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Supplemental information

CD63-mediated cloaking of VEGF in small extracellular vesicles contributes to anti-VEGF therapy resistance

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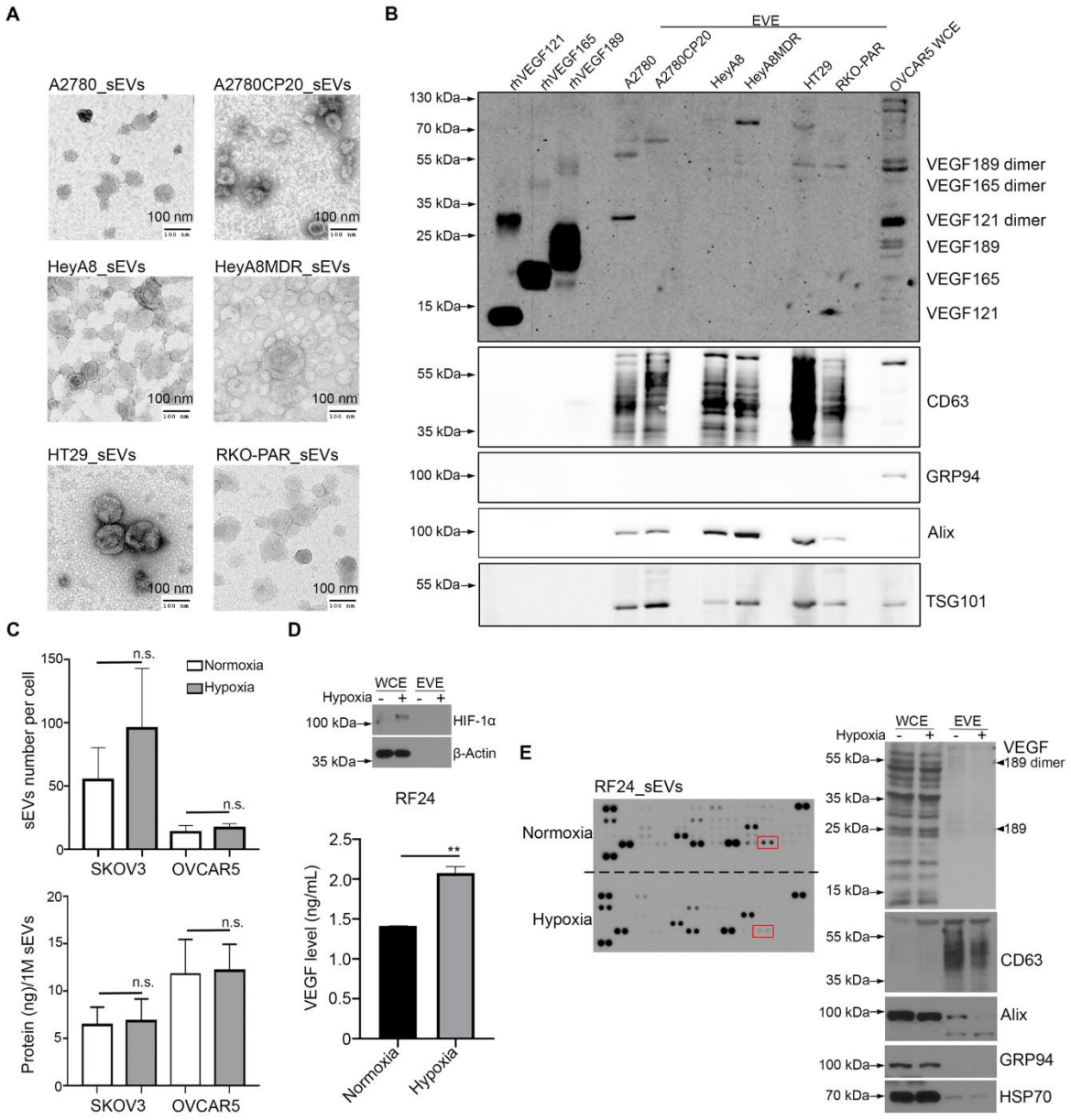

Figure S1. The presence of VEGF in small EVs, Related to Figure 1.

(**A**) Morphology of isolated small EVs determined by Transmission electron microscopy. Scale bar, 100 nm.

(**B**) VEGF isoforms in small EVs isolated from A2780, A2780CP20, HeyA8, HeyA8MDR, HT29, and RKO-PAR cells.

(**C**) The levels of secretion of small EVs from OVCAR5 and SKOV3 cells under normal and hypoxic conditions. Comparison of protein amounts in small EVs under normal and hypoxic conditions. n.s., not significant. P-values were determined by a Student's t-test for the comparison between two groups. Data are represented as mean ± SD.

(**D**) The induction of hypoxia-inducible factor-1α (HIF-1α) and secreted VEGF levels in cell culture supernatants were determined by a human VEGF ELISA kit. **, $p<0.01$. P-value was determined by a Student's t-test for the comparison between two groups. Data are represented as mean ± SD.

(**E**) Human angiogenesis array data for small EV extracts from RF24 cells. Red rectangles show the location of VEGF protein. Array membranes were developed at the same time. Western blotting shows the levels of VEGF in small EVs. Arrowheads point to the location of VEGF isoforms. sEVs, small EVs. WCE, whole-cell extract; EVE, small EV extract.

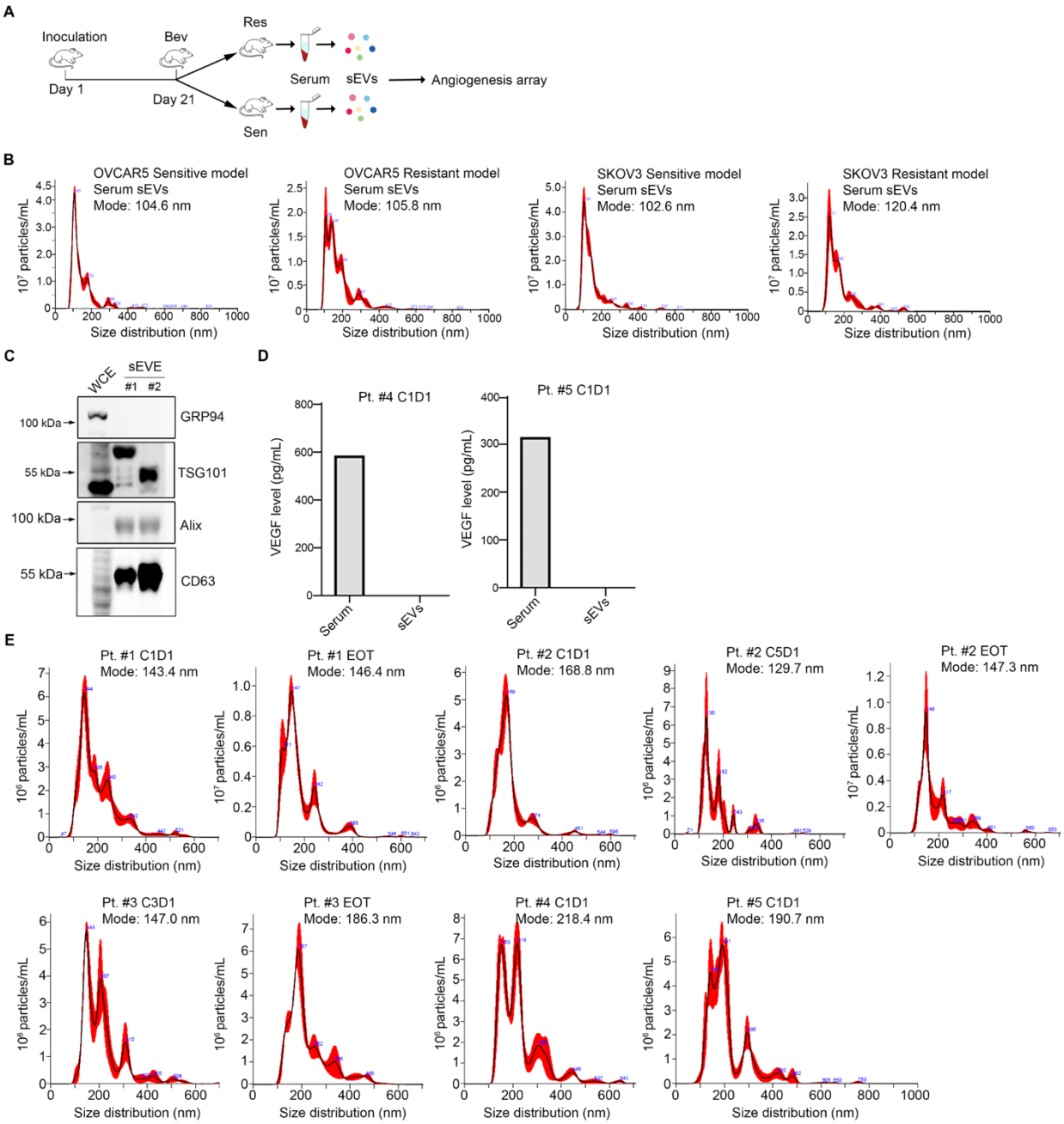

Figure S2. Characterization of small EVs isolated from serum samples, Related to Figure 2.

(**A**) The experimental schema for the *in vivo* experiment. Bev, bevacizumab; Res, resistant; Sen, sensitive.

(**B**) Particle analysis of serum small EVs isolated from OVCAR5 and SKOV3 xenograft mouse models.

(**C**) Examination of the small EV-positive markers CD63, Alix, and TSG101. GRP94 was used as a small EV-negative marker and SKOV3 WCE was used as a positive control for GRP94. Sample #1 was isolated from the SKOV3 mouse model, whereas sample #2 was isolated from the OVCAR5 mouse model.

(**D**) Translational study of eVEGF levels in serum samples from two patients treated with bevacizumab-containing therapy. C1D1, cycle 1 day 1.

(**E**) The size distribution of serum small EVs isolated from patient (Pt.) #1, Pt. #2, Pt. #3, Pt. #4, and Pt. #5. sEVs, small EVs. WCE, whole-cell extract; EVE, small EV extract.

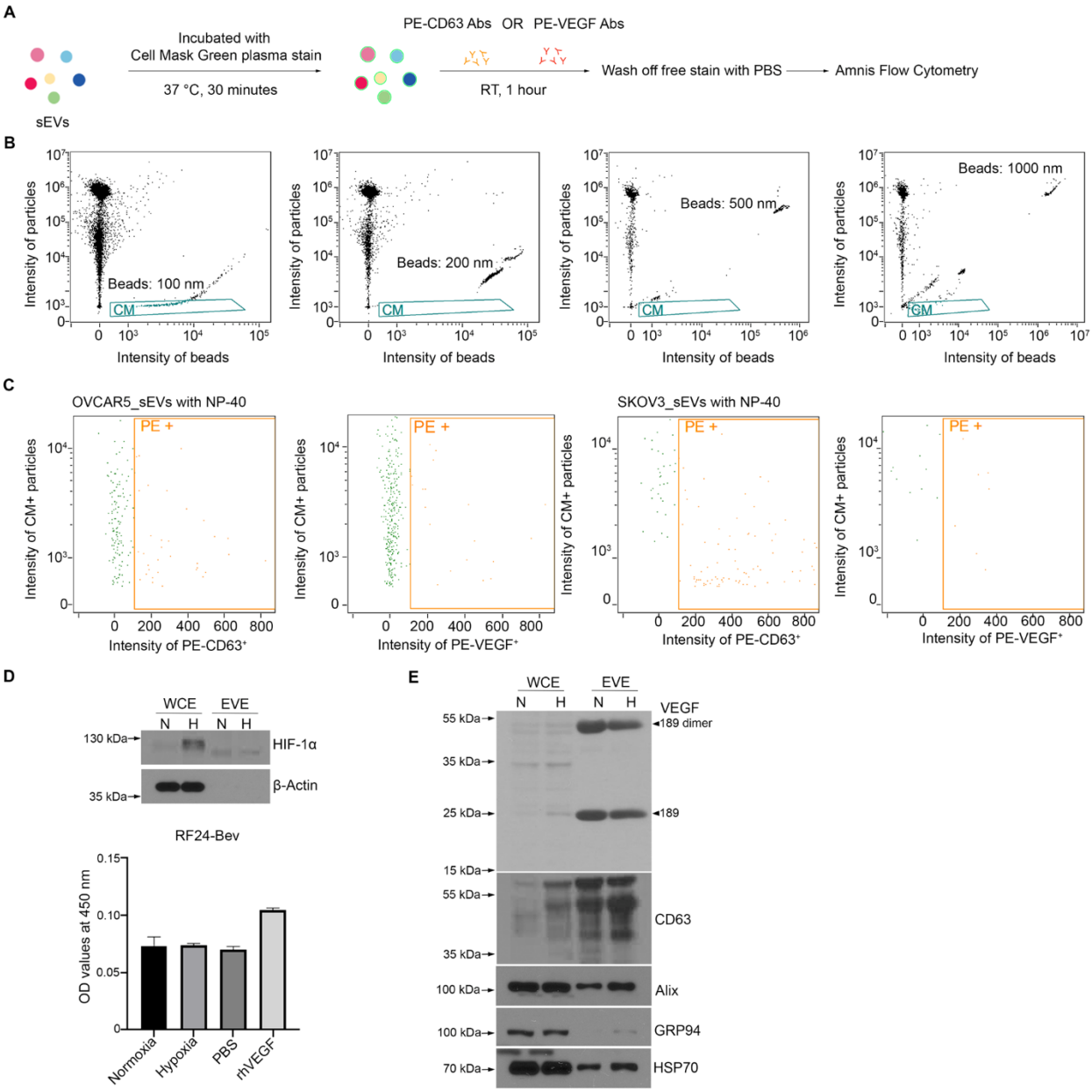

Figure S3. Process of Amnis image flow cytometry experiments, Related to Figure 3.

(**A**) Schematic of the experimental design. Anti-PE-conjugated CD63 (PE-CD63) antibodies (Abs) were used as positive controls.

(**B**) Various sizes of beads as size control for Amnis image flow cytometer.

(**C**) The numbers of small EVs after incubation with NP-40 detergent.

(**D**) Western blotting shows the induction of hypoxia-inducible factor-1α (HIF-1α). The OD (optical density) values at an absorbance of 450 nm of secreted VEGF levels in RF24-Bev cell culture supernatants was determined by a human VEGF ELISA kit compared to the negative control (PBS only) and positive control (15.6 pg/mL rhVEGF, the lowest dose of a standard curve). n.s., not significant. Data are represented as mean ± SD.

(**E**) eVEGF expression in RF24-Bev cells in the presence of 1 μg/μL bevacizumab. Arrowheads point to the location of VEGF isoforms. sEVs, small EVs. WCE, whole-cell extract; EVE, small EV extract.

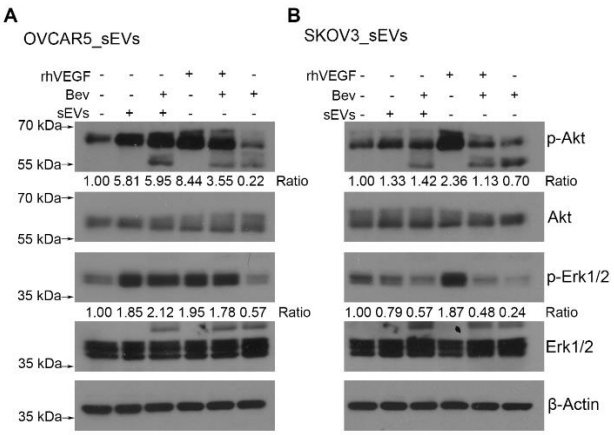

Figure S4. Activation of Akt/Erk signaling by cancer cells-derived small EVs, Related to Figure 5.
(**A and B**) Western blotting results of RF24 cells treated with small EVs from (**A**) OVCAR5 and (**B**) SKOV3 cells. Recombinant human VEGF protein (rhVEGF) was used as a positive control. Bev, 1 μg/μL bevacizumab. sEVs, small EVs. Protein expression ratio was calculated by dividing the pixel density of phosphorylated proteins with total proteins and normalized to the control group (lane 1).