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Differentiation of Tumour Promoting Stromal Myofibroblasts by Cancer Exosomes.

Running Title: Exosome activation of Cancer Stroma

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Abstract

Activation of myofibroblast rich stroma is a rate-limiting step essential for cancer progression. The responsible factors are not fully understood, but TGF β 1 is probably critical. A proportion of TGF β 1 is associated with extracellular nano-vesicles termed exosomes, secreted by carcinoma cells, and the relative importance of soluble and vesicular TGF β in stromal activation is presented. Prostate cancer exosomes triggered TGF β 1 dependent fibroblast differentiation, to a distinctive myofibroblast phenotype resembling stromal cells isolated from cancerous prostate tissue; supporting angiogenesis in vitro and accelerating tumour growth in vivo. Myofibroblasts generated using soluble-TGF β 1 were not pro-angiogenic or tumour-promoting. Cleaving heparan sulphate side chains from the exosome surface had no impact on TGF β levels yet attenuated SMAD-dependent signalling and myofibroblastic differentiation. Eliminating exosomes from the cancer cell secretome, targeting Rab27a, abolished differentiation and lead to failure in stroma-assisted tumour growth in vivo. Exosomal-TGF β 1 is therefore required for the formation of tumour promoting stroma.

Keywords: exosomes, prostate, stroma, fibroblast, TGF β

Introduction

In prostate, and other, carcinomas the interstitial stroma is often abnormally rich in myofibroblastic cells (1, 2) capable of supporting tumour growth, partly through encouraging angiogenesis (3-5). Stromal gene signatures can predict treatment response in breast cancer (6), and more generally, high stroma to tumour ratios appears indicative of poor prognoses (1, 7, 8). Therapeutics targeting stromal activation may therefore be attractive, but there remain many unanswered questions about how cancer cells communicate with and co-opt stromal cells into participating in the pathological process.

An assortment of epigenetic regulators, matrix components, cytokines and other factors are implicated in myofibroblast-induction, and phenotypic regulation. TGF β , however, is of critical importance in generating tumour-reactive stroma (2, 9). Stromal differentiation requires engagement of the TGF β type I (ALK5) and type II receptors, driving SMAD dependent and independent intracellular signalling events. This modulates transcription of a host of target genes (9, 10) including alpha-smooth muscle actin (α SMA) which, together with cell contractility, is the principal myofibroblast marker (11).

Although TGF β 1 is secreted at high levels as soluble homodimers by carcinomas; a proportion is also secreted in association with nanometre-sized extracellular vesicles, termed exosomes, which can trigger both canonical and non-canonical TGF β signalling pathways (12, 13). This phenomenon, however, is not yet widely appreciated. Exosomes are typically less than 150nm in diameter, originating from within multivesicular endosomes (14). They harbour a complex molecular repertoire of lipids (15), proteins (16) and RNA (17), and can modulate cellular responses by a variety of molecular mechanisms. Cancer exosomes exhibit well documented immune regulatory functions (18), many of which have been attributed to exosomal-TGF β 1 (12, 19-21). Exosomes influence other aspects of cancer biology, including metastasis (22, 23) and angiogenesis (24-27), yet few studies have investigated the function(s) of exosomes in the context of tumour-stroma activation.

We recently showed that cancer exosomes can trigger fibroblast to myofibroblast differentiation. This was dependant on exosomal-TGF β 1 and the heparan sulphate proteoglycan, Betaglycan (12). The relative potency, however, of soluble TGF β 1 compared to its exosome-associated form is unknown. Similarly, the physiological relevance of exosomal-TGF β 1 in modulating cancer stroma has not been well explored. In this report, we hypothesized that cancer-derived exosomes are a component of tumour to stromal cell communication required for generating a tumour-supporting stromal phenotype.

We present novel data demonstrating the necessity for cancer exosomes to drive stromal differentiation to a disease-associated phenotype. This phenotype is distinct from that generated by soluble TGF β 1, as it exhibits pro-angiogenic and tumour growth-promoting characteristics. We show heparan sulphate side-chains, at the exosome surface, control the qualitative nature of exosomal-TGF β 1 delivery, as loss of such chains attenuates SMAD-dependent TGF β signalling and abrogates stromal differentiation without impacting the TGF β 1 dose presented. Furthermore, exosome-deficient cancer cells fail to gain stroma-mediated growth advantage *in vivo*. Together these data highlight unique functional properties of exosomal-TGF β 1, and implicate exosomes as key modulators of stroma in prostatic cancer, and likely other scenarios.

Results

Cancer exosomes trigger distinctive fibroblast differentiation.

As an initial model, we treated lung fibroblasts with Du145 exosomes, characterised in Figure S1 and shown previously as TGF β -positive (12). Exosomes or dose-matched soluble TGF β 1 (sTGF β 1) significantly elevated expression of the principal myofibroblast marker, α SMA, plus other characteristic features, including EDA-Fibronectin. A myofibroblast-specific antibody, PR2D3, (28)

demonstrated positive expression following exosome or sTGF β 1 treatment. Inhibition of TGF β signalling, using an ALK5 inhibitor (SB431542), attenuated this exosome effect. In contrast, the mesenchymal marker, Vimentin, was expressed constitutively, and was not altered by treatment. The data reveal a myofibroblast phenotype that was similar following exosome or sTGF β 1 treatment (Fig1A), consistent with our previous report (12).

We next explored possible differences in secreted factors following exosome or sTGF β treatment. Using a low density protein array, we examined expression of 55 proteins of known associations with angiogenesis (Fig1B, C), revealing several differences. For example uPA, and HGF were preferentially elevated by exosomes (Fig1C, dark bars), whilst PDGF-AA and IGFBP-3 were better elevated by sTGF β 1 (Fig1C). We confirmed some of these results by ELISA, comparing exosomes with sTGF β 1 at several matched doses; demonstrating superior exosome-mediated elevation of uPA, HGF, FGF2, and VEGF-A (Fig1D). Confirmatory ELISA of purified exosomes, in the absence of fibroblasts, showed low levels of some of these factors were detectable (specifically 556pg/ml uPA, 0pg/ml HGF, 15.2pg/ml FGF2, 581pg/ml VEGF, when adding exosomes at an equivalent TGF β 1 dose of 1.5ng/ml). These were too low to account for the levels measured in fibroblast-culture medium, pointing to the onset of de novo production of these factors. Real-time PCR revealed elevated mRNA for these factors following exosome treatment, with relative expression above that of sTGF β 1 stimulation. Exosomally-induced mRNA was inhibited in the presence of the ALK5 inhibitor SB431542 (Fig1E). Protein array data comparing exosome treatment *vs* exosome & SB431542 also revealed most of the exosome-mediated alterations were TGF β 1 sensitive, with HGF, FGF2 and VEGF inhibited by 50% or greater by SB431542 (Fig1F). Exosomes trigger TGF β 1-dependent differentiation of fibroblasts into distinctive myofibroblasts compared to the classical stimulus of soluble TGF β .

Exosome-induced myofibroblasts modulate endothelial cells.

Many of the elevated growth factors are pro-angiogenic, so we performed a series of experiments to evaluate the myofibroblast influence on various aspects of endothelial cell biology *in vitro*. Human umbilical vein endothelial cells (HUVEC) were stimulated with fibroblast conditioned media, collected 72h after treatment with exosomes or sTGF β . After 24h, HUVEC highly expressed CD31 and CD105, and this was unaltered by treatment. There was some increase in ICAM-1 and VCAM-1 expression (Fig2A), by exosome-treated fibroblast culture medium, but this was not inhibited by SB431542, and therefore a TGF β 1-independent effect. Examining proliferation revealed strong stimulation by exosome-treated fibroblast culture medium. This was, however, inhibited by SB431542. In contrast, culture medium from sTGF β -treated fibroblasts had a much weaker influence (Fig2B). At 72h, there was no difference in the proportion of viable HUVEC in the differentially treated fibroblast culture medium (Fig2C), suggesting little difference in terms of HUVEC survival.

HUVEC monolayers were subjected to a scratch (Fig2D), prior to addition of fibroblast culture medium. The rate of scratch closure, monitored microscopically, was accelerated by culture medium from exosome-treated fibroblasts. This was partially attenuated by SB431542. Myofibroblasts, generated by sTGF β 1 had no impact on endothelial migration rate, matching the rate of spontaneous closure.

In a vessel formation assay (29), HUVEC co-cultured with untreated monolayers of fibroblasts had little effect, and supported only occasional CD31-positive cell clusters (Fig2E). Exosome-treated fibroblasts, however, supported formation of elaborate networks of vessel-like structures, forming multiple cross-bridges with neighbouring vessels. In contrast, fibroblasts treated with exosomes and SB431542 supported structures that were narrower with less branching, whilst stimulation with sTGF β 1 exhibited sparse CD31-positive structures akin to untreated fibroblasts. In summary, exosome-generated myofibroblasts, but not those generated using sTGF β , can positively influence multiple properties of endothelial cell function, and are pro-angiogenic in nature.

Exosomal heparan sulphate chains are required to drive such distinctive myofibroblast differentiation.

We next investigated how exosomes might mediate these TGF β -dependent yet distinctive effects on fibroblasts. Our previous study demonstrated a role for exosomally expressed Betaglycan in binding TGF β 1, so we hypothesised that the heparan sulphate side chains of this transmembrane proteoglycan were involved in exosome-fibroblast interactions. We treated exosomes with heparinase-III, cleaving at the 1-4 linkages between hexosamine and glucuronic acid residues in heparan sulphate, liberating mainly disaccharides from proteoglycan protein backbones. As evidence of successful digestion, western blotting using an anti- Δ HS antibody, recognising the remaining glycan stubs attached to the protein backbone (30), reveals distinct protein bands corresponding to various exosomally expressed proteoglycan protein cores (Fig3A). Without enzyme digestion, or treatment with heat inactivated enzyme, no staining was apparent yet bands of comparable intensity were seen when staining for GAPDH or TSG101.

We next determined the impact of such digestion on exosomal-TGF β 1, revealing enzymatic treatment, followed by two ultracentrifugation washes, made no difference whatsoever in the level of exosomal-TGF β , measured by ELISA (Fig3B), confirming direct association of TGF β with the protein backbone of Betaglycan. The ability of exosomes to drive SMAD3-dependent TGF β signalling was, however, attenuated following heparinase-III digestion (Fig3C), demonstrating that whilst HS-side chains play no role in tethering TGF β to the exosome surface they are vital for signalling-competent delivery of TGF β to the target cell. Heparinase-III digested exosomes are, therefore, unable to induce fibroblast α SMA expression (Fig3D). Furthermore, fibroblasts treated with enzyme digested exosomes failed to support angiogenesis, demonstrated by total inhibition of CD31-positive structures in the vessel formation assay (Fig3D, E). An additional control involved ultracentrifugation washing (x2) of heparinase-III only, prior to addition of any pelleted material to exosomes followed by adding to fibroblasts. This revealed no enzyme-related effects, consistent with

having effectively removed trace enzyme by this washing approach (Fig3D, E). We also explored levels of growth factors present in fibroblast CM following such treatments, showing enzyme treatment of exosomes significantly diminished HGF and VEGF secretion by fibroblasts, whilst deactivated enzyme had a weaker (VEGF) or negligible (HGF) effect (Fig3F). These experiments demonstrate heparan sulphate side chains on the surface of exosomes are required for functional TGF β 1 delivery to fibroblasts, as without these side chains, differentiation into pro-angiogenic fibroblasts doesn't occur.

Exosomes activate normal prostate stroma to become disease-like, in phenotype and function.

Fresh tissue was obtained from prostate cancer patients undergoing radical retropubic prostatectomy. Tumour-associated cores were obtained from patients with pathology results indicating disease in only one side of the prostate (Fig4A, right). Additional cores from regions with no sign of macroscopic disease were also taken from the same patient (Fig 4A, left). The tissue sections were examined by an independent histopathologist, and for the example shown, classified as cancerous (Gleason score 7) or normal, respectively.

Stromal cell cultures (Fig4B) established from these specimens were positive for Vimentin, and negative for Desmin, indicating a fibroblastic rather than a smooth-muscle phenotype. Cells from the normal stroma were α SMA negative, but could be stimulated to differentiate, becoming strongly positive for α SMA following treatment with exosomes or sTGF β 1 (Fig4B, C). In contrast, cells isolated from diseased tissue were constitutively but heterogeneously positive for α SMA, exhibiting a myofibroblastic-like phenotype without requiring stimulation (Fig4B, C).

We examined soluble factors secreted by stromal cultures, both constitutively and following stimulation with exosomes or sTGF β 1. Exosome treatment stimulated elevated VEGF-A, uPA, HGF and a modest rise in FGF2 by normal stroma (Fig4D), whilst sTGF β 1 gave weaker elevation in

VEGF-A and uPA with HGF and FGF2 remaining unchanged. These are similar to the findings using the lung fibroblasts. Diseased stroma constitutively produced high levels of VEGF-A, uPA, HGF and, to some degree, FGF2 (Fig4D). Activation of normal stromal cells by exosomes, therefore, generated a myofibroblast cytoskeletal and secretory phenotype resembling those of diseased tissue.

The angiogenic function of primary stromal cells was explored using the vessel formation assay. The data show untreated normal stroma was poor at supporting formation of vessels. Activation with sTGF β 1 failed to enhance this, as staining for CD31 remained low (Fig5A, B), although there was strong elevation in α SMA expression (Fig5A, B), indicating differentiation of stroma had occurred. Exosome treatment generated extensive and particularly thick vessel-like structures (Fig5A, C), and this effect was partly attenuated by SB431542, leading to long but narrow structures (Fig5A, C). Diseased stroma was constitutively able to generate extensive, thick vessel-like structures like those achieved by exosome-stimulated normal stroma (Fig5A).

To ascertain the importance of stromal-derived factors in supporting vessel formation, we added a range of inhibitors to the system after 72h stimulation of stroma, together with HUVEC, and monitored vessel formation as above. Inhibitors included blocking antibody against VEGF-A (31) or FGF2 (32), together with isotype-matched control antibody. To inhibit uPA, we added recombinant PAI (its natural inhibitor; (33)), and to inhibit the effects of HGF we used a small molecular inhibitor of its receptor-cMet (34). Vessel length and width was quantified, revealing the pattern and magnitude of the inhibitory effects were comparable between exosome-activated normal stroma and untreated diseased stroma (Fig5D). This highlights a requirement for these myofibroblast-derived factors in this assay, and further underlines the similarities between exosome-activated normal stroma, and constitutive diseased stroma in the angiogenic influence they impart.

We next explored the ability of differentially generated primary stromal cells to promote tumour growth *in vivo*, using a xenotransplantation model system with immune deficient mice. Du145 tumour cells were administered subcutaneously, alone or together with different types of primary stromal cells, and tumour growth was measured for up to 43 days. Co-administration with normal

prostate stroma slightly accelerated tumour growth, with a mean tumour volume 1.9-fold higher at day 43 ($p<0.05$) (Fig5E). With diseased stromal cells, however, there was clear promotion of growth, with a volume that was 3.3 times larger ($p<0.001$). Even though we expected tumour cells to activate co-injected stromal cells *in situ*, we found that pre-treating the normal stroma for only 3-days with exosomes prior to grafting gave a sufficient “head-start”, and enhanced tumour growth 2.4 fold ($p<0.001$). However, 3 day pre-activation using sTGF β 1 in a similar fashion, drastically reversed this stromal influence, resulting instead in tumour retardation. These *in vivo* findings support our *in vitro*-observations and emphasise the profound differences between exosomally-generated and sTGF β -generated myofibroblasts.

Cancer exosomes are required to activate tumor-promoting stroma *in vivo*.

Inhibition of exosome secretion is an attractive approach to assess their influence *in vivo*. Targeting Rab27a; a regulator of exosome secretion, can generate cells selectively deficient in exosome secretion (35). We generated stable transfections of Du145 cells bearing empty vector alone (pEF6) or with an anti-Rab27a ribozyme transgene. Successful and selective Rab27a knockdown (Rab27a^{KD}) was confirmed by western blotting and qPCR (Fig6A). Confocal microscopy showed early endosomes, stained with anti-EEA1 antibody, were not affected, but late endosomes/lysosomes accumulated LAMP-2 in Rab27a^{KD} cells (Fig6B). Flow cytometric analysis (Fig6C), also showed intracellular accumulation of LAMP-2 and CD9, but not EEA-1 following Rab27a^{KD} (Fig6B, C). These data are consistent with having targeted late, and not early endosomal compartments and are similar to the findings previously reported (35). Importantly for subsequent *in vivo* studies, the cells remained comparably viable, with identical proliferation rates *in vitro* (Fig6D).

Secretion of soluble factors by Du145 cells, showed comparable levels of uPA or VEGF-A (Fig6E), but decreased TGF β 1 of around 20% in the Rab27a^{KD} cells. TGF β 1 transcription however was unaltered, indicating this was not an off target effect of Rab27a knockdown (Fig6F). Depletion of exosomes from the media, by ultracentrifugation, revealed a comparable TGF β 1 loss of around

15-20% (Fig6G). The impact of Rab27a^{KD} on total secreted TGFβ1 is likely due, therefore, to reduced exosomal-TGFβ1. The exosome-containing 100,000g pellets generated from control or Rab27a^{KD} culture medium showed 70-95% reduction in the exosome-associated proteins TSG101, ALIX and Lamp2 (Fig6H). Furthermore, an in-house ELISA was used to measure material within the 100,000g pellets. This was based on immobilised anti-CD9, to capture exosomes, and anti-MHC Class-I-biotinylated antibody as a means of demonstrating CD9/Class-I co-localisation in microplate wells. A standard curve, of sucrose-cushion purified Du145 exosomes was used as a means of quantitation. The assay revealed an 82% decrease in exosomes secreted by Rab27a^{KD} cells (Fig6I). Together, the data support successful Rab27a knockdown in Du145, with a concomitant selective impairment of exosome secretion.

In functional terms, culture medium from control Du145 cells drove stromal differentiation to an αSMA-positive myofibroblast phenotype, that supported formation of HUVEC vessel structures *in vitro* (Fig7A, B). Medium from Rab27a^{KD} cells failed to trigger differentiation and subsequently we saw a lack of angiogenic effect. Fibroblasts secreted significantly lower levels of uPA, VEGF-A, HGF and FGF2 when treated with Rab27a^{KD} culture medium (Fig7C). Because the full impact of Rab27a^{KD} on the cancer cell secretome remains incompletely explored, it was possible that the effects seen here are due to inadvertent loss of secreted factors by these tumour cells, rather than due to exosome-deficiencies. To address this, similar experiments were performed comparing Du145 CM with that of CM-following exosome depletion by ultracentrifugation. Such depletion abrogated the ability of Du145 CM to drive stromal differentiation and vessel formation. Whilst in contrast, reconstituting the exosome pellet in the original volume of fresh medium is sufficient to fully restore this functional property. Again, the data point to exosomes, and not soluble factors within the cancer cell secretome as the key driver of pro-angiogenic differentiation of stroma (Supplemental FigS2).

We next investigated the function of exosome-deficient tumour cells *in vivo*. Control or Rab27a^{KD} Du145 cells were administered to immune deficient mice alone or together with normal stromal fibroblasts, and tumour growth monitored for 32 days thereafter. Control DU145 cells showed accelerated growth in the presence of normal fibroblasts (Fig7D). In marked contrast, Rab27a^{KD}

Du145 cells showed no growth enhancement whatsoever in the presence of fibroblasts. This lack of stimulated growth upon stromal co-administration was highly significant ($p < 0.001$) in this experiment, and the findings are consistent with failure of Rab27a^{KD} Du145 cells to achieve activation of stroma *in situ*.

Discussion

We show a critical role for TGF β bearing cancer exosomes in generating tumour-promoting stroma. Exosomes achieve these effects through driving fibroblast differentiation to a myofibroblast-like phenotype that supports angiogenesis *in vitro*, and tumour growth *in vivo*. These exosome effects are TGF β 1-dependent, yet generate a myofibroblast that is absolutely distinct from that generated using sTGF β 1. The study emphasises that the biophysical format of this particular growth factor is crucial in directing the cellular response and subsequent pathological changes that arise.

TGF β 1 is documented as the central factor implicated in altering stroma at diverse sites of carcinoma (2). Our report doesn't dispute such findings, as TGF β 1 drives the principal effects we demonstrate with exosomes, however, stromal-myofibroblasts generated by sTGF β 1 or exosomal-TGF β 1 are clearly different. Whilst cytoskeletal changes do not well discriminate this, the analysis of secreted factors reveal major differences in levels of multiple angiogenic factors, including uPA, HGF, VEGF-A and FGF2. Using primary prostate stromal cells, the ability of exosomes alone to drive normal stroma to a pro-angiogenic disease-like phenotype is of particular interest; and is not possible using sTGF β 1. Whilst forming only ~20% of the total TGF β 1 present in the tumour cell secretome exosomal, not soluble, TGF β 1 dictates the nature of stromal differentiation that occurs. Rab27a knockdown, or ultracentrifugation-based depletion of exosomes, renders tumour cell conditioned media unable to trigger fibroblast differentiation. Such effects are recapitulated *in vivo*, where defective exosome secretion render tumour cells impervious to the otherwise positive growth promoting influence of stroma. A degree of caution is required here, however, as currently we do not

know if other factors are inadvertently abrogated by the Rab27a targeting approach in these cells. Nevertheless, adding purified exosomes, or removing exosomes by physical means or by modulating their secretion, gives a consistent effect with respect to stromal alterations *in vitro*, and to date the Rab27a targeting approach is among the most appropriate tools available to aid the study of exosome effects *in vivo*.

A key distinguishing feature of soluble vs exosomal-TGF β 1 delivery is the role of the TGF β -RIII (Betaglycan), and possibly other membrane-associated proteoglycans. Betaglycan is known to tether TGF β 1 to the exosome outer surface (12), and hence exosomes deliver Betaglycan-associated TGF β 1 to recipient stromal cells. As well as acting as a ligand-sink for TGF β 1, Betaglycan plays established functions in co-reception, controlling hand-over of TGF β 1 to the type-I and II receptors thereby facilitating signalling (36). We show that digestion of HS-side chains from exosomes, whilst having no impact on the delivered TGF β 1 dose, severely attenuates exosome induced SMAD3-dependent TGF β 1 signalling, resulting in failed stromal differentiation and subsequent loss of angiogenic response. Therefore, delivery of TGF β in the context of intact HS-side chains is essential for the effects we observe, and is a critical aspect that distinguishes soluble from vesicular delivery of TGF β .

In conclusion, we show the essential role of extracellular vesicles in communication between cancer cells and surrounding stroma and demonstrate that exosomal-TGF β 1 is required for directing stromal differentiation to a pro-tumourigenic phenotype. Pathways therefore related to exosome-biogenesis/secretion, or molecules like HSPGs directing TGF β -delivery or other physical interactions between exosomes and stromal cells, are highlighted as targets for future therapeutic approaches aimed at abrogating this critical process in tumour progression.

Materials and Methods

Cell culture.

Prostatic stromal cells, from four patients, were obtained from the Wales Cancer Bank. Cells were isolated from radical prostatectomy cores by homogenization and 15h collagenase I digestion (200U/ml, Lonza). Cells were cultured in Stromal Cell Basal Medium (Lonza) then, following first harvest, DMEM/F12 media (Lonza). Cultures were confirmed free of epithelial cells by immunofluorescence staining for Cytokeratin, and used at passage 2-4. Normal lung fibroblasts (Coriell Institute for Medical Research) were maintained in DMEM/F12. Primary HUVEC (Lonza), were cultured in Endothelial Growth Media (EGM)-2 (Lonza).

Exosome Isolation.

Du145 prostate cancer cells (ATCC, Teddington, UK), were grown in Integra bioreactor flasks (37). Exosomes were purified from cell conditioned media, based on their flotation properties (38), using the sucrose cushion method (39). Exosomes were resuspended in PBS, quantified using the BCA-protein assay (Pierce/Thermo), and stored at -80°C. Typical exosome characterisation (12, 39) is shown in FigS1. Unless stated otherwise, exosomes were used at 200 µg/ml which gives a TGFβ dose equivalent to 1.5 ng/ml (12).

Protein array & ELISA.

Stimulated cells were incubated with Golgi-Stop™ and Golgi-Plug™ (Becton Dickinson) 18h prior to lysis, to prevent cytokine secretion. Samples were corrected for protein differences and analysed using an angiogenesis protein array kit (R&D Systems). Quantitation of secreted proteins was performed using the DuoSet ELISA Development System; (R&D Systems). An exosome ELISA was developed in-house, using anti-CD9 antibody (R&D systems) coated ELISA strips (Greiner), BSA to block (R&D Systems), detected with anti-MHC Class-I biotin conjugate (Leinco Technologies) and streptavidin-Europium (PerkinElmer), using the Delphia assay and wash buffer

system (Perkin Elmer). ELISA strips were read on a Wallac Victor² 1420 (PerkinElmer) and counts compared to sucrose-cushion purified DU145 exosomes as standards.

Quantitative RT-PCR.

Extraction of cellular RNA and quantitative RT-PCR was performed as previously described (12).

Immuno-fluorescent microscopy and time-resolved fluorimetry.

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 (1µg/ml, Santa Cruz) for 1h. For time-resolved fluorimetry, detection was achieved using an anti-IgG-biotin conjugate (GE Healthcare), and europium-conjugated streptavidin (PerkinElmer) as described previously (12). This approach was used to examine heparinase-III digestion of exosomes, staining post-digest with anti-ΔHS (F69-3G10, Seikagaku Biobusiness Corporation) (30). For epifluorescence or confocal microscopy, primary antibodies were detected using goat anti-mouse IgG Fab' Alexa488 conjugated (Invitrogen) and nuclei stained with DAPI. Confocal images were acquired on a Leica SP5 confocal laser scanning microscope (Milton Keynes, UK) equipped with Argon (Alexa488) and Blue Diode (Hoechst33342) lasers, a 63x/1.4 numerical aperture oil-immersion objective and Leica LAS AF software. Shown are representative figures from z-axis sections overlaid to generate maximum projection images.

Analysis of cell proliferation and viability.

Endothelial cell proliferation was assessed by thymidine incorporation assay as described (40), and viability quantified using Guava ViaCountTM flow cytometric system (Millipore). Manual counts with Trypan blue exclusion was performed in quadruplicate for Rab27a^{KD} DU145 cells.

Endothelial vessel-formation assay.

A method similar to Sheldon *et al* (24) was used to assess formation of endothelial vessel-like structures, on fibroblast monolayers pre-treated as specified. Endothelial cells, starved of growth factors for 24h, were added to fibroblasts (5:1 fibroblasts:endothelial cells), in a 1:1 volume of DMEM/F12:EGM2, in the absence of exogenous factors. Co-cultures were maintained for 5-7 days then immuno-fluorescently stained for CD31. ImageJ software (National Institutes of Health, USA), was used to quantify vessel length and width (≥ 4 fields of view), for each of three replicate wells. Data shown is representative of 3 independent experiments using lung fibroblasts. Prostate stroma experiments were conducted using matched cells from 4 patients (each repeated at least twice). Data shown are representative of donor-1, used for *in vivo* experiments. Where stated, growth factors were inhibited using VEGF or FGF neutralizing antibody (2 μ g/ml, R&D Systems), uPA inhibitor rhPAI-1 (2 μ g/ml, Sigma), and MET kinase inhibitor (2 μ g/ml, Calbiochem) to attenuate HGF receptor-cMet activation (31-34). Inhibitor concentrations were determined based on the manufacturers neutralization dose (ND₅₀).

Xenotransplanted tumour growth.

Suspensions (100 μ l) containing 300,000 Du145 cells and 75,000 primary stromal cells, in 3mg/ml Matrigel were sub-cutaneously injected into the flanks of 4 – 6 week old athymic nude mice (CD-1; Charles River Laboratories, UK). Tumour size, 4 mice per group, was measured weekly. Animals were treated humanely in accordance with UK Home Office and the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines. At the experimental end point, or severity limits, animals were dispatched humanely by schedule 1. Tumour volume was calculated; tumor volume = $0.523 \times \text{width}^2 \times \text{length}$. For Rab27a^{KD} details are identical, except we administered twice the cell numbers, with 2 tumours per mouse (opposite flanks).

Rab27a knockdown.

Ribozyme transgenes specifically targeting human Rab27a were designed using Zuker's RNA mFold programme (41) and synthesized by Sigma (Sense 5'–

CTGCAGCACTAGTTTCAAAGTAGGGCTGATGAGTCCGTGAGGA–3’; Antisense 5’–
ACTAGTGAAAGAGGAG-GAAGCCATAGCACTCGCAGAGAAATATGGAATTTTCGTCCT-
CACGGAC–3’). Ribozyme transgenes were cloned into mammalian pEF6/V5-His TOPO plasmids
(Invitrogen), amplified in chemically competent *E. coli*, and plasmid uptake confirmed by resistance
to ampicillin (100µg/ml). Correct transgene orientation was confirmed by PCR, prior to overnight
colony expansion. Purified plasmid DNA (2.5µg) was transfected into 1x10⁶ Du145 cells by
electroporation (Flowgene, UK). Plasmid-positive cells were selected based on blastocycin (Sigma)
resistance (42).

Electrophoresis and immuno-blotting.

Rab27a knockdown was confirmed by immuno-blotting whole cell lysates with anti-Rab27a
(Santa Cruz) or anti-GAPDH (BioChain, Abingdon, UK) antibodies. The impact on exosome
secretion was examined by immuno-blotting of pellets obtained after 100,000g ultracentrifugation,
staining for TSG101, ALIX and LAMP-2 (Santa Cruz). Blotting was performed as previously
described (12). For HSPG analysis, immuno-blotting was performed on exosomes (20µg/lane)
following heparinase-III digestion, and HS-stubs detected using 1µg/ml of anti-ΔHS antibody (F69-
3G10; Seikagaku Biobusiness Corporation) (30).

TGFβ reporter assay.

TGFβ signalling was assessed by transfecting fibroblasts with the SMAD3-responsive promoter
construct (SBE)₄-Lux, as previously described (43). After 24h, cells were washed with PBS, and
treated with serum-free medium containing exosomes or rhTGF-β1 for 6h. Luciferase activity was
measured using the Dual-Glo luciferase activity kit (Promega).

Flow cytometric analysis.

Du145 cells were fixed/permeabilised, stained with monoclonal antibodies against EEA1 (Becton Dickinson), LAMP2 (Santa Cruz), or CD9 (R&D Systems), and analyzed on a FACSCanto cytometer running FACSDiva V6.1.2 software (Becton Dickinson).

Statistical analysis.

Statistic 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 migration and *in vivo* 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 \pm SE, from one representative experiment of at least three similar experiments, unless stated otherwise.

Conflict of Interest

The authors declare no conflict of interest.

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References

1. Yanagisawa N, Li R, Rowley D, Liu H, Kadmon D, Miles BJ, et al. Stromogenic prostatic carcinoma pattern (carcinomas with reactive stromal grade 3) in needle biopsies predicts biochemical recurrence-free survival in patients after radical prostatectomy. *Hum Pathol* 2007;38(11):1611-20.
2. Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci* 2010 November 16, 2010;107(46):20009-14.
3. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium. *Cancer Res* 1999 October 1, 1999;59(19):5002-11.
4. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of Transforming Growth Factor- β Activity Decreases Angiogenesis in a Human Prostate Cancer-reactive Stroma Xenograft Model. *Cancer Res* 2002 November 1, 2002;62(21):6021-5.
5. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive Stroma in Human Prostate Cancer. *Clin Cancer Res* 2002 September 1, 2002;8(9):2912-23.
6. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med* 2009;15(1):68-74.
7. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. *Eur J Surg Oncol* 2012(0).
8. Mesker WE, Liefers G-J, Junggeburst JMC, van Pelt GW, Alberici P, Kuppen PJK, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I–II colon cancer patients. *Cell Oncol* 2009;31(3):169-78.
9. Verona EV, Elkahoul AG, Yang J, Bandyopadhyay A, Yeh I-T, Sun L-Z. Transforming Growth Factor- β Signaling in Prostate Stromal Cells Supports Prostate Carcinoma Growth by Up-regulating Stromal Genes Related to Tissue Remodeling. *Cancer Res* 2007 June 15, 2007;67(12):5737-46.
10. Renzoni E, Abraham D, Howat S, Shi-Wen X, Sestini P, Bou-Gharios G, et al. Gene expression profiling reveals novel TGFbeta targets in adult lung fibroblasts. *Respir Res* 2004;5(1):24.
11. Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmoulière A, Varga J, et al. Recent Developments in Myofibroblast Biology: Paradigms for Connective Tissue Remodeling. *Am J Pathol* 2012;180(4):1340-55.
12. Webber J, Steadman R, Tabi Z, Mason MD, Clayton A. Cancer exosomes activate fibroblast to myofibroblast differentiation in a TGF β dependent manner. *Cancer Res* 2010;70(23):9621-30.
13. Gu J, Qian H, Shen L, Zhang X, Zhu W, Huang L, et al. Gastric Cancer Exosomes Trigger Differentiation of Umbilical Cord Derived Mesenchymal Stem Cells to Carcinoma-Associated Fibroblasts through TGF- β /Smad Pathway. *PLoS One*. [doi:10.1371/journal.pone.0052465]. 2012;7(12):e52465.
14. Raposo G, Nijman HW, Stoorvogel W, Leijendekker R, Harding CV, Melief CJM, et al. B Lymphocytes secrete Antigen-presenting Vesicles. *J Exp Med* 1996;183:1161-72.

15. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science* 2008 February 29; 319(5867):1244-7.
16. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Exp Rev Proteomics* 2009; 6(3):267-83.
17. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; 9:654 - 9.
18. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009; 9(8):581-93.
19. Junji W, Hideya O, Hiroyuki S, Akio Y, Shuntaro N, Takashi M, et al. Surface-bound TGF- β on Effusion-derived Exosomes Participates in Maintenance of Number and Suppressive Function of Regulatory T-Cells in Malignant Effusions. *Anticancer Res* 2010 September 2010; 30(9):3747-57.
20. Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z. Human Tumor-Derived Exosomes Down-Modulate NKG2D Expression. *J Immunol* 2008 June 1, 2008; 180(11):7249-58.
21. Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. Human tumour-derived exosomes selectively impair lymphocyte responses to Interleukin-2. *Cancer Res* 2007; 67(15):7458-66.
22. Hood JL, San RS, Wickline SA. Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis. *Cancer Res* 2011 June 1, 2011; 71(11):3792-801.
23. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 2012; 18(6):883-91.
24. Sheldon H, Heikamp E, Turley H, Dragovic R, Thomas P, Oon CE, et al. New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* 2010 September 30, 2010; 116(13):2385-94.
25. Gesierich S, Berezovskiy I, Ryschich E, Zoller M. Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. *Cancer Res* 2006; 66(14):7083-94.
26. Hood JL, Pan H, Lanza GM, Wickline SA. Paracrine induction of endothelium by tumor exosomes. *Lab Invest* 2009; 89(11):1317-28.
27. Nazarenko I, Rana S, Baumann A, McAlear J, Hellwig A, Trendelenburg M, et al. Cell Surface Tetraspanin Tspan8 Contributes to Molecular Pathways of Exosome-Induced Endothelial Cell Activation. *Cancer Res* 2010 February 15, 2010; 70(4):1668-78.
28. Richman PI, Tilly R, Jass JR, Bodmer WF. Colonic pericrypt sheath cells: characterisation of cell type with new monoclonal antibody. *J Clin Pathol* 1987; 40(6):593-600.
29. Hetheridge C, G. Mavria, Mellor. H. Uses of the in vitro endothelial-fibroblast organotypic co-culture assay in angiogenesis research. *Biochem Soc Trans* 2011; 39:1597-600.
30. Lories V, Cassiman JJ, Van den Berghe H, David G. Multiple distinct membrane heparan sulfate proteoglycans in human lung fibroblasts. *Journal of Biological Chemistry* 1989 April 25, 1989; 264(12):7009-16.
31. Hashizume H, Falcon BL, Kuroda T, Baluk P, Coxon A, Yu D, et al. Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. *Cancer Res* 2010 Mar 15; 70(6):2213-23.

32. Jones JL, Anderson JM, Phuah CL, Fox EJ, Selmaj K, Margolin D, et al. Improvement in disability after alemtuzumab treatment of multiple sclerosis is associated with neuroprotective autoimmunity. *Brain* 2010 Aug;133(Pt 8):2232-47.
33. Stefansson S, McMahon GA, Petitsclerc E, Lawrence DA. Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. *Curr Pharm Des* 2003;9(19):1545-64.
34. Ma PC, Jagadeeswaran R, Jagadeesh S, Tretiakova MS, Nallasura V, Fox EA, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. *Cancer Res* 2005 Feb 15;65(4):1479-88.
35. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 2010;12(1):19-30.
36. Massague J. Receptors for the TGF-beta family. *Cell* 1992 Jun 26;69(7):1067-70.
37. Mitchell JP, Court J, Mason MD, Tabi Z, Clayton A. Increased exosome production from tumour cell cultures using the Integra CELLLine Culture System. *J Immunol Methods* 2008;335(1-2):98-105.
38. Lamparski H, Metha-Damani A, Yao J, Patel S, Hsu D, Ruegg C, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods* 2002 December 15 2002;270(2):211-26.
39. Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, et al. Proteomic analysis of bladder cancer exosomes. *Molecular & Cellular Proteomics* 2010 March 11, 2010;6:1324-38.
40. Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z. Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production. *J Immunol* 2011 July 15, 2011;187(2):676-83.
41. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003 July 1, 2003;31(13):3406-15.
42. Jiang WG, Davies G, Martin TA, Kynaston H, Mason MD, Fodstad O. Com-1/p8 acts as a putative tumour suppressor in prostate cancer. *Int J Mol Med* 2006;18(5):981-6.
43. Webber J, Meran S, Steadman R, Phillips A. Hyaluronan orchestrates transforming growth factor-beta1-dependent maintenance of myofibroblast phenotype. *J Biol Chem* 2009 Apr 3;284(14):9083-92.

Figure Legends

Figure1. Cancer exosomes generate a distinctive myofibroblast phenotype. (A) Growth arrested (72h) lung fibroblasts were stimulated for 72h with exosomes (exo), exosomes with the ALK5 inhibitor SB431542 (exo SB) or as indicated, and expression of α SMA or other markers were measured by time resolved fluorimetry (TRF) (mean \pm SE, n=4). (B) Scanned image of an angiogenesis protein array performed on fibroblast cell lysates normalized for protein, following 72h stimulation with sTGF β 1 (at 1.5ng/ml) or matched dose of exosomal-TGF β . (C) Densitometry-based comparison of these treatments depicting proteins differentially expressed by exosomes vs TGF β treatment, representative of 3 such experiments. (D) ELISA of fibroblast conditioned media collected 72h post stimulation with sTGF β 1 (white bars at 0, 0.375, 0.75, 1.5, 3 ng/ml) or matched doses of exosomal-TGF β (black bars) (mean \pm SE, n=3). (E) Quantitative PCR analysis of fibroblast mRNA at 72h following treatment with exosomes with or without SB431542 or as indicated, showing relative expression compared to untreated fibroblasts (mean \pm SE, n=3). (F) As for (B) and (C), an angiogenesis protein array was performed, comparing fibroblasts stimulated with exosomes in the presence or absence of SB431542, revealing the TGF β sensitive nature of exosome-driven changes.

Figure2. Exosome-generated myofibroblasts are pro-angiogenic. (A) Conditioned media from fibroblasts stimulated for 72h with exosomes or as indicated, were added to HUVEC monolayers for 24h, and expression of CD31 and other factors measured by time resolved fluorimetry (TRF) (Mean \pm SE, n=3). (B) HUVEC treated as indicated were pulsed with 3H-Thymidine for the last 18h of a 72h incubation, and as a measure of proliferation, 3H-Thymidine uptake was measured (mean \pm SE, n=4). (C) HUVEC viability was assessed at 72h using Guava ViaCount (Mean \pm SE, n=3). (D) A HUVEC monolayer scratch assay was performed in the presence of conditioned media from fibroblasts previously treated with exosomes or as indicated. The scratch width was measured at multiple points along the scratch (examples in black line (D), scale bar= 100 μ m). Line graph depicts measurements over a 24h period. (E) Fibroblasts were stimulated with exosomes or as indicated for 72h, and HUVEC were added. After 6d of co-culture, cells were fixed, and examined for CD31

positive structures by immuno-fluorescence. Typical images show differences in vessel-like structures across the treatments (scale bar=100 μ m). Bar graphs depict the vessel width and vessel length, from 4 fields of view for each of 3 replicates (Mean \pm SE). Representative of three such experiments.

Figure3. Cancer exosomes require GAG chains for TGF β delivery to fibroblasts. (A) Du145 exosomes were untreated (No enzyme), treated with active heparinase-III for 1h, or with heat inactivated heparinase-III. Treated exosomes (20 μ g/lane) were examined by SDS-PAGE/Western blot, staining with the anti- Δ HS antibody, or anti-TSG101 or anti-GAPDH as indicated. (B) Similarly treated exosomes were subjected to 2x ultracentrifugation washes, to remove solubilised material, and analysed using a TGF β 1 ELISA (Mean \pm SE, n=3). (C) Similarly prepared exosomes were added to lung fibroblasts for 6h and SMAD3-dependent TGF β signalling was compared to that of untreated fibroblasts, measured by luciferase-based reporter assay (Mean \pm SE, n=4). (D) Fibroblasts treated with washed, heparinase-III digested exosomes, or as specified, were stained at day-3 for α SMA expression (left), or HUVEC added for a further 7 days, and stained for CD31-positive structures (right). Typical images show differences in myofibroblast differentiation and vessel-like structures across the treatments (scale bar=100 μ m). (E) Bar graphs depict the average vessel width and length, from 4 fields of view for each of 3 replicates. Representative of three such experiments. In (D) and (E), an additional control where active enzyme was subject to ultracentrifugation washes (x2) prior to adding any pelleted material to exosomes, and then to fibroblasts, to account for potential trace enzyme in the system impacting fibroblasts directly. (F) Cell media collected from similarly treated fibroblasts at 72h, were analysed by ELISA for levels of HGF (left) and VEGF-A (right) (Mean \pm SE, n=3).

Figure4. Cancer exosomes can generate a diseased-like stromal phenotype. (A) Biopsies taken from a radical prostatectomy of a prostate cancer patient, from a non-involved and from a diseased region of the prostate. H&E stain reveals gross differences in tissue architecture consistent with normal (left) or disease with high Gleason score (right). (B) Cultures established from these tissues

were analyzed by immuno-fluorescence for the myofibroblast marker α SMA, with either no treatment, or following exosome or sTGF β 1 treatment for 72h (scale bar=100 μ m). (C) Parallel experiments were used to examine relative changes in α SMA, Vimentin or Desmin expression by time resolved fluorimetry (TRF) (mean \pm SE, n=3). (D) Conditioned media from these primary stromal cells, collected at 72h following stimulation as indicated, were examined by ELISA for VEGF-A, uPA, HGF and FGF2. (Mean \pm SE, n=3). The data are representative of isolates from four patients.

Figure5. Normal primary stromal cells become pro-angiogenic following cancer exosome

treatment. (A) Primary stromal cells, taken from histologically confirmed normal or cancerous tissue, were stimulated at passage 3, with exosomes or as indicated, and at 72h HUVEC cells were added. After 6 days co-culture, formation of CD31-positive vessel-like structures was assessed by immuno-fluorescence microscopy. Parallel wells were setup to examine α SMA expression by stromal cells. (B) The relative levels of α SMA or CD31 expression were measured by time resolved fluorimetry (TRF) (Mean \pm SE, n=3). (C) Measurements of vessel width (left) or vessel length (right) were taken from 4 fields of view for each of 3 replicates (mean \pm SE). (D) In a similar assay, inhibitors against VEGF, FGF, uPA (its inhibitor; PAI-I) or HGF (inhibitor of its receptor; cMET) were added together with HUVEC to exosome-activated normal stroma or untreated diseased stroma. Graphs depict vessel width or length, taken from 4 fields of view for each of 3 replicates (mean \pm SE). (E) Du145 tumour cells (3×10^5) were administered subcutaneously to immune deficient mice in the absence or presence of human prostatic stromal cells (7.5×10^4), and tumour volume was measured at intervals up to 43 days (mean \pm SE, n=4/group). Normal stromal cells were either untreated, or pre-treated for 72h with exosomes or sTGF β 1, prior to administering. As a comparator, untreated diseased stroma (from the same patient) was used. Stroma in the absence of tumour cells did not grow a measurable volume (not shown).

Figure6. Inhibition of Du145 cell exosome secretion, by targeting Rab27a. (A) Cell lysates of control or Rab27a^{KD} cells were examined by western blot, staining for Rab27a or GAPDH as indicated. Densitometric analysis of blots from three independent experiments (mean \pm SE, n=3),

confirms successful Rab27a protein knockdown. Confirmation of selective decrease in Rab27a-mRNA (line graph) assessed by qPCR. (B) Confocal images of control or Rab27a^{KD} cells, with immuno-fluorescent staining for the early endosomal marker EEA-1 or for LAMP-2 (scale bar= 25µm) (C) Flow cytometric analysis of fixed vs fix/permeabilised cells stained for EEA1, LAMP-2 or CD9, comparing control and Rab27a^{KD} cells (MFI=median fluorescence intensity, based on 30,000 events). (D) Cell and viability counts, by trypan blue exclusion performed serially for 2 weeks comparing parallel cultures of control or Rab27a^{KD} cells (mean±SE, n=4). (E) Cell conditioned media from control and Rab27a^{KD} cells (corrected for cell number), was analyzed by ELISA for secreted uPA, VEGF-A and TGFβ1, with data normalized for control cells set to 100% (mean±SE, n=4). (F) Q-PCR for TGFβ mRNA isolated from control and Rab27a^{KD} cells, showing relative expression normalized to control cells (mean±SE, n=4). (G) Du145 culture media was spun at 100,000g/1h, and the media pre- and post- centrifugation analyzed for TGFβ1 by ELISA, with data normalized for control cells set to 100% (mean±SE, n=4). (H) Western blots, of material which pellets at 100,000g, from control or Rab27a^{KD} cells, normalized for cell number, staining for TSG101, ALIX and LAMP2, representative of 3 such experiments (bar graphs shows densitometric analysis, mean±SE, n=3) (I) Resuspended 100,000g pellets, obtained from control or Rab27a^{KD} cells, corrected for cell number, were analyzed by an in house exosome-ELISA based on capturing with anti-CD9 antibodies and detecting using anti-MHC Class-I antibodies, (mean±SE, n=4).

Figure7. Exosome secretion is required for recruitment of pro-angiogenic and tumor

promoting stroma. (A) Conditioned media (CM) from control or Rab27a^{KD} cells were added to growth arrested normal stromal cells, and after 72h cells were either fixed and stained for αSMA, or HUVEC added for a further 5 days, followed by fixation and staining for CD31. Untreated stromal cells were included as a negative control (scale bar= 100µm). (B) Measurements of vessel width or vessel length were taken from 4 fields of view for each of 3 replicates (mean±SE). (C) Soluble factors were measured in stromal-cell conditioned media by ELISA, following 72h treatment with CM from control or Rab27a^{KD} cells (mean±SE, n=3). (D) Control or Rab27a^{KD} DU145 cells (6x10⁵) were added in the absence or presence (+Fb) of normal stromal cells (1.5x10⁵), subcutaneously to immune deficient mice, and tumour volume measured at intervals up to 32 days (mean±SE, n=8/group).

Legend for Supplemental Fig s1. Exosome Characterisation. (A). A continuous sucrose gradient was used to determine the density of vesicles secreted by the Du145 prostate cancer cell line. The refractive index of collected fractions was measured, and the density calculated as described (39). After washing by ultracentrifugation, fractions were immobilised to protein-binding ELISA-plates, and stained with anti CD9, CD63 or isotype control antibodies, using an indirect staining protocol, with a biotinylated secondary antibody. Signal intensity was determined by measuring the binding of streptavidin-Europium conjugate, by time resolved fluorimetry (TRF). (B) Based on this flotation characteristic, exosome were isolated by a simplified sucrose cushion-approach throughout the study, and such purifications exhibited characteristic enrichment of certain exosomal-makers compared to cell lysates, demonstrated by western blot (10 μ g protein / lane) and as a measure of purity, Calnexin is shown poorly detected in exosomes. (C) Size distribution histogram of sucrose cushion purified exosomes, measured by Nano-particle tracking analysis, revealing a population of small particles with a mode of 115nm. Shown are the mean of 6 replicates (grey line) with \pm SE- depicted by the dotted black lines (C, inset) presence of vesicular structures by TEM (D). Sucrose cushion purified exosomes, immobilised to protein-binding ELISA plates (at a dose of 1, 0.2 or 0 μ g/well), and stained for classical exosomal-surface markers as indicated using the same method as described for (A) above. Bars show mean \pm SE of duplicates.

Legend for Supplemental Fig s2. Exosome secretion is required for differentiation of pro-angiogenic stroma. (A) The concentration of nano-particles was determined by nano-particle tracking analysis, on conditioned media (CM) from Du145 cells, before (Control) and following ultracentrifugation at 100,000g 1h (Exo-depleted CM). The exosome-containing pellet was resuspended in the original volume, and analysed again (Exo-pellet Reconstituted) (Mean \pm SE, n=6). (B) Stromal cells were left untreated or treated with Du145 CM, exosome-depleted CM or reconstituted pellet (in the original volume of fresh medium). At 72h stromal differentiation was assessed examining expression of α SMA. HUVEC were then added to parallel wells, for an additional 5 days, at which point formation of vessels was examined by staining for CD31 (scale bar= 100 μ m).