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Supplemental Information

Tumor-Derived Extracellular Vesicles Require β1 Integrins to Promote Anchorage-Independent Growth

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SUPPLEMENTARY INFORMATION

TRANSPARENT METHODS

Cell lines and transfectants

PC3 PrCa cells (from a male patient) were maintained as described previously (DeRita et al., 2017). Stably transfected PC3 cells with shRNA to β1 ("shβ1") and mock vector-transfected ("mock") were generated as described previously (Goel et al., 2010). PC3 shβ1 and mock cells were further transfected with pECE vector (used as negative control) and pECE-chicken-β1 (full length chicken β1) (Hayashi et al., 1990) using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) at a ratio of 3 μL Lipofectamine/μg of DNA. After 48 hours, the transiently transfected cells were processed for cell surface expression of chicken β1 using FACS analysis with the CSAT Ab specific to chicken β1 (10 μg/mL) or mouse IgG (negative control). Samples were then incubated with an Alexa Fluor 488 goat anti-mouse secondary Ab (20 μg/mL), washed in PBS (3X) and the data were analyzed using the BD Celesta flow cytometer (BD Biosciences).

Animals

All male TRAMP, $\beta1^{pc-/-}$ /TRAMP and Wild-Type mice were generated as described earlier (Goel et al., 2013). For TRAMP: n = 6; for Wild-Type: n = 6; for $\beta1^{pc-/-}$ /TRAMP: n = 8. No female mice were analyzed in this study. Care of animals was in compliance with standards established by the office of laboratory animal welfare, Department of Health and Human Services at NIH. Experimental protocols were approved by the Institutional Animal Care and Use Committee.

Immunoblotting

Immunoblotting of EV and EV lysates were performed as reported earlier (Sayeed et al., 2013; Trerotola et al., 2015). In brief, we separated sample lysates on sodium dodecyl sulfate PAGE, and transferred to 0.45 μm polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 5% milk and incubated with primary and secondary antibodies.

Antibodies (Ab)

Blocked membranes were probed with either rabbit polyclonal antibodies (pAbs) to human and mouse calnexin (sc713), human and mouse c-Src (sc18), human and mouse FAK (sc558), human and mouse AKT (sc8312), and human and mouse ERK1/2 (sc292838) from Santa Cruz, human and mouse α5 integrin (#4705) from Cell Signaling, rabbit monoclonal Abs (mAbs) to human and mouse TSG101 (ab125011) and mouse CD63 (ab193349) from Abcam, mouse mAbs to human CD81 (ab23505) and human CD63 (ab8219) from Abcam, human and mouse β1 integrin (C-18) from BD Pharmingen, chicken β1 integrin (CSAT) from DSHB of University of Iowa, and human CD9 (sc13118) from Santa Cruz, or a rat mAb to mouse CD9 (Santa Cruz sc18869).

EV isolation

EV isolation from cells in culture was performed using differential ultracentrifugation (100,000 x g), as described previously (DeRita et al., 2017; Fedele et al., 2015). At 20 weeks of age, when palpable tumors have formed, intracardiac blood withdrawal from animal subjects and EV isolation were performed as previously described via PEG precipitation (Exoquick™) (DeRita et al., 2017), followed by density gradient separation (see next paragraph).

Density gradient isolation of small (sEVs)

The resulting pellet from 500 μL of mouse plasma or 10 confluent 150 mm dishes of PC3 cells after either ultracentrifugation or PEG precipitation was resuspended in 100 μl PBS and then subjected to density gradient separation to isolate the sEVs from any non-vesicular material co-precipitated during ultracentrifugation or PEG precipitation. We used a modified version of the

protocol described by Kowal et al (Kowal et al., 2016). In short, 100 (or up to 300 µl if pooling EV samples from the same condition) of the EV suspension was mixed with 500-700 µl tris-sucrose buffer (total 800 µl) (Kowal et al., 2016). This mixture was then combined with 800 µl 60% stock Iodixanol solution (Sigma) to make a 30% solution. On top of this 700 µl of 20%, then 700 µl 10% iodixanol solutions were carefully layered (Iodixanol solutions were diluted with the tris-sucrose buffer) to a total volume of 3 mL. The discontinuous gradient was then ultracentrifuged at 350,000 x g for 1 hour at 4 °C using the sw55Ti rotor (Beckman). Then, ten 260 µl fractions were taken sequentially from the top. The density of each fraction was measured (DeRita et al., 2017; Singh et al., 2016). To remove sEVs from iodixanol, each fraction was then subjected to 100,000 x g spin, resuspension in PBS, another 100,000 x g spin, and final resuspension in 75 µl PBS. These samples were stored at -80 °C.

Nanoparticle Tracking Analysis (NTA) of EVs

EVs were resuspended in PBS and diluted 1:1000. Small EV (sEV) fractions obtained from the iodixanol gradient were diluted 1:300 for *in vitro* derived PC3 EVs and 1:30 for mouse plasma-derived sEVs after density gradient separation. The samples were analyzed as previously described using the NTA 3.1 Build 3.1.46 software and the NS 300 instrument (Malvern Instruments, MA) (DeRita et al., 2017).

Transmission Electron Microscopy

A 3 µl volume of each sample was applied to a holy carbon grid that was glow discharged for 30 seconds. A solution of 2% uranyl acetate was freshly made in deionized water. Each sample was then stained twice with 3 µl of 2% uranyl acetate. Excess stain and sample were blotted away with a Whatman filter and the grid was let to dry until imaged. TEM micrographs were collected using Tecnai TF20 FEG TEM microscope and the images were recorded on Falcon III direct electron detector.

Anchorage-independent growth assay

PC3 cells after EV treatment were measured for anchorage-independent growth. PC3 cells were plated in 6-well plates to 75% confluency and serum-starved for 5 hours. After starvation, cells were treated with either PEG-precipitated EVs from mouse plasma (100 µg/mL, with a range of 9.6×10^7 - 2.6×10^{10} EVs/mL), gradient-purified EVs from mouse plasma (7.5×10^7 sEVs/mL), ultracentrifugation-isolated sEVs from shβ1 or mock PC3 cells (9×10^{10} EVs/mL), gradient-purified EVs from PC3 cells (21 µg/mL, with a range of 12.6×10^8 – 6.3×10^9 EVs/mL), or vehicle (PBS). For β1 integrin inhibition, the EVs were pre-treated with 100 µg/mL ATN-161 ($AcPHSCNNH_2$) (Tocris) or control GRGESP peptide (Gibco) at 4 °C for 45 minutes prior to cell treatment. EV concentration was always matched between samples for each experiment. EV treatment was overnight for 16-18 hours in serum-free media. The next day, new 6-well plates were then coated with 0.8% agarose to create a basement layer. Treated cells were trypsinized and 5,000 cells from each well were resuspended in 2 ml of 2X RPMI containing 10% FBS. The cell suspension was then mixed with 0.2 ml of 3% agarose; 2 ml of this mixture was layered gently on top of the basement layer to seed cells in a final concentration of 0.3% soft agar matrix. After solidification, 0.5 ml full media was added to prevent drying. Each condition was performed in duplicate. After two weeks (or four weeks for results in Figure 3A), all colonies were counted and classified by size via the NIS-Elements-F software and Nikon Eclipse TS100 microscope. Ten to twelve random optical fields were counted per experimental condition at 40x magnification. The number of colonies ≥ 25 µm out of the total number of colonies per field (325 x 250 µm) was quantified and reported as a percentage after 2 weeks in soft agar.

Confocal microscopy and EV transfer

DU145 prostate cancer cells grown on fibronectin coated (10 µg/ml) glass coverslips were serum-starved for 24 h followed by incubation with PKH26 red dye (Mini26, Sigma-Aldrich) labeled EVs (20 µg/ml, ~10^{11} vesicles) from PC3-Mock and PC3-shβ1cells or PKH26 red dye in PBS alone for 24 h. The cells on coverslips were then washed with PBS (2 washes), fixed with 4% PFA for 15 min at room temperature, washed with PBS (3 washes), quenched with 50 mM NH4Cl for 15 min, washed with PBS (2 washes), permeabilized with 0.1% Triton X-100 for 10 min, washed with PBS (3 washes), blocked with 5% BSA, stained with FITC-conjugated phalloidin (2 µg/ml, Sigma-Aldrich, Cat. # P-5282) for 1 h at room temperature, washed with PBS (3 washes), and mounted on glass slides using ProLong™ diamond antifade mountant with DAPI (Invitrogen). The cells on coverslips were imaged by Nikon A1R confocal microscope. A Z-stack image analysis was done by NIS Elements Viewer software (version 4.11.0) to evaluate PKH26 red dye labeled EV internalization into DU145 cells.

Statistical Analysis

Unless otherwise indicated, data in the figures are presented as mean ± SEM, and significant differences between experimental groups were determined using the 2-tailed Student's t test. A two-sided P value of less than 0.05 was considered statistically significant.

SUPPLEMENTARY FIGURES AND LEGENDS

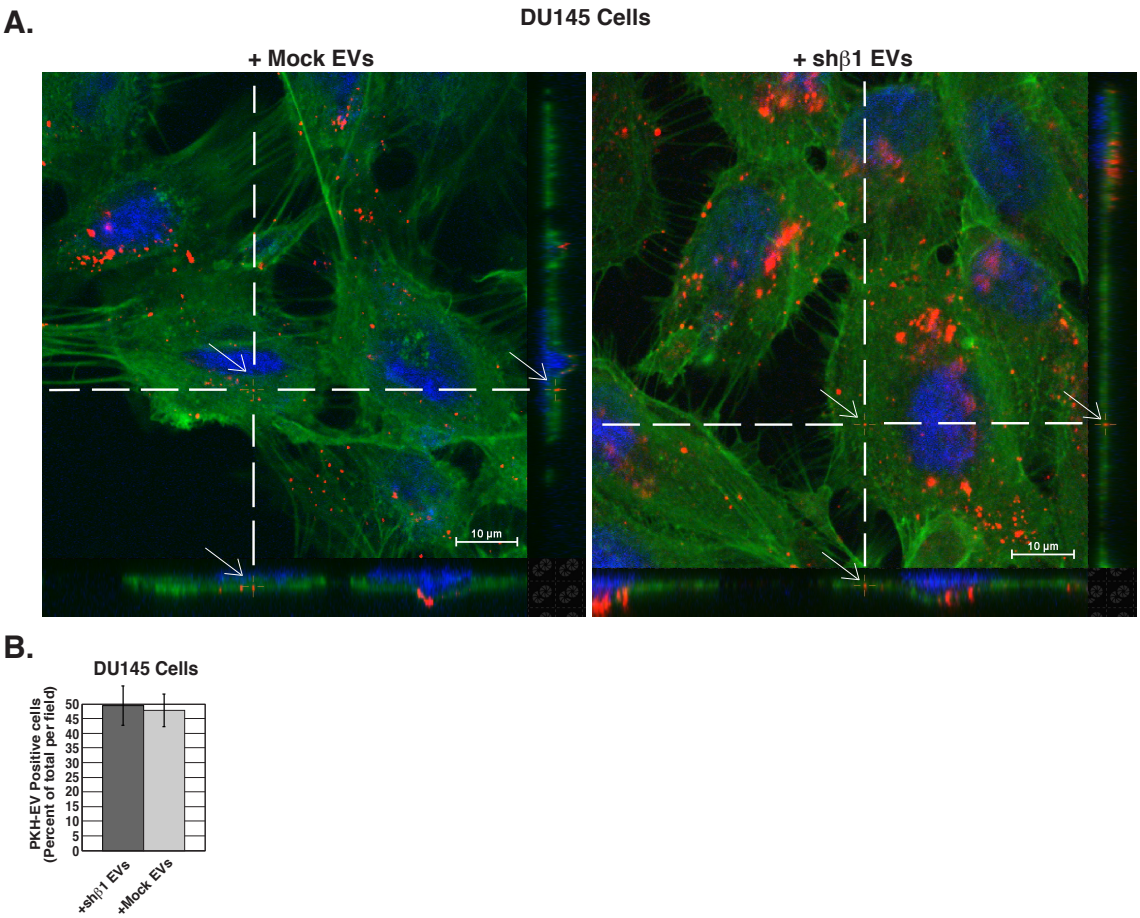

Figure S1: Down-regulation of β1 does not affect extracellular vesicle internalization, related to Figure 3. (A) DU145 cells were incubated with PKH26 - labeled EVs from either shβ1 or mock PC3 cells for 24 hours and confocal microscopy was carried out to evaluate EV internalization. DAPI was used to detect nuclei (blue), FITC-labelled Phalloidin was used to label actin (green), and PKH26 Red was used to label the EVs (red). Z-stack analysis was used to determine the presence of EVs inside the cells versus the cell surface. (B) The percent of total cells showing internalized EVs was quantified and reported.