Rab35-dependent extracellular nanovesicles are required for induction of tumour supporting stroma

V Yeung[a†], J.P. Webber[a†], E.A Dunlop[a], H Morgan[b], J Hutton[b], M Gurney[c], E Jones[c], J Falcon-Perez[d], Z Tabi[a], R Errington[a], A Clayton[a].

Communication between diseased cells and the microenvironment is a complex yet crucial element in progression of varied pathological processes. Recent studies in cancer highlight an important role for small extracellular nanovesicles secreted by cancer cells as modulators of cancer-associated stroma, leading to enhanced angiogenesis and metastatic priming. The intrinsic factors regulating extracellular nanovesicle biogenesis and secretion are therefore relevant in studies of nano-communication in the cancer milieu. We generated prostate cancer cells bearing stable knockdown of several candidate vesicle regulating factors and examined the impact on cell health, vesicle secretion and on communication with fibroblastic stromal cells. We highlight that RAB11B and RAB35 regulate phenotypically distinct nanovesicle populations, each accounting for only around 20% of the total. Depleting RAB35, but not RAB11B leaves a remaining population of vesicles whose phenotype is insufficient for driving fibroblast to myofibroblast differentiation, leading to attenuated motile behaviours in 3D in vitro models. Co-implantation of tumour cells with stromal fibroblasts in xenografts similarly showed that RAB11B knockdown had little effect on growth rates in vivo. In contrast, significant attenuation in growth, and attenuation of myofibroblasts at the tumour site was evident when using RAB35-knockdown cells. The study concludes that a RAB35 regulated nanovesicle sub-population is particularly important for communication between cancer and stromal cells, and is required for generating a tumour-supportive microenvironment.

1 Introduction

In prostate and other carcinomas, the formation of neoplastic lesions is accompanied by changes in the microenvironment, which are reciprocally supportive [1]. The release of paracrine factors from tumour cells activate the peri-tumoural stroma, resulting in a modified, myofibroblastic stromal phenotype. These are associated with enhanced growth, vascularisation and invasive behaviours. Ultimately, this is a rate-limiting step in the progression of the disease [2-4].

The composition of the cancer cell secretome is complex, and multifactorial [5], including small nanometre sized extracellular vesicles whose influence in orchestrating microenvironmental alterations is attracting attention [6-8]. Small 100nm or so vesicles, are thought principally to derive from late multivesicular endosomal (MVE) compartments, and are often referred to as exosomes [9]. MVEs may traffic to the plasma membrane with concomitant release of the vesicular cargo into the extracellular space [9]. The molecular machinery responsible for exosome biogenesis, intracellular trafficking and eventual secretion remains incompletely understood, as reviewed [10, 11].

Our previous studies emphasised the presence of nano-vesicle associated Transforming growth factor-beta (TGFβ1) [12], and highlighted their roles in generating tumour-promoting stromal myofibroblasts [13, 14]. Myofibroblasts generated by vesicles had a phenotype and function akin to those cells educated in vivo by the tumour [14-16]. Such nano-vesicles are important elements of the microenvironment driving changes towards exacerbating tumour growth [17].

Better understanding of vesicle manufacture and secretion processes can provide tools to manipulate their secretion, and thereafter study the impact in terms of loss of function. For example, trafficking of endosomal compartments involve some of the 70 Rab GTPases, and several are implicated in controlling MVE traffic and fusion events. Abrogating RAB11B function attenuates exosome secretion in an erythroleukaemic cell line (K562) [18, 19]. Inhibiting RAB35 activity results in accumulated endosomal compartments, and reduced exosome secretion in oligodendrocytes [20]. In the cervical carcinoma HeLa line, screens silencing RAB2B, RAB9A and RAB27A/B showed attenuated secretion of tetraspanin-positive (CD63, CD81) vesicles [21]. RAB5 is an early endocytic marker [22], yet several studies have indicated a role in exosome expulsion [21, 23]. We [14] and others [24, 25] have since confirmed the effect of RAB27 silencing in the context of vesicle secretion in cancer, but acknowledge such targets may also function in regulating general secretion in some cells [21, 25].

Members of the Endosomal Sorting Complex Required for Transport (ESCRT) are involved in MVE formation, but Knockdown of some ESCRT components does not always correlate with abrogated MVE formation [26] or exosome secretion [27], but silencing of HRS, TSG101 and STAM1 however may reduce the quantity of exosome vesicles and/or modify their protein composition [27]. Tetraspanin family members such as CD81 or CD63 [28, 29], and lipid-regulating enzymes such as neutral sphingomyelinase [30] and phospholipase D2 [31] are implicated in ESCRT independent mechanisms of exosome formation.

The docking and fusion of the MVE to the plasma membrane is the final step prior to nano-vesicles release from the cell. RAB27A/B and RAB35 facilitate docking [20, 21], and SNARE proteins (soluble N-Ethylalemide sensitive fusion attachment (SNAP) receptors) mediate diverse membrane-fusion events [32], including syntaxin1, or YKT6 in drosophila [33]. YKT6 targeting attenuates exosome secretion by as much as 80% [34]. However, it is important to note that all of the above findings may differ across different cell types [10]. Given the assortment of regulating factors

which influence exosome secretion in some cell types, and not in others, teasing out a pathway describing biogenesis, traffic and release remains a challenge to the vesicle field [10].

In this report, we used a well-established vesicle generating model system, based on prostate cancer cells, and we induced knockdown of some aforementioned candidate regulators of exosomes in these cells. We targeted the tetraspanin- CD9, the GTPases RAB5A, RAB11B, RAB35, the epithelial SNARE protein-VAMP7 [35] and the ESCRT-associated VPS25 which is essential for MVE formation [36], and examined the impact on vesicle secretion and vesicle-based communication with stromal cells. We demonstrate RAB11B and RAB35 as elements contributing towards a modest proportion of secreted vesicles, and revealed phenotypically similar yet distinct nano-vesicles arise from these pathways. We identify the RAB35-derived vesicles but not the RAB11B vesicles are required for fibroblast to myofibroblast differentiation, and subsequent growth promoting effects *in vivo*. The study highlights the existence and functional importance of distinct, naturally produced nano-vesicle subpopulations in controlling the aggressiveness of the tumour microenvironment and highlights the relative importance of RAB35-regulated vesicles in these effects.

2 Materials and methods

2.1 Cell culture

Prostate cancer cells (Du145) from ATCC were maintained in RPMI-1640 media. Primary fibroblasts of lung origin (AG02262) from Coriell Institute for Medical Research, were used at passage <10 and maintained in DMEM/F12 media (Lonza). The media were supplemented with 2mM L-Glutamine (Lonza) and 1Ul/ml Penicillin/Streptomycin (Lonza). All cell cultures were maintained in 10% FBS that had been depleted of bovine exosomes by overnight ultracentrifugation at 100,000 x g (Ti70 rotor, κ-factor of 110.5, LE80-ultracentrifuge, Beckman Coulter) followed by 0.2 μm and then 0.1 μm vacuum filters (Millipore).

2.1 Lentiviral Transduction of Du145 Cells

Du145 cells (5 x 10^3 per well) were plated in 96-well flat-bottom plates (Greiner Bio-One). At 24h, cells were transduced with MISSION® lentiviral particles (Sigma) at a multiplicity of infection of 20 in the presence of hexadimethrine bromide (8μg/ml; Sigma-Aldrich). At 24h, infected cells were selected for puromycin resistance (1.25μg/ml; Sigma-Aldrich) and viral particle-containing medium was replaced. Cells were considered free of lentiviral particles, post-passage 6.

2.3 Quantitative RT-PCR, and western blotting

Extraction of cellular RNA and quantitative RT-PCR was performed as previously described [13]. TaqMan gene expression assays for CD9, RAB5a, RAB11B, RAB35, VAMP7, VPS25, MMP1, MMP13 and TIMP3 were from ThermoFisher Scientific (assay ID's; CD9 Hs00233521_m1, Rab5a-Hs00991290_m1,RAB11B-Hs00188448_m1, Rab35-Hs00900055_m1, VAMP7-Hs00194568_m1, VPS25-Hs00260613_m1, MMP-1 Hs00899658_m1, MMP-13 Hs00233992_m1, TIMP-3 Hs00165949_m1). For westerns, cell lysates were made in RIPA buffer (Santa Cruz) and protease inhibitor cocktail, and protein quantified by Bradford Assay. Equal proteins (20μg) were added per well. For vesicle concentrates, these were not normalised per well, instead the pelleted material was loaded as a measure of potential differences in vesicle quantity. Procedures used are as described [14]. Antibodies included; GAPDH (BioChain, Abingdon, UK), CD9 (R&D Systems), RAB11B and RAB35 (Thermo Fisher), VAMP7 (R&D Systems) RAB5A, VPS25, TSG101, ALIX, Hsp70, Hsp90 and LAMP-1/2 were from Santa Cruz.

2.4 Analysis of cell proliferation

After 6 passes, the selected lentivirus transduced Du145 cells were seeded at identical density in 96 well plates, and images of general morphology taken by bright field microscopy using a 10x objective (Zeiss Observer Microscope). The WST-1 assay (Roche) was performed in quadruplicates, according to manufacturer's instructions at 24, 48 and 72 hours, and absorbance (420nm) measured on a PHERAstar plate reader (BMG Labtech, Germany).

2.5 Nanoparticle Tracking Analysis

Cell conditioned media (CM) were corrected for differences in cell number at harvest time, i.e. the volume of CM was adjusted by addition of fresh RPMI/FBS (exosome-depleted) to account for the differences in cell number. Particle size and counts were measured using the NanoSight™ nanoparticle tracking system (Malvern Instruments, Malvern, UK). For these measurements, samples were diluted in particle free water (Fresenius Kabi, Runcorn, UK) to concentrations up to $2x10^9$ particles/ml, within the linear range of the instrument. Analysis was performed on a NanoSight™ NS300 system with a 488nm laser and temperature set to 25°C. Six videos of 60s were taken in light scatter mode with controlled fluid flow with a pump speed set to 80. Videos were analysed using the batch analysis

tool of NTA 3.1 software (version 3.1 build 3.1.54), where minimum particle size, track length and blur were set at "automatic". The area under the histogram for each triplicate measurement was averaged and used as a particle concentration measurement. Background measurements of culture media that had not been exposed to cells contained negligible particles. Data represent three such experiments.

2.6 Vesicle Concentrates

Du145 prostate cancer cells, bearing control or shRNA against targets were seeded at identical cell densities, and allowed to condition the medium for 7 days. Typical experiments involved 6x T75cm^3 flasks per condition. Cell conditioned medium (CM) was harvested, and manual cell counts were performed. CM were rendered free of cells and debris by centrifugation (400 x g, 8 min and 2000 x g 15 min) and filtration through a 0.22μm filter (Millipore). The volume of CM was adjusted by addition of fresh RPMI/FBS (exosome-depleted) to account for the differences in cell number. The CM (60ml) was subjected to ultracentrifugation (200,000 x g for 2h, 70Ti rotor, κ-factor of 110.5, in Bell-top Quick Seal tubes in an LE-80K Ultracentrifuge, Beckman Coulter, High Wycombe, UK) to concentrate exosome-vesicles, or in some experiments CM were used directly to stimulate fibroblasts. Vesicle concentrates (pellets) were resuspended in 20μl of SDS-sample buffer, for western blotting, or PBS for protein array or fibroblast stimulation experiments. Particle to protein ratio was determined as described [37].

2.7 Cryo-electron microscopy

Concentrated vesicles were adsorbed onto glow-discharging holey carbon 200-mesh copper grids (Quantifoil Micro Tools GmbH). Grids were vitrified with the aid of a Vitrobot (Maastricht Instruments BV). Vitrified samples were imaged at liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL) equipped with a field emission gun and operated at an acceleration voltage of 200 kV. Six microscopic fields were assessed per sample, and the diameter of vesicle structures in each field was measured using Image-J.

2.8 Protein array & ELISA

Vesicle concentrates from control, RAB11B or RAB35 knockdown cells (corrected for protein) were subject to lysis (RIPA Buffer) and analysis by a sensitive proximity ligation assay configured in the form of a 92-plex array. Alternatively, cell conditioned medium normalised by protein, was used. The Proseek Inflammation Panel was used (Olink Bioscience, Upsalla, Sweden). The analyte menu is depicted (supplemental Fig. S3). The data were filtered to remove analytes reporting with signal below the assay limits of detection (LOD), and comparisons between control and knockdowns were made based on fold change and t-test. The LOD was defined as 1.5 standard deviations above background and values below this limit were reported as <LOD. Assays of vesicle concentrates or fibroblast-conditioned media were also performed by ELISA (DuoSet ELISA Development System; R&D Systems), for VEGF-A or HGF with modifications from the manufacturers protocol substituting a colourimetric readout with that of a time-resolved fluorescence as described [14]. To assess CD9-or CD81 levels in vesicle concentrates, samples were immobilised onto protein-binding 96 well plates as described [38], and stained with CD9 antibody (R&D Systems) or CD81 antibody (Millipore), and an Europium-based detection system. Signal was measured by time resolved fluorimetry. The quantity of MMP-1 and MMP-13 present in cell CM of 3D spheroids were assayed using the RayBio® sandwich ELISA kits (RayBioTech) according to manufacturer's instructions. All plate-based assays were read on a PheraStar FS plate reader (BMG, Germany).

2.9 Immuno-fluorescent microscopy

Following treatment, cells were fixed with ice cold acetone:methanol (1:1) for 5 min, dried and blocked in 1%BSA/PBS for 1h, then stained with primary antibodies. To assess fibroblast differentiation, primary antibody was against alpha smooth muscle actin (αSMA) (1μg/ml, Santa Cruz) for 1h. To examine changes in endosomal compartments, primary antibodies included anti-CD63, anti-CD81 (Bio-rad), anti-LAMP1 (Santa Cruz) or anti EEA1 (early endosomal antigen-1, from Becton Dickinson) and fluorescence microscopy performed with structured illumination using the Zeiss Apotome system, using a 63x/1.4 numerical aperture oil-immersion objective. Shown are representative figures from z-axis sections overlaid to generate maximum projection images (Zeiss Z1 Observer, Cambridge, UK, running Zen Pro Software).

2.10 Fibroblast differentiation assay

Fibroblasts were cultured in chamber slides or 24 well plates and at 80% confluence were serum-starved for 72h. Fresh DMEM/F12 media, soluble recombinant human TGFβ1 (1.5ng/ml, from Promocell), purified Du145 exosomes (200 μg/ml), vesicular concentrates (or CM) from lentivirally transduced Du145 cells was added for 72h. After washing, cells were fixed and immuno-fluorescently stained for αSMA as described [13]. Fibroblast-conditioned culture medium at 72h was assayed for HGF and VEGF by ELISA.

2.11 Tumour/Stroma Spheroid escape assays

Tumour cells (Control, RAB11B or RAB35 knockdown) were incubated alone or together with fibroblasts at a ratio of 4 tumour cells : 1 fibroblasts, in 96-well u-bottom cell-repellent surface plate (Greiner Bio-One). This ratio was chosen based on our previous in vivo experiments [14]. The medium consisted of RPMI in 10% exosome-depleted FBS. The total number of cells was 10^4 per well. At day 4, spheroids were transferred to Matrigel™ (Corning, UK) coated 24-well plates, and microscopically monitored over a further 96h time course. For each sphere/outgrowth (6 replicates per group), the area occupied within the cell outgrowth region was manually measured (using Image J), and is presented as relative to control (tumour only) spheres at 4 days, in which no outgrowth was evident.

2.12 In Vivo Xenotransplantation

In vivo experiments were conducted in accordance with the UKCCCR guidelines for the welfare of animals in experimental settings. Du145 control, RAB11B or RAB35 knockdowns were administered together with fibroblasts in a 100µl bolus of Matrigel™ using a 21 gague needle (Becton Dickinson, Oxford, UK) to the dorsal flank of CD1 athymic mice. A total of $5x10^6$ cells were injected subcutaneously, with a ratio of 4 tumour cells to 1 fibroblast, and there were 4 mice per experimental group. Tumours became palpable, in all mice at around day 7, and measurements using Vernier calipers were taken every 2 to 3 days thereafter to determine the volume. Tumour volume was calculated; tumour volume= 0.523 x width2 x length. Total body weight was also taken, and this did not change for any mouse during the procedures. Data show the mean tumour volume (±SE) per group at each time point up severity limits at 46 days. Tumours were excised and photographed, embedded in O.C.T. (Optimal Cutting Temperature compound, Agar Scientific, Essex, UK) and cryo-sectioned. Sections were thawed, fixed in acetone:methanol, dried, and stained with the same protocol as used for cells. Images were collected using a 20x objective (Zeiss Observer).

2.14 Statistical analysis

Statistical analyses were performed using Prism-4 software V4.03 (Graph Pad, San Diego, CA). In experiments with more than two experimental groups 1-way ANOVA with Tukey's post-test was used, except for kinetic experiments where a 2-way ANOVA with Bonferroni post-test was used. Experiments with two experimental groups were evaluated using students t test. P values less than 0.05 are considered significant *p<0.05, **p<0.01, ***p<0.001. Graphs depict mean±SE, from one representative experiment of at least three similar experiments, unless stated otherwise.

3 Results

3.1 Specific knockdown of candidate vesicle regulators

We used an established method based on lentiviral-delivery of shRNA to generate knockdowns. For each target (CD9, RAB5A, RAB11B, RAB35, VAMP7 and VPS25), we tested 5 shRNA-sequences. These are detailed in Table S1. Virally transduced cells were maintained in a predetermined lethal puromycin dose for six passes, before assessing the level of target mRNA and protein. Lentivirus delivering a shRNA sequence targeting a non-mammalian gene was used as the control. Using TaqMan™ gene expression assays, the levels of target-mRNA were compared to control cells. The 5 shRNA sequences targeting CD9 showed variable mRNA knockdown, from 45% to 87%. For all other targets, mRNA knockdown of at least 71% and up to 97% was achieved (Fig 1A, bars). Analysis of whole cell lysates by western blotting demonstrated attenuated expression of target proteins, with good agreement with the level of mRNA knockdown. The selection of sequences for follow-on analyses were based on a combination of diminished RNA and protein from these assays, and those chosen are denoted (Fig 1A, symbol-†). Selected cells maintained in continuous culture for 15 weeks, longer than any experiments conducted in the study, demonstrated stability of attenuated target-mRNA levels (Fig S1). We examined some properties of these cells for possible cytopathic consequences of the knockdowns. The cells exhibited a similar morphology regardless of target-protein attenuation, retaining typical cobblestone appearance (Fig 1B). Although seeded identically, the VPS25-knockdowns showed fewer attached cells, and were slower to reach confluence. When assessing proliferation, using a colorimetric mitochondrial activity assay (WST-1 assay) the impact of knockdown was negligible for CD9, RAB11B and RAB35 at 72h. There were, weak changes of proliferation seen with RAB5A and VAMP7 knockdown, but notable (~30%) attenuated proliferation for VPS25- knockdown (Fig 1C). Most of the knockdowns, therefore had limited negative effects on cell viability except for VPS25.

3.2 The consequence of knockdown on exosome secretion

We used multiple approaches to assess the outcome of target knockdown on secreted vesicles. The panel of cells were seeded identically and allowed to secrete factors into the medium for 72h. Cell counts were performed at medium harvest time, and cell-derived nanoparticles were quantified by nanoparticle tracking analysis (NTA) using the NanoSight™ (NS300) system. The size distribution of particles was similar irrespective of knockdown, and revealed a population of predominantly small particles of <200nm in diameter, with few events exceeding 300nm (Fig 2A). The histogram mode of the control cells was around 110nm and was similar throughout the panel of cells, with a tendency for larger particles however in the VAMP7 and VPS25 cells (Fig 2B, left). Significant differences were apparent in the quantity of particles produced per cell. Here the RAB5A knockdown elevated the number of detected particles ($p<0.01$), whilst RAB11B, RAB35, VAMP7 and VPS25 knockdowns demonstrated modest levels of reduced particulate. Changes following CD9 Knockdown showed no significant effects.

The NTA method does not discriminate vesicles from non-vesicular nano-aggregates of proteins/cell debris and hence supporting data to show possible impact on vesicle changes are needed. We used ultracentrifugation to concentrate vesicular material present in the cell-conditioned medium, to examine relative levels of known vesicle associated proteins. As above, cell counts were used as a basis for correcting for differences in cell numbers at the medium harvesting time (7d), and the medium was cleared of cell-debris by low speed centrifugation and filtration (0.22μm). Vesicles were concentrated from cell-conditioned medium by a 2h ultracentrifugation at 200,000xg and used for western blotting, and 70% of the pellet was used per lane (Fig 2C). Membranes were probed for the ESCRT-related protein TSG101 for the lysosome associated membrane proteins-1 (LAMP1), and for Hsp70 and 90 often associated with small vesicles[39-41]. Band intensity was variable across the cell panel, revealing elevation with RAB5A, for most of these proteins, except perhaps for TSG101. Coupled with the NTA data above, these point to a general elevation in nanovesicles positive for typical exosome-proteins arising following RAB5A knockdown. This set of proteins appeared reduced in intensity with RAB11B and RAB35 knockdowns, again in agreement with the NTA data. The pattern for CD9 and VAMP7 knockdowns was less consistent, where changes in band intensity seemed target protein dependent (Fig 2C). Whilst the blot showed reduced vesicle-related proteins with the VPS25 knockdown, this treatment also showed potential toxicities (Fig 1C), and hence this casts doubt on the vesicle-specific impact of VPS24 downregulation. We also immobilised the remainder of the ultracentrifuged material (30% of the pellet) onto protein-binding plates, and stained for the presence of surface-associated Tetraspanins CD9 or CD81. The assay revealed a clear-cut diminished signal for CD9 from all the knockdown cells ($p<0.01$), except for RAB5a, where signal was elevated almost 2-fold ($p<0.001$) (Fig 2D). The pattern was similar for CD81 staining, although not quite reaching significance.

Because knockdowns of RAB11B or RAB35 consistently pointed to attenuated vesicle production we performed a confirmatory experiment, examining an additional shRNA-sequence for RAB11B (sequence #558, Table 1) and RAB35 (sequence #796, Table 1) by NTA. This confirmed a modest attenuation of particles of between 20 and 30% (Fig S2).

Previous reports have documented changes in size or distribution of endo/lysosomal compartments following RAB27a [21] or RAB35 [20] knockdown. We therefore briefly examined the consequence of RAB11B or RAB35 knockdown in relation to this aspect, by immuno-fluorescent microscopy, staining for CD63 (LAMP-3), CD81 and the lysosome associated membrane protein-1 (LAMP-1). We also included early endosome antigen (EEA1) a marker of early/recycling endosomes. There was clear evidence for an elevation in intracellular CD63, LAMP1 and CD81 compared to control for both RAB11B and RAB35 knockdowns. The weak and diffuse punctuate staining of CD63 (Fig S3A), LAMP1 (Fig S3B) or CD81 (Fig S3C) in control cells became more intense, with particular peri-nuclear accumulation. In contrast, staining for EEA1 (Fig S3D) remained punctate and diffuse within the cytosol with no evidence of altered intensity or distribution, suggesting the early endosomes remained unaffected by the knockdowns.

We next questioned whether genuine vesicle structures were still being produced under these conditions. The ultracentrifuged pellets were examined by Cryo-electron microscopy revealing the presence of lipid-bounded nano-vesicles typical of exosomes in all specimens, and hinted at diminished vesicle counts with knockdown of RAB11B or RAB35 (Fig 2E). This was not formally quantified because detachment from the grids during vitrification cannot be fully prevented. We also observed small, electron dense structures of around 10-20nm diameter present following RAB35 knockdown (Fig 2E, arrows). These are likely beyond the detectable range of NTA, whose limits of detection is around 40-45nm [42], and the nature or importance of these structures is currently unknown. Measurements across 6 microscopic fields per condition reveal the underrepresentation of small vesicle-structures by the NanoSight platform as reported, and indicate a somewhat smaller modal size (49nm) for Control and Rab11b vesicles, but this was smaller still for RAB35 knockdown cells (~26.7nm). The data collectively indicate perturbation of late, but not early endosomes following knockdown of RAB11B or RAB35, which leads to a modest decrease of around 20% (by NTA) in the quantity of vesicle secreted, and may increase the relative proportion of very small vesicles upon RAB35 knockdown.

3.3 The loss of RAB11B or Rab35 alters the composition of the remaining secreted vesicles.

We next examined the protein profiles of concentrated vesicle-rich pellets, to determine if the vesicle phenotype was altered as a consequence of RAB-knockdown. For completion, we also examined the general cell secretome in the same fashion. We initially evaluated potential difference in the protein content of vesicles generally across the three conditions. Using the particle to protein measure [37] there was no significant difference across the three specimens (Fig 3A). We next employed a sensitive proximity-ligation array for this experiment (Proseek Multiplex Inflammation Panel) measuring the relative levels of 92 analytes to sub picogram sensitivity. The assay material was normalised based on protein-input, and hence acts as a measure of vesicle quality irrespective of differences in secreted vesicle quantity. Signal above limits of detection was present for 46 analytes when analysing the total secretome. With respect to the vesicle-pellet, an additional 8 analytes were undetectable, giving information on a total of 38 factors (Fig S4).

We compared the profile of the vesicle pellet from control cells to pellets arising from RAB11B and RAB35 knockdowns. Figure 3B depicts this as a fold-change compared to control cells, and reveals 18 analytes exhibit greater than ±2-fold changes in relative abundance following knockdown. Whilst the magnitude of change was dissimilar, the direction of change was the same for both RAB11B and RAB35, for all except for three instances. For example CXCL6 was >3.8 fold decreased in pellets from RAB11B knockdown cells, and >2 fold increased in pellets from RAB35 knockdown. The factors significantly ($P<0.05$) different compared to control cells are indicated in the volcano plot (Fig 3B), highlighting elevations in VEGF-A, TRAIL, IL18R1, TNFRSF3, TGFA and CXCL6 and reduction in MMP1 in the pellets from RAB35 knockdown. In contrast, pellets from RAB11B knockdown demonstrated elevation in 4E-BP1, with decreased levels of TWEAK, TRAIL, AXL1N and CXCL6. The data show similarity across the pellets, but also many examples where the vesicle population following RAB11B vs RAB35 knockdown is not identical. A search using the Vesiclepedia database [43] (search date 21st March 2018), reveals identification of 4E-BP1, MMP1, TWEAK, TNFRSF3, CXCL6 and VEGFA as vesicularly associated across several other studies [44-48]. In addition a proteomics profiling study of Du145 cells has previously identified exosomal expression of IL-18 receptor, and VEGFA [38]. This suggests such factors are known to be vesicle related, and are not assumed to be soluble contaminants in vesicle isolates.

Similar comparisons were made with cell free conditioned medium (Fig 3B), with several changes compared to controls. IL-6 (36-fold) and LIF (30-fold) were significantly elevated ($P<0.05$) in the secretome of RAB35-knockdown cells but not altered in the vesicle-pellet, indicating soluble factors like these are not pelleted by the vesicle concentration protocol used here. Alterations in CXCL6, IL18R1 (RAB35- knockdown) or CXCL6, 4E-BP1 (RAB11B-knockdown) however were altered by knockdown in both the general secretome and in the vesicle rich pellets. Profiles of the secretome therefore also highlight several examples of dissimilarity, and show the RAB11B and RAB35 knockdowns impart distinct effects on the general secretome, and these are not exclusive to their effects on vesicles.

3.4 Vesicles remaining following RAB11B or RAB35 knockdown lose stroma-activation potency

We previously showed exosomes from these, and other cancer cells can drive differentiation of stromal fibroblasts [14] or mesenchymal stem cells [15] into a form of myofibroblastic cell that is tumour promoting *in vivo*. The stromal cells become positive for the principle myofibroblast marker- alpha smooth muscle actin (αSMA), and secrete factors such as VEGF and HGF that were associated with a pro-angiogenic influence. We used these assays to examine if the nano-vesicles generated by RAB11B or RAB35 dependent pathways exhibited similar functional effects. Cell conditioned medium was harvested from identically treated control or knockdown cells, and the vesicle concentrates prepared by ultracentrifugation were normalised (as for Fig 3A/B) based on vesicle protein (200μg/ml). These were used to stimulate fibroblasts, which were analysed after 72h by immuno-florescence microscopy for the onset of αSMA. Fibroblasts treated with fresh media (without adding vesicles) exhibited negligible αSMA staining, but addition of vesicles from control cells strongly stimulated onset of αSMA (Fig 3C). Similarly, vesicles isolated from RAB11B knockdown cells also initiated expression of αSMA. In contrast, vesicles from RAB35 knockdowns revealed a poorly convincing differentiation response; whilst some αSMA was detectable (Fig 3C, arrows); this was similar to background staining seen in untreated cells. We conclude that vesicles remaining following perturbation of RAB35 are insufficient to initiate myofibroblastic differentiation. We also examined the culture medium conditioned by fibroblasts during this 72h stimulation for VEGF and HGF levels (Fig 3D), revealing the strong elevation in secreted VEGF/HGF mediated by control vesicles was significantly attenuated by either RAB11B or RAB35 knockdown. This shows onset of αSMA and growth factor secretion are not directly coupled during myofibroblast differentiation, and indicates a strong attenuation in both aspects when stimulating with vesicles from RAB35-knockdown cells.

3.5 Spheroid escape is attenuated by RAB35 knockdown

To avoid handling and transfer of concentrated vesicles, we explored the effects of tumour : stroma co-culture, employing 3D spheroid cultures. This gives a better representation of *in vivo* tumours by virtue of concentration gradients, hypoxic cores and the existence of cell-to-cell contacts.

Spheroids were established using control, RAB11B or RAB35 tumour cells with or without the inclusion of fibroblasts, at a ratio of 4-tumour cells per fibroblast. There were surprisingly little differences in the growth of the spheres over time (up to 24 days), regardless of their cellular composition (not shown). However, embedding 4-day established spheres into Matrigel™ pre-coated wells provided a modality to assess the extent/rate of extra-spheroidal escape. This acts as a surrogate measure of how well the microenvironment contains the tumour. This was monitored microscopically for a further four days.

Images at the end-point reveal the movement of cells, away from the central spheroid, and the extremity boundary is emphasised (Fig 4A, dotted line). The area occupied by the motile cells was not significantly different when spheres comprised tumour cells alone, irrespective of controls or knockdowns. However, in the presence of fibroblasts, control cells exhibited extensive spheroidal escape of approximately 7 fold the area of control cells alone ($P<0.001$). Spheroids comprising Rab11b knockdown cells with fibroblasts, behaved in a similar fashion with outgrowths only slightly smaller, (around 5 to 6 fold) ($P<0.05$). In contrast, spheres of Rab35-knockdown cells and fibroblasts failed to show extensive spheroid escape, and this would be consistent with defective cross-talk between cancer and stromal cells upon Rab35 deficiency (Fig 4A, B).

Parallel spheroids were collected at day 4, prior to outgrowth assays, and RNA was extracted for TaqMan™ gene expression assays, focussing on some selected matrix-modulating factors associated with invasive properties [49]. For matrix metalloproteinase-1 and -13, levels of mRNA was significantly reduced with either RAB11B or RAB35 knockdown (Fig 4D). In the presence of fibroblasts, there was a significant elevation in mRNA for these factors ($P<0.01$). This elevation, however was attenuated following knockdown of either Rab11b or Rab35, suggesting the cross talk between tumour and stroma is perturbed following either knockdown in relation to MMP. The presence of TIMP3 mRNA, was not detected in spheroids comprising tumour cells only, as this seems a property of fibroblasts. Nevertheless, knockdown of Rab11b or Rab35 resulted in enhanced mRNA levels for TIMP3 (Fig 4C). These data point to perturbations in the microenvironment due to RAB11B or RAB35 knockdown that attenuate aggressive, motile behaviours. The negative effect on motility, however were more marked with RAB35 attenuation.

3.6 The impact of Rab knockdown on in vivo tumour growth.

In order to examine if these effects on tumour to stroma interactions are also evident *in vivo*, we employed a xenograft model system, where control, RAB11B or RAB35 knockdown tumour cells were co-administered with stromal fibroblasts subcutaneously to the dorsal flank of immune deficient mice. The tumour volume was measured every 2-3 days until reaching severity limits. For each condition, tumours became palpable at 7d, with steady growth thereafter. RAB11B knockdown, showed little difference in growth rates compared to the control, reaching a large volume in excess of $330mm^3$ by 46 days compared to $389mm^3$ formed by controls (Fig 5A). When using RAB35 knockdowns however, tumours showed significant attenuation ($P<0.001$) of growth from day 35 onwards, and at 46 days the volume remained $<100mm^3$. Representative images of excised tumours are shown (Fig 5B), demonstrating restricted growth of the RAB35 knockdown tumours. The excised tissues were cryo-sectioned for subsequent histological staining or for RNA extraction. Sectioned tissue was examined for levels of αSMA-protein staining (Fig 5C). In tumours generated with control tumour cells, we observed frequent patches of αSMA-positivity, and a similar degree of αSMA positivity and distribution was evident with RAB11B-tumours. However, when examining the RAB35-tumours, there was a paucity of regions displaying αSMA suggesting that stromal fibroblastic cells within such tissues lacked this key characteristic of myofibroblasts. Extracted RNA from tissue sections, was subjected to TaqMan™ gene expression assays, revealing strong attenuation of αSMA -mRNA in the RAB35 knockdown tumours ($p<0.001$). Some attenuation was also evident for RAB11B-knockdowns, but only just reaching significance. Levels of mRNA for the matrix modulating proteases MMP1 and MMP13 were attenuated to a similar extent with wither RAB knockdown. In contrast, however, mRNA for TIMP3 was elevated around four-fold upon RAB35 knockdown but was unchanged with RAB11B knockdown. This suggests a decrease in metalloproteinases combined with an increase in their inhibition may occur within the smaller, RAB35 knockdown microenvironment. We conclude that the *in vitro* data revealing a role for RAB35-dependent vesicle mediated tumour to stromal communication, appears also to hold true in an *in vivo* tumour growth model, revealing an important role for RAB35 but less so for RAB11B in stroma regulation and tumour growth.

Discussion

Although we have known of the existence of different types and origins of extracellular vesicles for many years [50], there nevertheless remains a knowledge gap about which type of nano-vesicle population is of most relevance in diverse aspects of cancer or other diseases. Recent profiling studies have used OptiPrep™ gradients or affinity-based isolations as a modality for physically separating out different vesicle sub-types. These have highlighted particular markers that may identify vesicle-populations arising from the plasma membrane, or endosomally-derived exosome vesicles [51]. However, these studies also highlight an overlap in the repertoire of molecular constituents across diverse vesicle sub-types. Even within the small exosome-type of vesicle, the existence of structural and molecular heterogeneity is well established but the cellular machinery responsible for their biogenesis and secretion and the functional relevance of nanovesicle heterogeneity remains underexplored to date. The concept that there may be several forms of MVEs producing different types of nano-vesicles is an attractive idea, first proposed by Vidal, Colombo and colleagues [10, 18, 52], raising the possibility of attenuating the release of particular vesicle sub-populations by targeting MVE trafficking. This was elegantly demonstrated for the first time in a leukaemia cell line, where vesicle release was diminished following transfection with a RAB11 dominant-negative [18]. However, the relevance in terms of how the remaining vesicle-subsets function, was not explored. Other studies in different cell systems have followed, identifying RAB27 [21, 25] and also RAB35 [20] as regulators of vesicle secretion. In murine breast cancer and other lines, perturbation of RAB27 led to a major decrease in vesicle secretion, of around 80% [14, 24, 25]. This is expected to give rise to an obvious deficiency in vesicle-mediated functions. Indeed examples include reduced metastatic spread in melanoma [24], or in breast cancer models [25]. However, trafficking machinery in different cell types may well be subject to alternative regulators, and the dramatic effects of RAB27 attenuation is not uniformly apparent [25].

In our presented study, we explored a set of candidate vesicle regulators aiming to elicit a more modest impact on vesicle secretion; without fully abrogating vesicle secretory capacity. Those candidates taken forward; RAB11B and RAB35, served this function well, and highlighted these Rab-proteins are not essential for maintaining cell viability or proliferation. This is fortuitous, because assessing vesicle secretion by cells experiencing major toxicities is very difficult as they produce a different variety of cell blebs and debris potentially confounding analyses of endosome-derived nano-vesicles. RAB11B or RAB35 did reveal, however, alterations in late endosomes; accumulating LAMP1, CD81 and CD63 in peri-nuclear compartments, but with normal EEA1-stained early endosomes. Similar observation have been made in other cell types as a consequence of attenuated RAB27a [14, 21] or RAB35 [20], and is perhaps consistent with a diminished capacity to expel these proteins out of the cell in the form of vesicles, and their subsequent late endosomal or lysosomal accumulation. We measured around 20% decrease in exosomes by nanoparticle tracking analysis following either knockdown, and this was confirmed for 2 different shRNA sequences. The strength of the shRNA-based knockdown was around 95% (by RNA), yet the effect on vesicles is rather minor. It strongly suggests the existence of other pathways for vesicle expulsion in which RAB11B or RAB35 are redundant in these cells, supporting the premise that distinct MVE exist and are differentially regulated.

Following target knockdown, the vesicles that continue to be secreted were briefly protein profiled. Most of the analytes were at broadly comparable levels in control and knockdown cells, and this agrees with our expectation of considerable overlap in constituents across vesicle sub-populations [51]. Several analytes were differentially expressed, however pointing to RAB11B and RAB35 regulating relatively minor yet distinct vesicle secretion pathways in these cells. Examining functional differences *in vitro* between vesicles dependent on RAB11B or RAB35 was performed by normalising any deficiency in vesicle quantity, and the observed effects therefore reflect the difference in vesicle phenotypes. This has revealed an important difference between RAB11B and RAB35- in terms of regulating the differentiation of stromal fibroblasts to myofibroblasts.

The onset of myofibroblast accumulation in solid cancers is associated with worsening, more aggressive disease [2-4], and we indicate in 3D-culture assays and in vivo the relative importance of the RAB35-dependent pathway in controlling motility, growth and local differentiation. These data agree with the general premise of myofibroblast signatures of aggressivity, and show how vesicle traffic regulators can modulate the microenvironment including the available protease milieu, significantly; with a particularly vivid example in terms of tumour growth. They also highlight the relative weak (in vitro) or negligible (in vivo) role for the RAB11b pathway, and that the features of vesicles controlled by this regulator do not well correlate with tumour growth.

We previously highlighted that nanovesicles deliver assorted stimuli including TGFβ1 in complex with Heparan sulphate proteoglycans to elicit tumour-promoting myofibroblast differentiation [14]. The detailed mechanisms by which vesicles drive this particular form of myofibroblast however are not yet fully elucidated, and whilst TGFβ1 and intact Heparan sulphate side chains are essential for activating the SMAD-dependent signalling pathway, and subsequent αSMA onset [14] the relevance of the co-delivered vesicle cargo remains unclear. Our report identifies that functional αSMA-induction requires only a small proportion of the total available vesicles; those which are regulated in a RAB35 dependent manner. Certainly a greater understanding of this vesicle sub-population, its contents and interactions with stromal cells is now warranted, and studies are ongoing to distinguish the critical elements which dictate potency in stromal differentiation.

We have not yet fully evaluated the importance of some of the other targets provisionally described in this report, including CD9, RAB5a and VAMP7. The data at hand show RAB5a may be a candidate for enhancing the extracellular vesicle content of the secretome, either through accelerated secretion, or conversely perhaps modulating the capacity of the cell to take-up and process exosomes in an autocrine fashion. It highlights some of the complexities in deciphering the machinery regulating the amount of vesicles available in the extracellular space, and studies are ongoing to ascertain in more detail the precise role of RAB5a in exosome expulsion and uptake.

Conclusions

We evidence herein that RAB11B or RAB35 control minor yet distinct nanovesicle secretion pathways, resulting in vesicle subpopulations with distinct effects on stromal cell differentiation, and microenvironmental support for tumour growth. The RAB35 regulated pathway is identified here as an element contributing to an aggressive tumour-environment, supporting motility and growth, and hence may provide a selective therapeutic target for perturbing nano-communication between cancer cells and stromal fibroblasts. The activation of tumour-associated stroma is a common feature seen across a spectrum of solid cancer types, and similar stromal phenotypes are also evident in numerous lethal fibrotic conditions. The identification of a vesicle subpopulation driving such changes has implications that are likely beyond cancer. Deciphering in more detail the molecular components of this vesicle sub-population will be important in future studies. This will not only provide insight into mechanistic basis underlying the activity of this subpopulation on stromal cells, but may reveal unique features that could be utilised to directly block such interactions and subsequent effects. Moreover, as this subset of vesicles signify a capacity for tumours to strongly activate stromal cells; their identification in biofluids of cancer patients may be informative, and indicate the likelihood of developing aggressive, stromally supported tumours.

Our study emphasises the inherent complexity of vesicle-mediated communication in pathological settings, and proposes the need for greater understanding of nanovesicle heterogeneity and their functional importance in pathological processes.

Figure1. Validation and selection of lentivirus transduced DU145 cells. DU145 prostate cancer cells were transduced with Mission lentiviral particles, delivering shRNA sequences against six specified targets. For each target, five different shRNA sequences were tested, as annotated (#). After 24h, the cells were expanded in the presence of puromycin (1.25μg/ml). After 6 passes, cells were analysed by Taq-Man™ PCR assays for the levels of target mRNA. These are presented as expression relative to those found in identically treated cells but with an irrelevant non-mammalian shRNA sequence (CTR). Graphs represent mean±SD of triplicate measurements for each sequence, and the degree of knockdown (%) is indicated in each case (A, Bars). Cell lysates were prepared and analysed by western blot for the relevant target protein and for GAPDH, as a loading control. Selected cells (†) were examined for general morphology, with typical epithelioid cobble-stone appearance (B). As a surrogate assessment of viable cell numbers, identically seeded cells were subjected to the WST-1 reagent at 24h, 48h and 72h and the means of quadruplicate absorbance values (at 420nm) are shown and represents of two such experiments (C) (*P<0.05, **P<0.01, ***P<0.001, 2-way Anova with Bonferroni post test).

Figure 2. Impact of knockdowns on extracellular vesicles. Control or knockdown cells were cultured for 72h and harvested medium analysed by NTA analysis. The size distribution histogram of six replicate measurements for each sample is shown (A). The mode of the histogram (B, left bars) and the number of particles, adjusted for cell number at harvest time (B, right bars) is shown (Mean±SD, of 6 replicates (*P<0.05, **P<0.01, ***P<0.001, 1-way Anova with Tukey's post test). These data are from a contiguous experiment and are representative of three such experiments. Cell conditioned medium was adjusted for differences in cell number, and pre-cleared of debris and large particles by centrifugation before ultracentrifugation; allowing focussed analysis of nano-vesicles. Pellets (70% of total) were analysed by western blotting for typical exosome-associated proteins as indicated. Representative of three experiments (C). Remainder 30% of pellets were immobilised to protein-binding plates, and stained for CD9 or CD81. Bars show signals relative to those of control-cells (mean±SD of triplicates, *P<0.05, **P<0.01, ***P<0.001, 1-way Anova with Tukey's post test), and represent two such assays (D). The pellets from control, *RAB11B* or *RAB35* cells were examined by cryo-electron microscopy. Representative fields are depicted and example structures at higher magnifications are shown. White arrows highlight very small ~10nm spherical structures. Bars=100nm (E, left). Measures of vesicle diameter across 6 fields per condition, and the number of analysed events, and mode is depicted (E, right).

Figure 3. Knockdown of *RAB11B* or *RAB35* alters the protein profile and function of vesicles. Conditioned medium from control or knockdown cells were concentrated by 200,000xg ultracentrifugation and pellets examined for global differences in protein content per vesicles using the particle to protein ratio, plot shows three preparations per sample type (mean±SD) (A). Pellets were normalised for protein, and analysed using the Proseek Multiplex proximity ligation array. The analysis represent differences in 38 analytes reporting with signals above the assay limits of detection (LOD). Comparing the vesicle-pellets derived from *RAB11B* or *RAB35* relative to control, the volcano-plot (B, left) identifies differentially expressed analytes with a fold change>2 and a p<0.05. Similarly, cell conditioned media was analysed showing 48 analytes reporting with signals above the LOD (B, right). Growth arrested fibroblasts were stimulated with fresh media or vesicle concentrates at equivalent protein concentration (200μg/ml) each, and 72h cells were fixed and stained for αSMA, shown is

representative of three experiments (C). Fibroblast conditioned media were collected at 72h, and assayed for growth factors, where bars represent measurements from triplicate wells, representing three such experiments. mean+S.D (*P<0.05, **P<0.01, ***P<0.001, 1-way Anova with Tukey's post test) (D).

Figure 4. Knockdown of *RAB11B* or *RAB35* results in changes in the microenvironment and motility in 3D speroids. Tumour cells (Control, RAB11B or Rab35 knockdown) were incubated alone or together with fibroblasts at a ratio of 4 tumour cells : 1 fibroblasts, in 96-well u-bottom cell-repellent surface plate (Greiner Bio-One). This ratio was chosen based on our previous *in vivo* experiments [14], the total number of cells was 10^4 per well. At day 4, established spheroids were transferred to freshly Matrigel™ (Corning, UK) coated 24-well plates, and microscopically monitored for a further 96 h. The circumference of the region of extra-spheroidal cell boundary (dotted line) is shown, scale bar=100μm or 200μm for tiled images (A) and the area occupied by escaping cells, relative to control tumour-only spheres was measured. Whiskers plot represent relative area at the 96 hour timepoint (showing median, 25th to 75th percentiles, and min & max values, of 6 replicates and *P<0.05, ***P<0.001, 1-way Anova with Tukey's post-test) (B). Six parallel spheroids per condition at day 4 were harvested, pooled and extracted mRNA examined by TaqMan™ gene expression assays for MMP-1, MMP-13 and TIMP-3 (C). Bars show relative levels of target mRNA compared to control tumour cells alone, of triplicate measurements, representing two such experiments. (*P<0.05, **P<0.01, ***P<0.001, 1-way Anova with Tukey's post-test).

Figure 5. *RAB35* but not *RAB11B* knockdown attenuates growth in vivo. A total of $5x10^6$ cells comprising tumour cells with fibroblasts, at a ratio of 4:1 respectively were administered subcutaneously to the dorsal flank of CD1 athymic mice. Three groups were compared; including control tumour cells, *RAB11B* or *RAB35* knockdowns. When palpable, at day 7 onwards, tumour growth was assessed every 2 to 3 days and the plot depicts tumour volume for up to 46 days (Mean±SE, n=4, ***P<0.001, 2-way Anova with Bonferroni post test) (A). Tumours were excised, and photographed to represent the differences in volume at the experiment end point (B). Cryosectioned tissue was stained for αSMA (green) or DAPI (blue), bar=100μm (D) or used for RNA extracts, followed by TaqMan™ gene expression assays. Bars show mean+S.D of triplicates (*P<0.05, **P<0.01, ***P<0.001, 1-way Anova with Tukey's post test).

Supplemental Figures

Figure S1. Stability of lentivirus transduced DU145 cells. Lentivirally transduced cells were maintained in continuous culture with two-passages per week. At 6 weeks, or 15 weeks, TaqMan™ gene expression assays performed for the specified targets. Bars represent triplicate measures ±S.D., and compare relative target expression in non mammalian control (NM Ctrl) cells to targeted shRNA knockdown cells.

Figure S2. Confirmation of shRNA targeting of RAB11B and RAB35. Secretion of nano-vesicle was tested for an additional shRNA sequence targeting RAB11B or RAB35. NTA analysis revealed a similar, small mostly <200nm particulate population (A) with no significant difference in histogram mode (B). Examining particles per cell confirmed perturbation of secretion with RAB11B (shRNA sequence #558) or RAB34 (sequence #796) of 26% or 21% respectively. Bars represent mean±S.D of 6 replicates (*P<0.05, 1-way Anova with Tukey's post test).

Figure S3. Perturbations in late but not early endosomes. Du145 cells (NM Ctrl, RAB11B or RAB35 knockdowns) were seeded identically onto glass coverslip chamber slides, and at 48h were fixed and stained for markers of endosomes CD63 (A), LAMP1 (B), CD63 (C) or a marker of early/recycling endosomes EEA1 (D). Images were taken using structured illumination, using a 63x/1.4 numerical aperture oil-immersion objective. Shown are representative figures from z-axis sections overlaid to generate maximum projection images (Zeiss Z1 Observer, Cambridge, UK, running Zen Pro Software).

Figure S4. Protein Profiling Array. Samples were examined for differences in protein expression levels using a 92-plex protein array covering an assortment of growth factors, chemokines and other components associated with inflammation (Proseek Inflammation Panel, Olink). In cell conditioned media 46 analytes reported with signal below the assay limits of detection (LOD), and a further 8 fell below LOD for the vesicle concentrates. The Venn diagram, and array coverage lists identify those detected and not detected in each category.

Conflicts of interest

There are no conflicts to declare

Acknowledgements

The in vivo experiments were conducted by Axis Bioservices Ltd, and the authors are grateful to Dr Jenny Worthington. Funding support was from a Life Science Research Network Wales PhD. Studentship (for VY) and from the Division of Cancer and Genetics, School of Medicine, Cardiff University.

Notes and references

‡
§
§§

1. K. Kaminska, C. Szczylik, Z. F. Bielecka, E. Bartnik, C. Porta, F. Lian and A. M. Czarnecka, *J Cell Mol Med*, 2015, 19, 283-296.
2. J. A. Tuxhorn, G. E. Ayala, M. J. Smith, V. C. Smith, T. D. Dang and D. R. Rowley, *Clin Cancer Res*, 2002, 8, 2912-2923.
3. A. Orimo, P. B. Gupta, D. C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V. J. Carey, A. L. Richardson and R. A. Weinberg, *Cell*, 2005, 121, 335-348.
4. A. F. Olumi, G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty and G. R. Cunha, *Cancer Res*, 1999, 59, 5002-5011.
5. S. Patel, A. G. Ngounou Wetie, C. C. Darie and B. D. Clarkson, *Adv Exp Med Biol*, 2014, 806, 409-442.
6. L. Milane, A. Singh, G. Mattheolabakis, M. Suresh and M. M. Amiji, *J Control Release*, 2015, 219, 278-294.
7. J. Webber, V. Yeung and A. Clayton, *Semin Cell Dev Biol*, 2015, 40, 27-34.
8. S. R. Baglio, T. Lagerweij, M. Perez Lanzon, D. Xuan Ho, N. Leveille, S. A. Melo, A. M. Cleton-Jansen, E. S. Jordanova, L. Roncuzzi, M. Greco, M. A. van Eijndhoven, G. Grisendi, M. Dominici, R. Bonafede, S. Lougheed, T. D. de Gruijl, N. Zini, S. Cervo, A. Steffan, V. Canzonieri, A. Martson, K. Maasalu, S. Koks, T. Wurdinger, N. Baldini and D. M. Pegtel, *Clin Cancer Res*, 2017, DOI: 1078-0432.CCR-16-2726 [pii].
9. G. Raposo, H. W. Nijman, W. Stoorvogel, R. Leijendekker, C. V. Harding, C. J. M. Melief and H. J. Geuze, *J Exp Med*, 1996, 183, 1161-1172.
10. M. Colombo, G. Raposo and C. Thery, *Annu Rev Cell Dev Biol*, 2014, 30, 255-289.
11. L. Blanc and M. Vidal, *Small GTPases*, 2017, DOI: 10.1080/21541248.2016.1264352 [doi], 1-12.
12. A. Clayton, J. P. Mitchell, J. Court, M. D. Mason and Z. Tabi, *Cancer Res*, 2007, 67, 7458-7466.
13. J. Webber, R. Steadman, M. D. Mason, Z. Tabi and A. Clayton, *Cancer Res*, 2010, 70, 9621-9630.
14. J. P. Webber, L. K. Spary, A. J. Sanders, R. Chowdhury, W. G. Jiang, R. Steadman, J. Wymant, A. T. Jones, H. Kynaston, M. D. Mason, Z. Tabi and A. Clayton, *Oncogene*, 2015, 34, 290-302.
15. R. Chowdhury, J. P. Webber, M. Gurney, M. D. Mason, Z. Tabi and A. Clayton, *Oncotarget*, 2015, 6, 715-731.
16. J. P. Webber, L. K. Spary, M. D. Mason, Z. Tabi, I. A. Brewis and A. Clayton, *Oncotarget*, 2016, 7, 20124-20139.
17. H. Zhao, L. Yang, J. Baddour, A. Achreja, V. Bernard, T. Moss, J. C. Marini, T. Tudawe, E. G. Seviour, F. A. San Lucas, H. Alvarez, S. Gupta, S. N. Maiti, L. Cooper, D. Peehl, P. T. Ram, A. Maitra and D. Nagrath, *Elife*, 2016, 5, e10250.
18. A. Savina, M. Vidal and M. Colombo, *J Cell Sci*, 2002, 115, 2505-2515.
19. J. L. Johnson, J. He, M. Ramadass, K. Pestonjamasp, W. B. Kiosses, J. Zhang and S. D. Catz, *J Biol Chem*, 2016, 291, 3423-3438.
20. C. Hsu, Y. Morohashi, S.-i. Yoshimura, N. Manrique-Hoyos, S. Jung, M. A. Lauterbach, M. Bakhti, M. Grønborg, W. Möbius, J. Rhee, F. A. Barr and M. Simons, *The Journal of Cell Biology*, 2010, 189, 223-232.
21. M. Ostrowski, N. B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C. F. Moita, K. Schauer, A. N. Hume, R. P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M. C. Seabra, F. Darchen, S. Amigorena, L. F. Moita and C. Thery, *Nat Cell Biol*, 2010, 12, 19-30.
22. J. P. Gorvel, P. Chavrier, M. Zerial and J. Gruenberg, *Cell*, 1991, 64, 915-925.
23. M. F. Baietti, Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, Y. Ivarsson, F. Depoortere, C. Coomans, E. Vermeiren, P. Zimmermann and G. David, *Nat Cell Biol*, 2012, 14, 677-685.
24. H. Peinado, M. Aleckovic, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. Garcia-Santos, C. Ghajar, A. Nitadori-Hoshino, C. Hoffman, K. Badal, B. A. Garcia, M. K. Callahan, J. Yuan, V. R. Martins, J. Skog, R. N. Kaplan, M. S. Brady, J. D. Wolchok, P. B. Chapman, Y. Kang, J. Bromberg and D. Lyden, *Nat Med*, 2012, 18, 883-891.
25. A. Bobrie, S. Krumeich, F. Reyal, C. Recchi, L. F. Moita, M. C. Seabra, M. Ostrowski and C. Théry, *Cancer Res*, 2012, 72, 4920-4930.
26. S. Stuffers, C. Sem Wegner, H. Stenmark and A. Brech, *Traffic*, 2009, 10, 925-937.
27. M. Colombo, C. Moita, G. van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L. F. Moita, C. Thery and G. Raposo, *J Cell Sci*, 2013, 126, 5553-5565.
28. D. Perez-Hernandez, C. Gutierrez-Vazquez, I. Jorge, S. Lopez-Martin, A. Ursa, F. Sanchez-Madrid, J. Vazquez and M. Yanez-Mo, *J Biol Chem*, 2013, 288, 11649-11661.
29. J. R. Edgar, E. R. Eden and C. E. Futter, *Traffic*, 2014, 15, 197-211.
30. K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brugger and M. Simons, *Science*, 2008, 319, 1244-1247.
31. R. Ghossoub, F. Lembo, A. Rubio, C. B. Gaillard, J. Bouchet, N. Vitale, J. Slavik, M. Machala and P. Zimmermann, *Nat Commun*, 2014, 5, 3477.

32. Y. Wang, L. Li, C. Hou, Y. Lai, J. Long, J. Liu, Q. Zhong and J. Diao, *Semin Cell Dev Biol*, 2016, DOI: S1084-9521(16)30204-X [pii].
33. K. Koles, J. Nunnari, C. Korkut, R. Barria, C. Brewer, Y. Li, J. Leszyk, B. Zhang and V. Budnik, *J Biol Chem*, 2012, 287, 16820-16834.
34. M. Ruiz-Martinez, A. Navarro, R. M. Marrades, N. Vinolas, S. Santasusagna, C. Munoz, J. Ramirez, L. Molins and M. Monzo, *Oncotarget*, 2016, DOI: 10.18632/oncotarget.9862 [doi].
35. S. K. Rao, C. Huynh, V. Proux-Gillardeaux, T. Galli and N. W. Andrews, *J Biol Chem*, 2004, 279, 20471-20479.
36. D. Teis, S. Saksena, B. L. Judson and S. D. Emr, *EMBO J*, 2010, 29, 871-883.
37. J. Webber and A. Clayton, *Journal of Extracellular Vesicles*, 2013, DOI: http://dx.doi.org/10.3402/jev.v2i0.19861, 19861
38. J. Webber, T. C. Stone, E. Katilius, B. C. Smith, B. Gordon, M. D. Mason, Z. Tabi, I. A. Brewis and A. Clayton, *Mol Cell Proteomics*, 2014, 13, 1050-1064.
39. T. Takeuchi, M. Suzuki, N. Fujikake, H. A. Popiel, H. Kikuchi, S. Futaki, K. Wada and Y. Nagai, *Proc Natl Acad Sci U S A*, 2015, 112, E2497-2506.
40. A. Clayton, A. Turkes, N. Hossein, M. D. Mason and Z. Tabi, *J Cell Sci*, 2005, 118, 3631-3638.
41. G. Lancaster and M. Febbraio, *J Biol Chem*, 2005, 280, 23349 - 23355.
42. V. Kestens, V. Bozatzidis, P. J. De Temmerman, Y. Ramaye and G. Roebben, *J Nanopart Res*, 2017, 19, 271.
43. H. Kalra, R. J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V. C. Bond, F. E. Borràs, X. Breakefield, V. Budnik, E. Buzas, G. Camussi, A. Clayton, E. Cocucci, J. M. Falcon-Perez, S. Gabrielsson, Y. S. Gho, D. Gupta, H. C. Harsha, A. Hendrix, A. F. Hill, J. M. Inal, G. Jenster, E.-M. Krämer-Albers, S. K. Lim, A. Llorente, J. Lötvall, A. Marcilla, L. Mincheva-Nilsson, I. Nazarenko, R. Nieuwland, E. N. M. Nolte-'t Hoen, A. Pandey, T. Patel, M. G. Piper, S. Pluchino, T. S. K. Prasad, L. Rajendran, G. Raposo, M. Record, G. E. Reid, F. Sánchez-Madrid, R. M. Schiffelers, P. Siljander, A. Stensballe, W. Stoorvogel, D. Taylor, C. Thery, H. Valadi, B. W. M. van Balkom, J. Vázquez, M. Vidal, M. H. M. Wauben, M. Yáñez-Mó, M. Zoeller and S. Mathivanan, *PLoS Biol*, 2012, 10, e1001450.
44. S. N. Hurwitz, M. A. Rider, J. L. Bundy, X. Liu, R. K. Singh and D. G. Meckes, Jr., *Oncotarget*, 2016, 7, 86999-87015.
45. A. Janowska-Wieczorek, M. Wysoczynski, J. Kijowski, L. Marquez-Curtis, B. Machalinski, J. Ratajczak and M. Z. Ratajczak, *Int J Cancer*, 2005, 113, 752-760.
46. B. S. Hong, J. H. Cho, H. Kim, E. J. Choi, S. Rho, J. Kim, J. H. Kim, D. S. Choi, Y. K. Kim, D. Hwang and Y. S. Gho, *BMC Genomics*, 2009, 10, 556.
47. B. Liang, P. Peng, S. Chen, L. Li, M. Zhang, D. Cao, J. Yang, H. Li, T. Gui, X. Li and K. Shen, *J Proteomics*, 2013, 80, 171-182.
48. D. B. Peterson, T. Sander, S. Kaul, B. T. Wakim, B. Halligan, S. Twigger, K. A. Pritchard, Jr., K. T. Oldham and J. S. Ou, *Proteomics*, 2008, 8, 2430-2446.
49. S. Jodele, L. Blavier, J. M. Yoon and Y. A. DeClerck, *Cancer Metastasis Rev*, 2006, 25, 35-43.
50. S. J. Gould and G. Raposo, *J Extracell Vesicles*, 2013, 2.
51. J. Kowal, G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach and C. Thery, *Proc Natl Acad Sci U S A*, 2016, 113, E968-977.
52. A. de Gassart, C. Geminard, D. Hoekstra and M. Vidal, *Traffic*, 2004, 5, 896-903.