets of activated fibroblasts that carry out cancer stage-specific functions. We show that exosomes can support subsets of activated fibroblasts that execute specialized functions and display distinct cellular and secretory protein profiles.

3.1 Isolation of exosomes from primary and metastatic cells lines

First, we re-validated SW480 and SW620 cells as a model of CRC progression representing early and late stage, respectively (**Figure S1**). In accordance with a previous report^[39], SW480 cells displayed epithelial morphology with tight cell-cell junction, while SW620 cells were rounded with a fibroblast-like appearance (**Figure S1A**).^[22, 39] Moreover, SW620 cells compared to SW480 cells displayed elevated expression of invasion and metastasis-related oncoprotein Met^[40] (**Figure S1B**). In contrast, SW480 cells expressed higher level of cell surface glycoprotein CD44 implicated in cell adhesion and migration^[41]. Consistent with previous reports^[22, 39], SW480 cells displayed higher migratory and matrix adhesive capacity, whereas SW620 displayed relatively higher proliferative, anchorage-independent growth and invasive capacity (**Figure S1C-G**). Thus, SW480 and SW620 cells display features consistent with early (primary) and late stage (metastatic) of cancer.

To isolate exosomes, SW480 and SW620 cells were cultured in CELLLine™ Classic bioreactor flasks and the conditioned media (CM) was isolated as previously described^[24]. The CM was subjected to an exosome isolation and purification strategy (using differential centrifugation coupled to isopycnic (iodixanol-density) ultracentrifugation) as previously described^[22] (**Figure 1A**). Purified exosomes expressed protein markers ALIX and TSG101 (**Figure 1B**), displayed spherical morphology (Figure 1C) and were 30-200 nm in size (**Figure S2**), (David W. Greening et al. 2015; Rai et al. 2019). SW480 and SW620 cell-derived exosomes will be referred to as SW480-Exos and SW620-Exos, respectively.

3.2 Primary and metastatic cell-derived exosomes trigger activation and cellular transformation of quiescent fibroblasts

We next questioned whether early and late stage cancer exosomes can activate quiescent fibroblasts. Although there are several markers of activated fibroblasts and/or CAFs, α -SMA that assembles into contractile filamentous stress fibres running longitudinally along the cell body^[1, 42, 43], is routinely used a marker of activated fibroblasts and/or CAFs^[1, 8]. We next investigated whether SW480/SW620-Exos are able to trigger activation of human fibroblasts (neonatal human foreskin fibroblasts, neoHFFs) by monitoring α -SMA expression. To demonstrate uptake by fibroblasts, SW480-Exos and SW620-Exos were labelled with lipophilic tracer DiI and incubated with neoHFFs. Isopycnic (iodixanol-density) ultracentrifugation was employed to separate unbound dye from labelled exosomes. Fluorescent microscopy revealed that both SW480-Exos and SW620-Exos were readily taken up by neoHFFs within 2 h (**Figure 2A**). Next, we investigated whether cancer exosomes are capable of activating quiescent fibroblasts. To induce quiescence in neoHFFs, we maintained neoHFFs monolayers in serum-free medium for 72 h as previously described^[10]. Next, we stimulated neoHFFs monolayers with SW480-Exos or SW620-Exos or PBS vehicle for 72 h. Fluorescent microscopy revealed that both SW480-Exos and SW620-Exos induced expression of α -SMA filamentous structures in neoHFFs (**Figure 2B**). In contrast, vehicle-treated control neoHFFs supported only sparse α -SMA filaments. This suggests that SW480 and SW620-Exos are capable to triggering activation of quiescent fibroblasts.

An important trait of CAFs that normal quiescent fibroblasts acquire upon activation is anchorage-independent growth capacity (an indicator of transformed phenotype) that is crucial for survival in the absence of external signals from extracellular matrix^[44, 45]. To test whether SW480-Exos or SW620-Exos activated fibroblasts acquire anchorage-independent growth capacity, we used soft agar colony formation assay^[46]. Fibroblasts stimulated with SW480-Exos or SW620-Exos or control fibroblasts treated with PBS alone were suspended in 0.6% agarose and allowed to form colonies. Microscopic examination after 10 days revealed that both SW480-Exos and SW620-Exos promoted significantly greater number of colonies formed by neoHFFs (**Figure 2C**), suggesting that fibroblasts activated by both primary and metastatic cancer-cell derived exosomes display a transformed phenotype.

Thus, our data suggests that cancer exosomes are able to activate (**Figure 2B**) and transform (**Figure 2C**) quiescent fibroblasts, consistent with previous reports^[9, 10]. Notably, our data show that exosomes derived from primary colon adenocarcinoma cell line SW480 -can mediate fibroblast activation. Indeed, CAFs are associated with not only advanced cancers but are also found in early stages of almost all solid cancers^[42, 43, 47], including CRC^[48]. In fact, CAFs are

also found in abundance even in developing colorectal adenomas^[49] and contribute towards colon tumourigenesis⁶⁴.

3.3 Fibroblasts activated by primary and metastatic cancer cell-derived exosomes are functionally distinct

We next questioned whether the fibroblasts activated by primary and metastatic cancer cell-derived exosomes are functionally distinct. Because different subsets of CAFs are speculated to perform different functions^[1, 8], we next investigated fibroblasts activated by primary and metastatic cancer cell-derived exosomes differ for their pro-proliferative^[45, 50], pro-angiogenic^[45, 51] and pro-invasive^[52, 53] capacities.

Fibroblasts activated by primary cancer cell-derived exosomes display pro-proliferative and pro-angiogenic phenotype

To investigate the ability of activated fibroblasts to support cancer cell proliferation, neoHFF monolayer activated with SW480-Exos, SW620-Exos or control neoHFFs monolayer were overlaid with SW480 GAP GFP cells (**Figure 3A**). The growth of SW480 GAP GFP cells were then monitored using fluorescence microscopy. By day 6, neoHFFs treated with SW480-Exos, as compared to control fibroblasts or neoHFFs treated with SW620-Exos, supported significantly ($p < 0.0005$) larger SW480 GAP GFP colonies (**Figure 3A, lower panel**). This suggests that quiescent fibroblasts activated by early stage cancer exosomes, in contrast to metastatic cancer exosomes, display pro-proliferative phenotype.

To investigate pro-angiogenic capacity of Exos-activated fibroblasts, human umbilical vascular endothelial cells (HUVECs) were overlaid onto a neoHFFs monolayer treated with SW480-Exos or SW620-Exos or control neoHFFs monolayer, and monitored for their ability to assemble into microvascular networks (**Figure 3B**). Compared to control neoHFFs or neoHFFs treated with SW620-Exos that supported sparse endothelial tubules (visualised based on staining with endothelial cell marker CD31), SW480-Exos activated fibroblasts supported well defined microvascular networks (**Figure 3B**). These vascular networks comprised significantly longer tubules (vessel length) that established multiple cross-bridges with neighbouring vessels (branch points) ($p < 0.005$) (**Figure 3B, lower panel**). This demonstrates that fibroblasts activated by early stage cancer exosomes, compared to late stage cancer exosomes, display greater pro-angiogenic capacity.

Fibroblasts activated by metastatic cancer cell-derived exosomes display invasive phenotype

Another important function of CAFs is to degrade extracellular matrices to support invasion of cancer cells into surrounding tissues that facilitates metastasis^[52, 53]. To test whether fibroblasts activated by primary and metastatic cancer cell-derived exosomes differed in their invasive capacity, neoHFFs stimulated with SW480-Exos or SW620-Exos were overlaid onto Transwell™ inserts coated with Matrigel™ matrix and assayed for their invasive potential. Compared to neoHFFs activated with SW480-Exos, neoHFFs activated with SW620-Exos showed significantly greater level invasion through the Matrigel™ matrix (**Figure 3C**). This shows that fibroblasts activated by metastatic cancer exosomes display pro-invasive phenotype.

Collectively, our data show that fibroblasts activated by exosomes from different stages of cancer specialise to perform different functions. While primary cancer cell-derived exosomes activate fibroblasts to support tumour expansion (by supporting cell proliferation and angiogenesis), metastatic cancer cell derived exosome activated fibroblasts display invasive capacity that facilitates invasion into surrounding tissues and metastasis. In line with these observations, SW620-Exos (compared with SW480-Exos) were significantly enriched in proteins important in invasion and metastasis, including MET, TNC, and S100A8/A9^[22]. This highlights the role of cancer exosomes in activating quiescent fibroblasts into distinct functional subtypes that potentially provide context-dependent fitness, redirecting TME focus from supporting tumour growth to metastatic dissemination.

3.4 Fibroblasts activated by primary and metastatic cancer cell-derived exosomes have distinct protein profiles

As a first step towards understanding the molecular events associated with fibroblast activation by primary and metastatic cancer cell-derived exosomes, we examined SW480- and SW620-Exos treated fibroblasts protein profiles and their soluble secretome (SS). PBS-vehicle treated neoHFFs were used as control fibroblasts. GeLC-MS/MS identified a total of 605, 622 and 631 proteins in whole cell lysate (WCL) of control neoHFFs, neoHFFs treated with SW480-Exos or SW620-Exos, respectively (**Figure 4A, Table S1, Table 1**). A total of 237, 265 and 253 proteins were identified in SS of control neoHFFs, neoHFFs treated with SW480-Exos or neoHFFs treated with SW620-Exos, respectively (**Figure 4B, Table S1, Table 2**). Inspection of protein expression profile (represented as a heatmap) revealed a striking reprogramming of

cellular (**Figure 4C**) as well as SS (**Figure 4D**) of fibroblasts following treatment by primary or metastatic cancer cell-derived exosomes. Venn diagram in **Figure 4E** indicates the number of proteins that are dysregulated (stimulated or suppressed) following treatment with SW480- or SW620-Exos.

Glucose and amino acid metabolism related proteins altered in neoHFFs activated with SW480-Exos or SW620-Exos

DAVID-based GeneOntology (Biological Processes) analyses of WCL proteins or SS proteins (in italics) whose expression was upregulated or downregulated in neoHFFs following treatment with either SW480- or SW620-Exos (**Figure 4E, upper panel**) revealed their involvement in “actin cytoskeleton organization”, “muscle contraction” and “extracellular matrix organization” (**Figure 4F**) consistent with cellular and extracellular changes that are hallmark of activated fibroblasts^[1]. Importantly, a major finding was their involvement in two important “metabolic processes”, namely “carbohydrate metabolic process” and “amino acid metabolic process” (**Figure 4F**). These processes are listed in **Tables S2-3**. As an orthogonal approach, DAVID-based KEGG pathway analysis also revealed pathways such as “metabolic pathway” and “carbon metabolism” (**Figure 4G**).

These proteins include glucosidase alpha acid (GAA), a lysosomal enzyme essential for glycogen degradation^[54]. Glycogen metabolism results in free glucose that sustains cancer cell growth, survival and prevents their premature senescence^[55]. Moreover, glucose metabolism in cancer tissues is also altered when it switches from oxidative phosphorylation to aerobic glycolysis (referred to as the ‘Warburg effect’)^[56]. Importantly, this metabolic switch is also observed in CAFs that undergo aerobic glycolysis compared to normal fibroblasts (that undergo oxidative phosphorylation)^[57]. This phenomenon of aerobic glycolysis in CAFs is induced in response to signals originating from tumour cells and is called the “Reverse Warburg Effect”^[57]. Interestingly, neoHFFs activated with SW480- or SW620-Exos displayed significant down-regulation of caveolin-1 (CAV1), a TGF- β type I receptor kinase inhibitor, loss of which results in Reverse Warburg Effect^[57]. Moreover, loss of CAV1 triggers fibroblast activation, support tumour progression, metastasis and predict poor clinical outcome^[57]. We also observed that neoHFFs activated with SW480- or SW620-Exos displayed upregulation of glucose transporter GLUT1 and monocarboxylate transporter MCT4. Because glycolysis is less efficient compared to oxidative phosphorylation for energy production, metabolically-reprogrammed CAFs often display elevated level of glucose transporters^[58]. This is particularly

important in increasing glucose uptake by CAFs in the TME where the glucose supply is limited due to competition for uptake between cancer and other cells^[58]. On the other hand, the energy-rich metabolites such as lactate and ketone bodies produced during glycolysis are released by CAFs in the TME, through monocarboxylate transporters such as MCT4, which can in turn fuel growth of tumour cells^[59].

Proteins implicated in amino acid metabolism was also identified that include enzymes that mediate amino acid biosynthesis (Serine hydroxymethyltransferase (SHMT2), Isocitrate dehydrogenase IDH2) and amino acid transporters (SLC1A5 also known as ASCT2, SLC3A2). Compared to normal fibroblasts, CAFs produce and secrete large amounts of amino acids (such as alanine^[60] and glutamine^[61]) that cancer cells use to fuel macromolecular biosynthesis.

This suggests that tumour-derived exosomes metabolically alter the activated fibroblasts and potentially contribute towards metabolite sharing between tumour and CAFs that is critical for tumour progression^[62]. The role of cancer exosomes in metabolic reprogramming of stromal fibroblasts is only beginning to emerge^[21]. Breast cancer exosomes have been shown reprogram glucose metabolism in pre-metastatic cells to promote metastasis^[19]. Moreover, glucose flux is important during anchorage independent growth. Schafer *et al.*, reported that detachment from ECM results in loss of glucose transport, ATP deficiency and increased of ROS production that induce anoikis^[63]. Cells undergoing anoikis could be rescued by restoring glucose-flux^[63]. Thus, elevated GLUT transporter by SW480- or SW620-Exos in neoHFFs could represent a mechanism of acquiring anchorage independence. Thus, our data suggests that both primary and metastatic cancer cell-derived exosomes promote metabolic switch in activated fibroblasts.

Distribution of CAF markers in neoHFFs treated with primary and metastatic exosomes

CAF marker α -SMA was the most upregulated protein identified in neoHFFs treated with SW480 or SW620-Exos. In contrast, expression levels of fibroblasts markers^[1, 43] such as vimentin (VIM) and fibroblast activation protein alpha (FAP) remained unchanged (**Figure S3A**). Increased extracellular matrix deposition is also a hallmark of activated fibroblasts /CAF^s^[1]. Compared to neoHFFs, neoHFFs treated with SW480-or SW620-Exos displayed enhanced secretion of collagen, tenascin C/X and SPARC (**Figure S3A-B**). On the other hand, CAV1 expression was downregulated in neoHFFs treated with SW480-Exos or SW620-Exos (**Figure S3A**). Loss of CAV1 in CAFs is an indicator of metabolic reprogramming in CAFs^[57].

Western blotting-based validation of mass spectrometry data set for α -SMA, VIM and GAPDH is shown in **Figure 4H**.

3.5 Primary cancer exosomes activated fibroblasts display selective upregulation of angiogenesis- and cell proliferation-related proteins

We next investigated proteins that were uniquely upregulated or downregulated in neoHFF WCL and SS treated with SW480-Exos or SW620-Exos (**Figure 4E, middle panel, Table S1**). GO analysis of the dysregulated proteins revealed their involvement in “mRNA stability”, “cell cycle”, “amino acid metabolism”, “signal transduction” and “aorta development” (**Figure 4F, Table S4-5**).

In the WCL, several proteins specifically upregulated were proteins that alter the secretory profile, including, Rab10a and NDRG1. Rab10, a small GTPase belonging to the RAS superfamily, controls intercellular vesicle trafficking to regulate secretion of cytokines^[64]. Rab10a has been shown to regulate ECM composition^[64] important in tumour angiogenesis^[65]. N-myc downstream-regulated gene 1 (NDRG1) is a nickel and calcium-inducible gene^[66] whose elevated expression is linked to poor patient prognosis and tumour-associated angiogenesis in several malignancies including cervical adenocarcinoma^[67], gastric cancer^[68] and lung cancer^[69] and regulates secretion of angiogenic factors^[69]. This prompted us to question whether expression of pro-angiogenic factors in CAFs^[43] could be modulated by SW480-Exos. We employed a angiogenic protein array to monitor expression levels of various angiogenesis-related factors in SW480-Exo activated neoHFFs (**Figure S4**). neoHFFs activated with SW480-Exos or control neoHFFs were solubilized using TX-100 and overlaid onto nitrocellulose membranes that were spotted in duplicate with antibodies against 55 angiogenesis-related proteins. To prevent cytokine secretion, neoHFFs were treated with Golgi-Stop and Golgi-Plug 18 h before solubilization^[31]. Compared to vehicle-treated fibroblasts, exposure to SW480-Exos resulted in >17-fold increased expression of interleukin-8 (IL-8) (**Figure S4A**). IL-8 is a chemokine that acts on endothelial cells through CXCR receptors to promote angiogenesis^[70]. Elevated expression of IL-8 was validated in activated neoHFFs using fluorescent microscopy (**Figure S4B**). Indeed, both Rab10a and NDRG1 have been shown to promote secretion of IL-8^[69, 71]. Cancer cells release exosomes that stimulate stromal cells to secrete IL-8 that, in turn, modulates angiogenesis and leukemia cell survival^[72]. Importantly, IL-8 also plays a central role in the immunobiology of human malignancies^[73].

Whether, exosomes regulate immune repose in CRC through stromal secretion of IL-8 warrants investigation.

^[74]Inspection of SS from neoHFFs treated with SW480-Exos or SW620-Exos revealed unique upregulation of protein S100-A6 that is implicated in CAF^[75] and cancer cell proliferation and Farnesyl-diphosphate synthase (FDPS) that is implicated in proliferation^[76]. S100-A6 belongs to the A group of the S100 protein family of calcium- binding proteins^[77] and is upregulated in tumour tissues including colon cancer tissues^[78]. Exogenous supplementation of S100-A6 enhanced colon cancer cell proliferation in vitro and tumour growth in vivo through MAPK activation^[78]. FDPS is a key intermediate enzyme in the mevalonate pathway for lipid moieties synthesis that are critical for cell proliferation. Elevated FDPS activity are often detected in CRC tissues compared to normal mucosa and FDPS blockade significantly reduced cell proliferation^[79].

Thus, primary cancer cell-derived exosomes, compared to metastatic cancer cell-derived exosomes, reprogram fibroblasts protein profile that support angiogenesis and tumor cell proliferation.

3.6 Fibroblasts activated by metastatic cancer-cell derived exosomes display upregulation of invasion-related proteins

We next investigated proteins specifically upregulated or downregulated in the WCL and SS of neoHFFs treated with SW620-Exos or SW480-Exos. DAVID-based GO analysis revealed their involvement in “rRNA processing”, “apoptotic signalling pathway”, “translation” and “extracellular matrix disassembly” (**Figure 4F, Table S6-7**). Consistent with the invasive capacity of SW620-Exos treated neoHFFs, proteins uniquely upregulated in WCL and SS were those that can remodel the ECM that is critical during invasion. These include upregulation of cytoskeletal reorganizing proteins (PDML1 and MYO1B) in WCL and elevated secretion of extracellular matrix disassembly proteins (MMP11, ADAM10, EMMPRIN).

PDLIM1 is a member of PDZ and LIM protein family that binds to alpha-actinin-1 to form stress fibres and focal adhesions^[80]. Although PDLIM1 does not affect the rate of proliferation, anchorage independent growth or tumour growth, it interacts with actin cytoskeleton to promote invasive and metastatic behaviour of breast cancer cells^[81]. Moreover, PDLIM1

interacts with neurotrophin receptor p75^{NTR} in highly invasive patient-derived glioma stem cells/tumour-initiating cells to support cancer cell invasion^[82].

Myosins are molecular motor proteins that bind to actin to perform functions such as motility, contractility and invasion and metastasis in cancer^[83]. Myosin 1b is a member of class I myosin that promotes invasion in cervical cancer^[84], and lymph node metastasis of head neck squamous cell carcinoma^[85]. Myosin 1b promotes invasion through large protrusion formation of cell membranes^[85]. ROCK-dependant myosin organization results in protease-independent matrix remodelling during invasion^[86].

MMP11 (Matrix metalloproteinase 11, also known as stromelysin-3), a metalloproteinase enzyme which degrade the extracellular matrix, is specifically expressed in stromal cells surrounding invasive breast carcinomas^[87]. MMP11 is overexpressed in various invasive carcinomas including colon cancer and serum level of MMP11 associated with colon cancer aggressiveness and metastasis^[88].

Extracellular matrix metalloproteinase inducer (EMMPRIN) is a glycosylated transmembrane protein that belongs to the immunoglobulin superfamily^[89]. EMMPRIN stimulates production of MMPs to support invasion^[90]. Although a transmembrane protein, EMMPRIN could be released from tumor cells through EVs^[91] or through proteolytic shedding^[92].

A disintegrin and metalloprotease 10 (ADAM10) is a transmembrane metalloproteinase that cleaves membrane proteins at the cell surface to release their ectodomain into the extracellular space^[93]. ADAM10 is overexpressed in a variety of cancers and ADAM-mediated ectodomain shedding of diverse growth factors, cytokines, receptors and adhesion molecules and promotes tumour progression and metastasis in a variety of cancers^[93] including CRC^[94]. ADAM10 itself is also subject to ectodomain shedding (mediated by ADAM9 and ADAM15) which results in release of proteolytically active soluble ADAM10 that can potentially function in the TME^[95]. Importantly, ADAM10 can proteolytically cleave vascular endothelial cadherin (a major adhesion molecule of endothelial adherent junction) to induce vascular permeability^[96] that facilitates cancer cell extravasation into circulation and metastatic spread^[97].

Thus, it appears that SW620-Exos activated fibroblasts specialise in physically remodelling the extracellular matrix through cytoskeletal re-organization, membrane protrusion formation and secretion of matrix-remodelling proteins. This finding is consistent with the role of CAFs in fibroblast-led invasion of cancer cells and metastasis^[52].

4. Concluding remarks

It is long known that CAFs dynamically interact with cancer cells during multistage carcinogenesis. However, the importance of phenotypic and functional heterogeneity of CAFs, which changes spatially and temporally as cancer evolves, is only starting to unravel. Our data show that exosomes from different stages of cancer can activate quiescent fibroblasts to carry out specialized functions to provide context-dependant fitness to tumour cells (**Figure 5**). Co-evolution of tumour and its stroma is critical for tumour growth and metastasis^[98]. It is conceivable that within the primary tumour (dominated by SW480 clonal population), exosomes activate normal fibroblast towards CAFs that support tumour cell proliferation and angiogenesis required for tumour expansion. Accumulating mutations inadvertently gives rise to a new sub-clonal population of cancer cells^[99] that can potentially bring about a switch in how local fibroblasts are reprogrammed. Newly acquired specialised function of activated fibroblasts potentially facilitates regional invasion, induce vascular permeability, favour metastatic dissemination, which then culminates in metastasis. A conserved feature of CAFs in early and late stage appears to be their metabolic reprogramming. Although a large body of literature supports targeting CAFs as anti-cancer therapeutics, recent data suggests that their usefulness in the clinic as anti-cancer therapeutics requires a better understanding of CAF phenotypic and functional heterogeneity.

Collectively, our proteomic data indicate that primary and metastatic cancer cell-derived exosomes differentially regulate cellular and secreted protein profiles of fibroblasts. Whereas SW480-Exos-activated fibroblasts display elevated expression of proteins that support proliferation and angiogenesis, SW620-Exos activated fibroblasts display elevated expression of proteins that support invasive outgrowth and metastasis. In summary, this study provides an insight into the role of exosome's in regulating CAF activation and heterogeneity that facilitates a switch from pro-tumorigenic to pro-invasive phenotype critical to metastasis.

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Figure legends

Figure 1. Isolation and characterization of SW480 and SW620 cell-derived exosomes.

Experimental workflow used for isolation of exosomes from conditioned media of SW480 or SW620 cells using differential centrifugation coupled to OptiPrep™ density gradient centrifugation. B) Western blot analysis of purified exosomes using anti-ALIX and anti-TSG101 antibodies. C) Cryo-electron microscopic analysis of SW480- and SW620-Exos. Scale bar, 200 nm.

Figure 2. SW480- and SW620-cell derived exosomes activate and transform quiescent fibroblasts.

A) Live-fluorescent microscopic analysis of normal human foreskin fibroblasts (neoHFFs) incubated with DiI-stained SW480-Exos or SW620-Exos (5 µg, 2 h) (red). Nuclei were stained with Hoechst (blue). Scale bar, 10 µm. B) Fluorescent microscopic analysis of α -SMA (green) expression in neoHFFs exposed to SW480-or SW620-Exos (150 µg/mL) or vehicle (PBS) for 72 h. Nuclei were stained with Hoechst (blue). Scale bar, 100 µm. C) Bright-field microscopic analysis of soft-agar colony forming capability (over 10 days) of neoHFFs stimulated with SW480- or SW620-Exos (20 µg). Scale bar, 200 nm. Number of colonies were manually counted and plotted as a histogram (5 microscopic fields examined per well, n = 3; average \pm standard error of mean (s.e.m.)).

Figure 3. Pro-proliferative, pro-angiogenic and invasive capacity of fibroblasts treated with SW480- and SW620 cell-derived exosomes.

A) Pro-proliferative capacity. Fluorescent microscopic analysis of SW480 GAP GFP cells (green) that were overlaid onto neoHFFs monolayer stimulated with SW480- or SW620-Exos (150 µg/mL) or PBS alone. Scale bar, 100 µm. Inset is the image in higher magnification. Fluorescent images were quantified for colony size using Image J based on three biological replicates. B) Pro-angiogenic capacity. Immunofluorescence microscopic analysis of HUVECs (red) overlaid onto neoHFFs monolayer stimulated with SW480-Exos or SW620-Exos (150 µg/mL) or PBS alone. Scale bar, 100 µm. Vessel length and branch points were quantified (3 microscopic fields examined per well, n = 3; average \pm s.e.m.). C) Invasive capacity. Fluorescent microscopy analysis of neoHFFs treated with SW480- or SW620-Exos (30 µg/mL) or PBS alone were assessed for invasion across Matrigel™ using Transwell-Matrigel™ invasion assay. Scale bar, 50 µm. Nuclei of invading cells (stained with Hoechst stain) quantified and represented as average \pm s.e.m.

Figure 4. Protein profiling of fibroblasts activated with SW480- or SW620-derived exosomes. Venn diagram of proteins identified in A) whole cell lysate (WCL), B) soluble secretome (SS) of neoHFFs treated with SW480- or SW620-Exos. Heatmap illustrations of proteins identified in C) WCL, D) SS derived from neoHFFs, neoHFFs treated with SW480-Exos, and neoHFFs treated with SW620-Exos. Scale shown is the representation of label-free quantitation intensity. E) *Top panel.* Venn diagram of proteins whose expression was commonly dysregulated in neoHFFs activated with SW480- or SW620-Exos. Venn diagram of proteins whose expression was specifically dysregulated in neoHFFs following treatment with SW480-Exos (*middle panel*) or SW620-Exos (*bottom panel*). F) Gene Ontology (GO) terms for “Biological Processes” identified in proteins dysregulated in E. Italicised GO terms are enriched in SS proteins. G) Pie-chart showing KEGG pathway terms identified in proteins that were commonly dysregulated in neoHFFs activated with SW480-Exos or SW620-Exos. H) Relative abundance of α -SMA, VIM and GAPDH based on spectral counts (left panel). Western blot analysis (20 μ g) of WCL using anti- α -SMA, -VIM and -GAPDH antibodies (right panel).

Figure 5. Schematic representation of fibroblast reprogramming by primary and metastatic cancer exosomes.

Supplementary Figure Legends

Figure S1. Characterization of SW480 and SW620 cell lines. A) Bright-field microscopic analysis of SW480 and SW620 cells. B) Western blot analysis of SW480 and SW620 cells for expression of CD44, MET and actin (20 μ g WCL per lane). C) Adhesion of cells to different matrix proteins substrate (BSA, MatrigelTM or collagen) was compared after 2 h of seeding. Nuclei of attached cells were stained with Hoechst and imaged using fluorescent microscopy. Data presented as the average \pm s.e.m (n=3). $P<0.05$, $P<0.0005$, ANOVA. D) Migration of SW480 and SW620 cells were compared using Transwell-migration assay. After 16 h, nuclei of cells from lower side of the membrane were stained with Hoechst and imaged using fluorescent microscopy. Five fields of view were imaged for each insert. Data presented as the average \pm s.e.m (n=3). $P<0.05$, $P<0.0005$, ANOVA. E) A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cell proliferation assay of SW480 and SW620 cells over 3 and 6 days. (n=9). $P<0.05$, ANOVA. F) Bright field microscopic analysis of SW480 and SW620 colonies formed in soft agar colony formation assay over 5 days. Data presented as the average \pm s.e.m (n=3). $P<0.005$, ANOVA. G) Bright-field microscopic analysis of SW480 and SW620 colonies formed in MatrigelTM matrix over 5 days. SW620 showed a greater level of invasion into matrix compared to SW480. Data presented as the average \pm s.e.m (n=3). $P<0.005$, ANOVA.

Figure S2. Nanoparticle tracking analysis of exosomes. NTA of SW480-Exos (upper panel) and SW620-Exos (lower panel) using NanoSight, NS300. Size distribution (nm) representative of 3 analysis where each analysis consisted of 60 sec video captures with a 405 nm laser.

Figure S3. Distribution of cancer associated fibroblasts markers in neoHFFs activated with SW480- or SW620-Exos. A) Distribution of fibroblasts / cancer associated fibroblasts markers in neoHFFs activated with SW480- or SW620-Exos. Right panel is the histogram of indicated proteins (spectral counts). B) Distribution of ECM and MMPs in neoHFFs activated with SW480- or SW620-Exos. Right panel is the histogram of indicated proteins (spectral counts).

Figure S4. SW480-Exos trigger elevated expression of IL-8 in fibroblasts. A) Control neoHFFs or neoHFFs activated with SW480-Exos were profiled for expression of 55 target proteins using antibody-based angiogenesis array. Relative fold change represented as a histogram (right panel). B) Control neoHFFs or neoHFFs activated with SW480-Exos were subjected to immunofluorescence assay for IL-8 (green) and analysed by fluorescence microscopy. Nuclei were stained with Hoechst (blue). (Scale bar, 10 μ m).