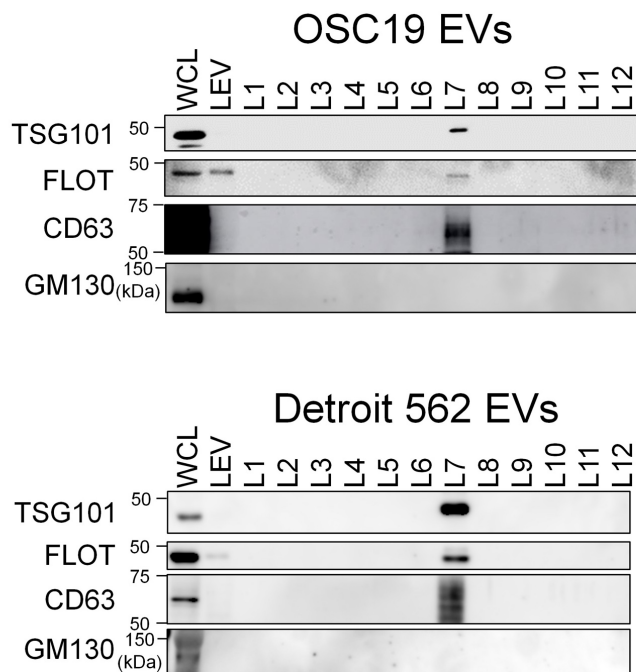
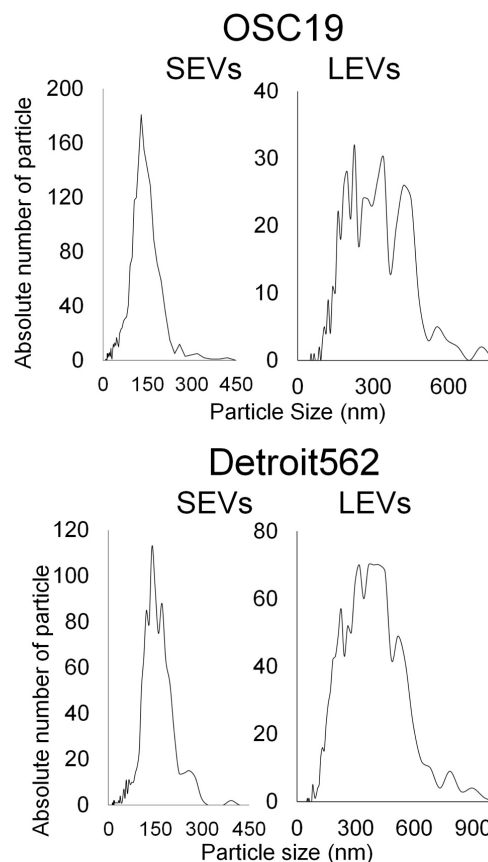
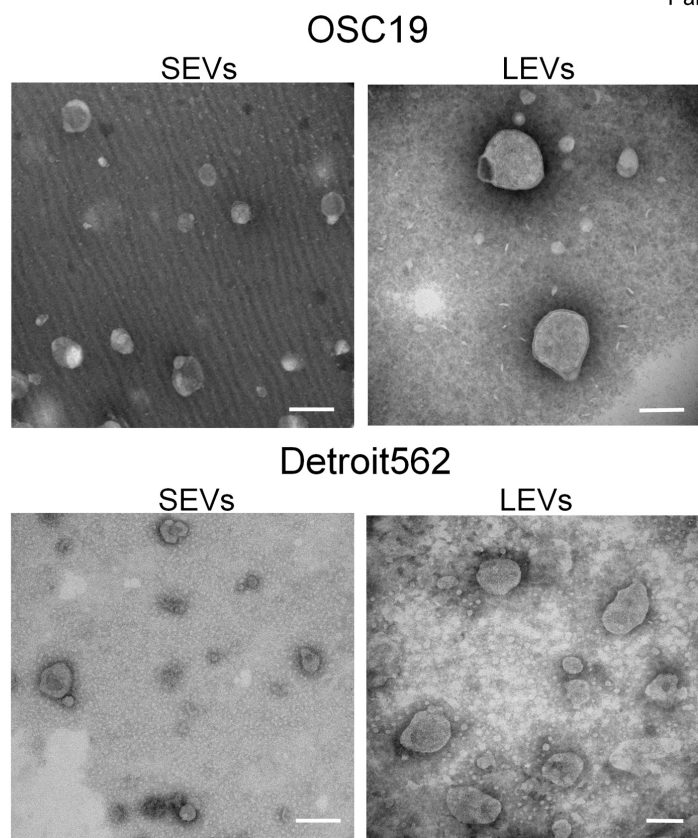


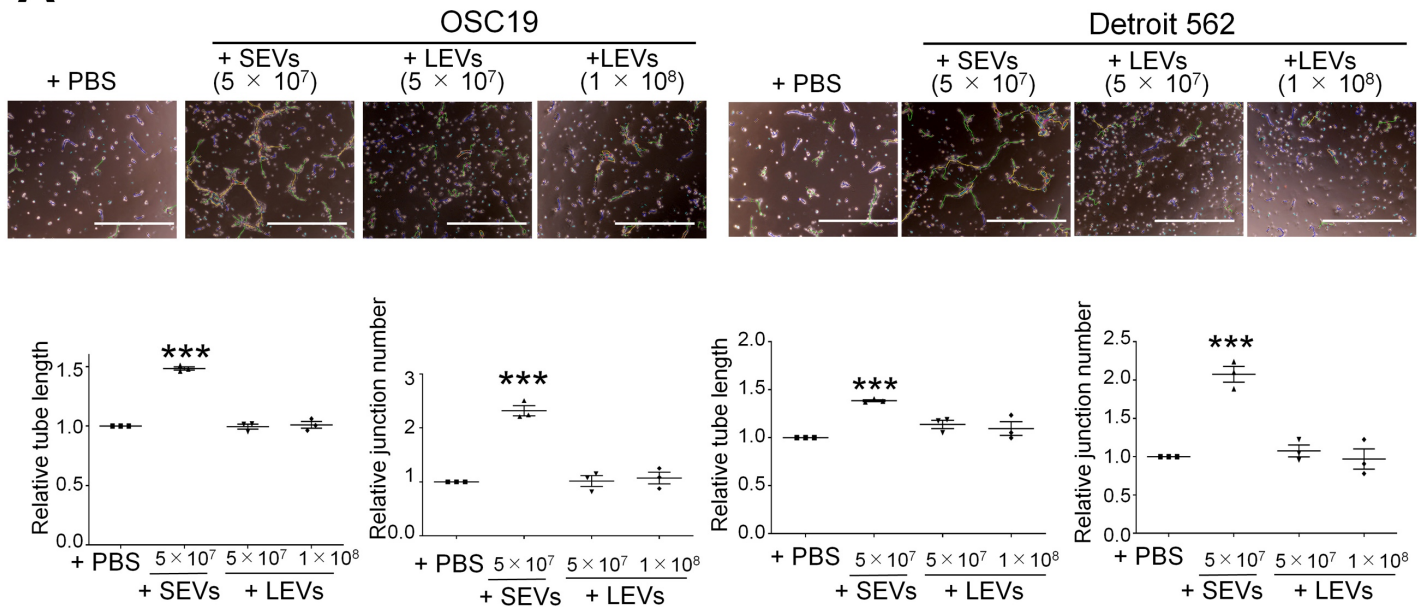
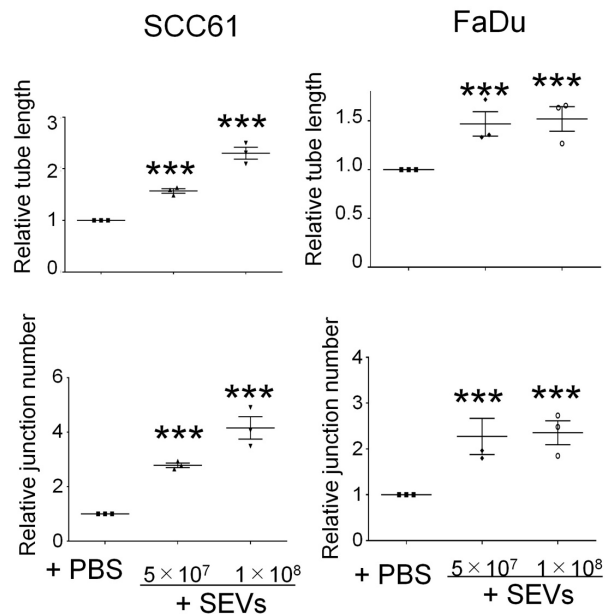
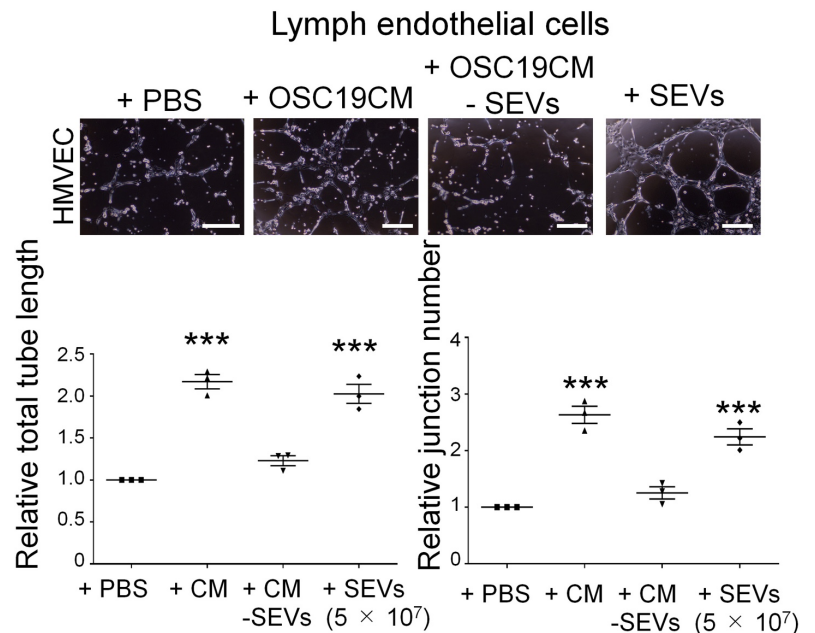
Supplementary Information Sato et al.

Includes 6 Supplementary Figures, 1 Supplementary method, and Titles for 3 Supplementary Excel Spreadsheet Tables.

A**B****C**

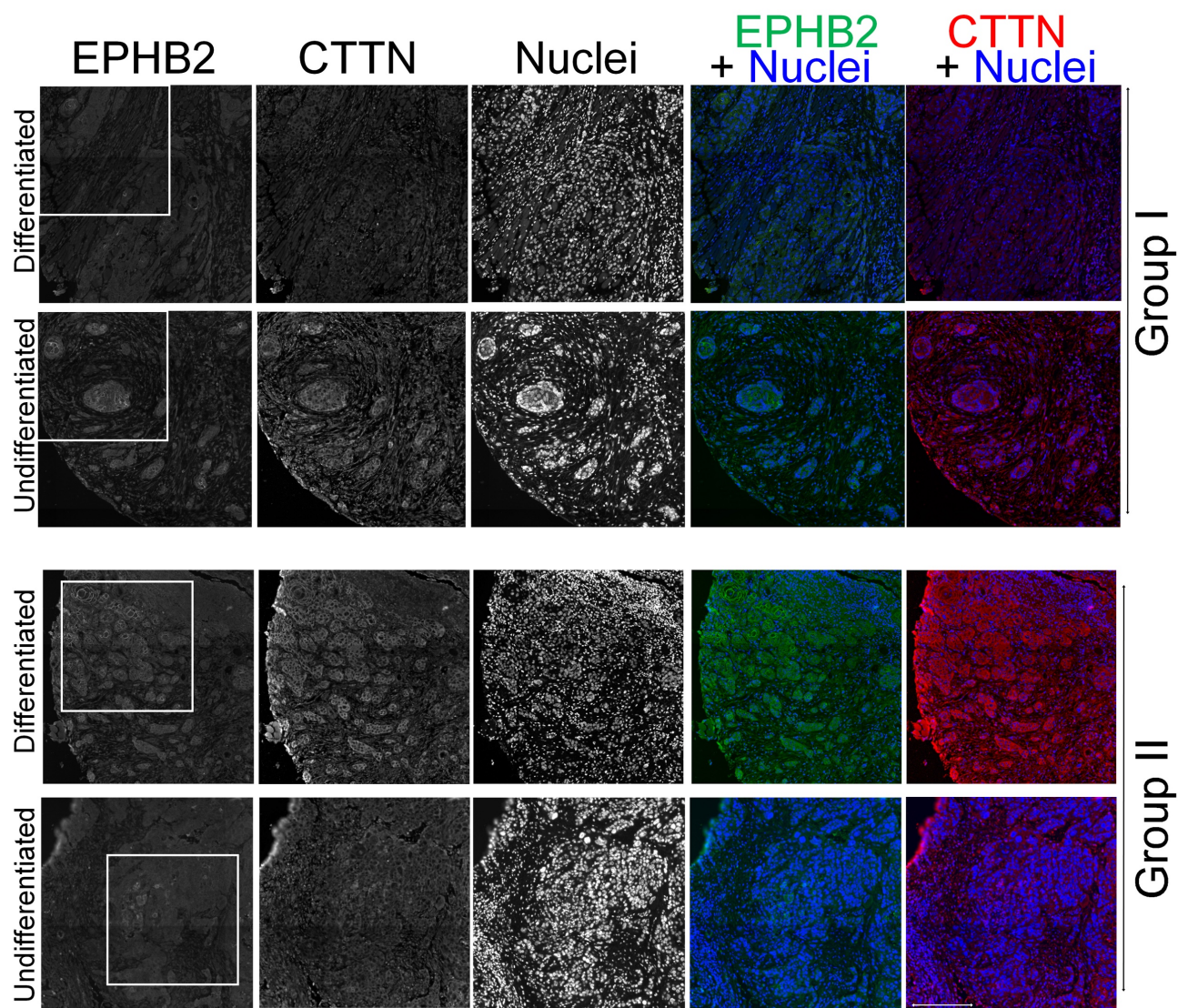
Supplementary Figure 1. Characterization of EVs purified from OSC19 and Detroit 562 cells.

(A) Representative Western blots of exosome markers (TSG101, Flotillin, CD63) and non-exosome marker (GM130) in OSC19 and Detroit 562 whole cell lysates (WCL), large EVs (LEVs), and density gradient (DG) fractions 1–12. (B) Representative nanoparticle tracking size distribution traces of OSC19 and Detroit 562 LEVs and SEVs purified from DG fraction 7. (C) Representative transmission electron microscopy images of negative stained LEVs and DG SEVs. Scale bars = 200 nm.

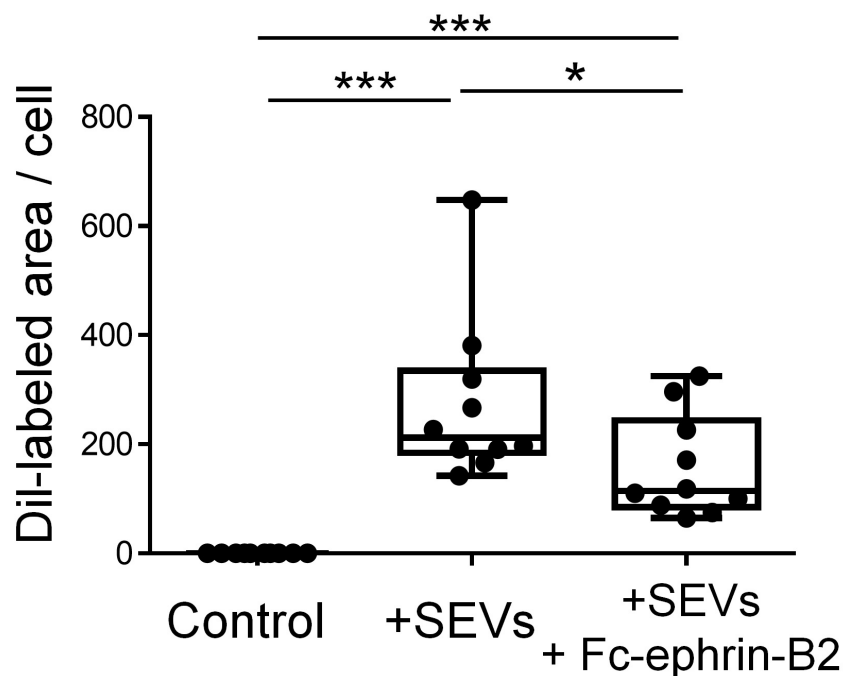
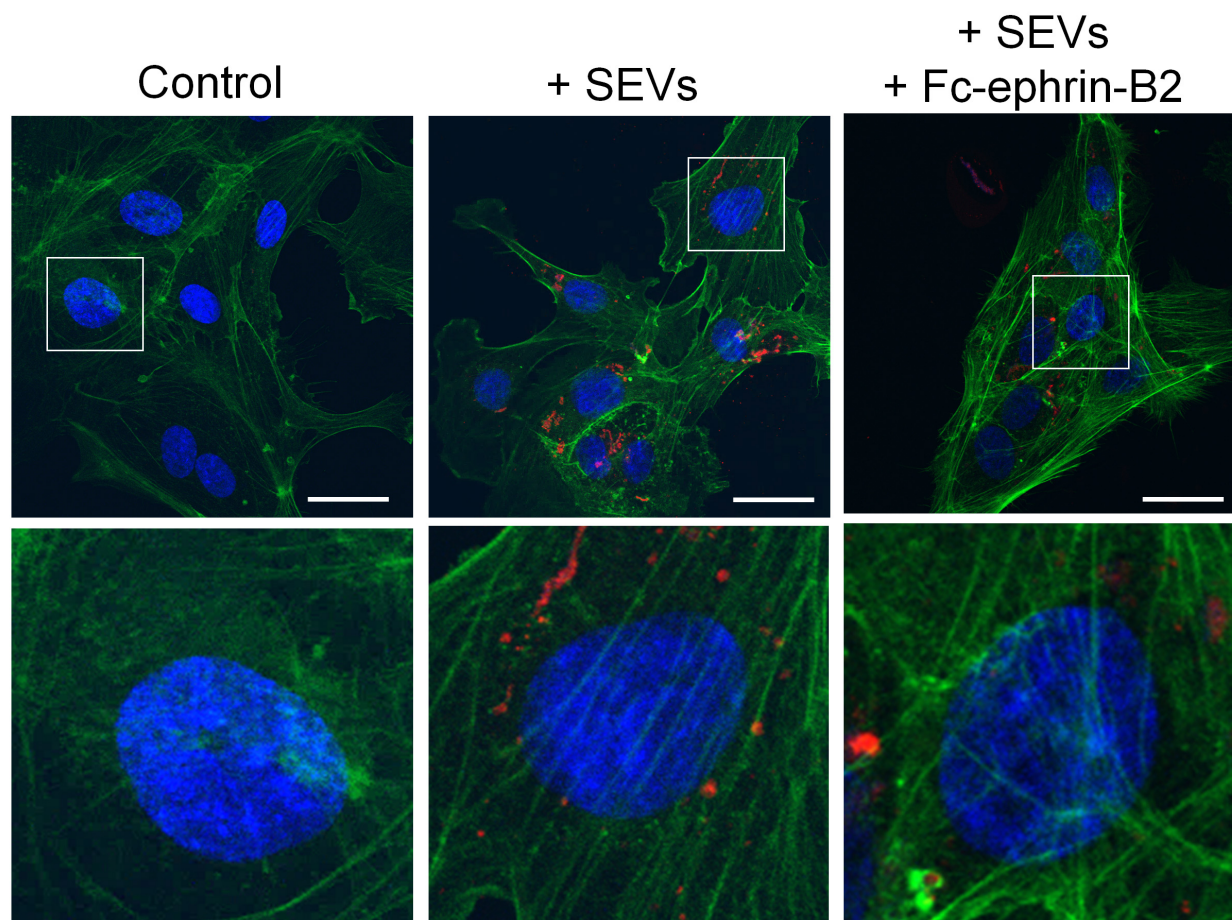
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Supplementary Figure 2. HNSCC SEVs drive tube formation of blood and lymph endothelial cells.

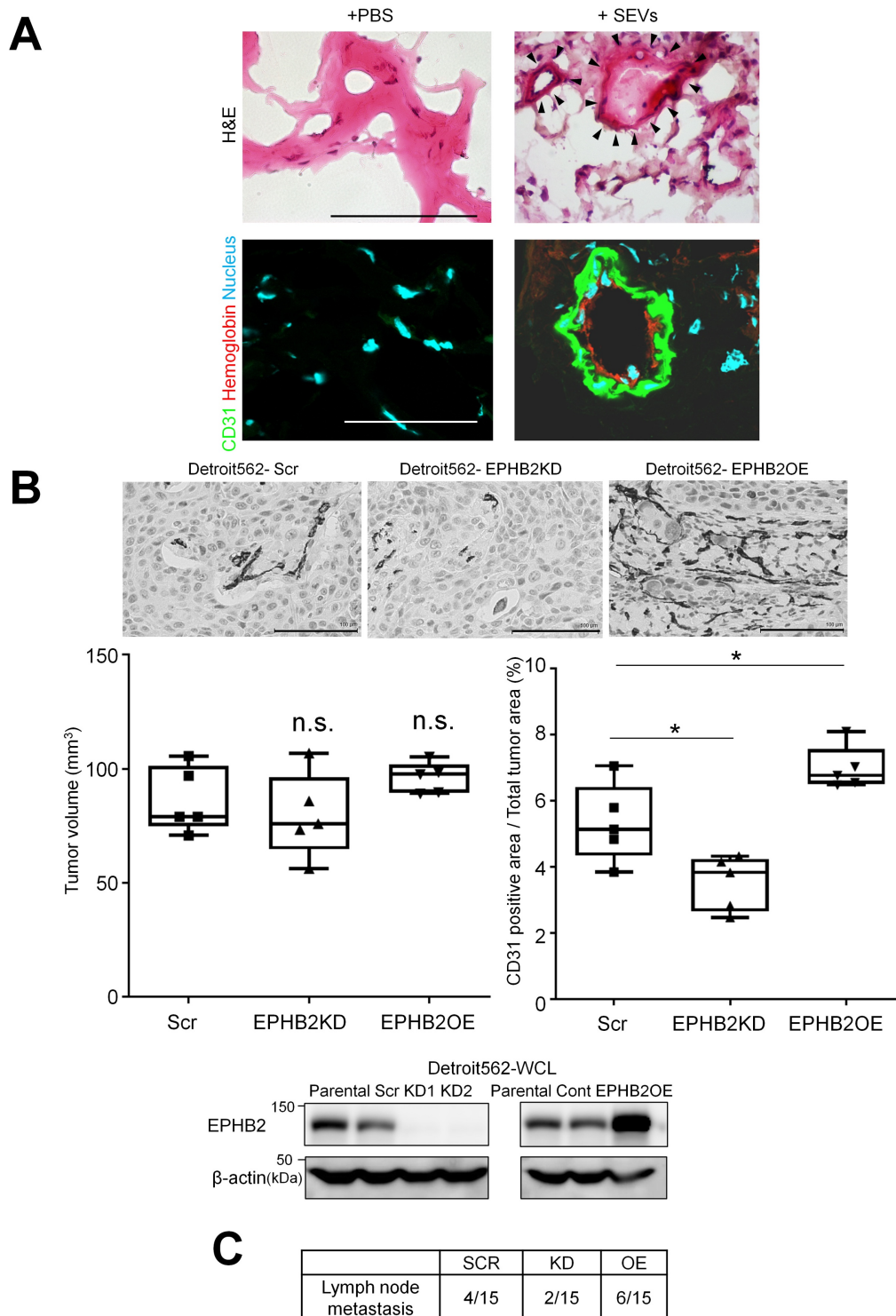
Results from tube formation assays with HUVECs (A) and (B) or with lymphatic endothelial HMVEC-dLyAd cells (C). (A) Representative images of HUVEC cells cultured with PBS, SEVs or LEVs purified from OSC-19 or Detroit 562 cells (number of EVs added/well is indicated). Scale bar = 500 μ m. (B) Quantification of relative total tube length and relative junction number in HUVECs cultured with PBS, SCC61 SEVs, or FaDu SEVs. (C) Representative images and quantitations from HMVEC-dLyAd cells cultured with PBS (control), conditioned media (CM), SEV-deprived CM (CM-SEV), or purified SEVs (5×10^7) from OSC19 cells. Scale bars = 500 μ m. For all graphs, dot plots represent mean $\pm$ SE. Dunnet method was used for statistical analysis using the average of ≥ 3 technical replicates per condition for each n value from ≥ 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to +PBS condition.



Supplementary Figure 3. Representative images of immunofluorescent staining for EPHB2 and Cortactin. Representative staining for tumors from patients in groups I and II is shown for differentiated and undifferentiated HNSCC tissue areas with single channel (EPHB2, Cortactin (CTTN), and nucleus), and merged images (EPHB2 + nucleus, and CTTN + nucleus). Boxes depict the regions shown in Main Fig. 2E. Scale = 200 μ m.

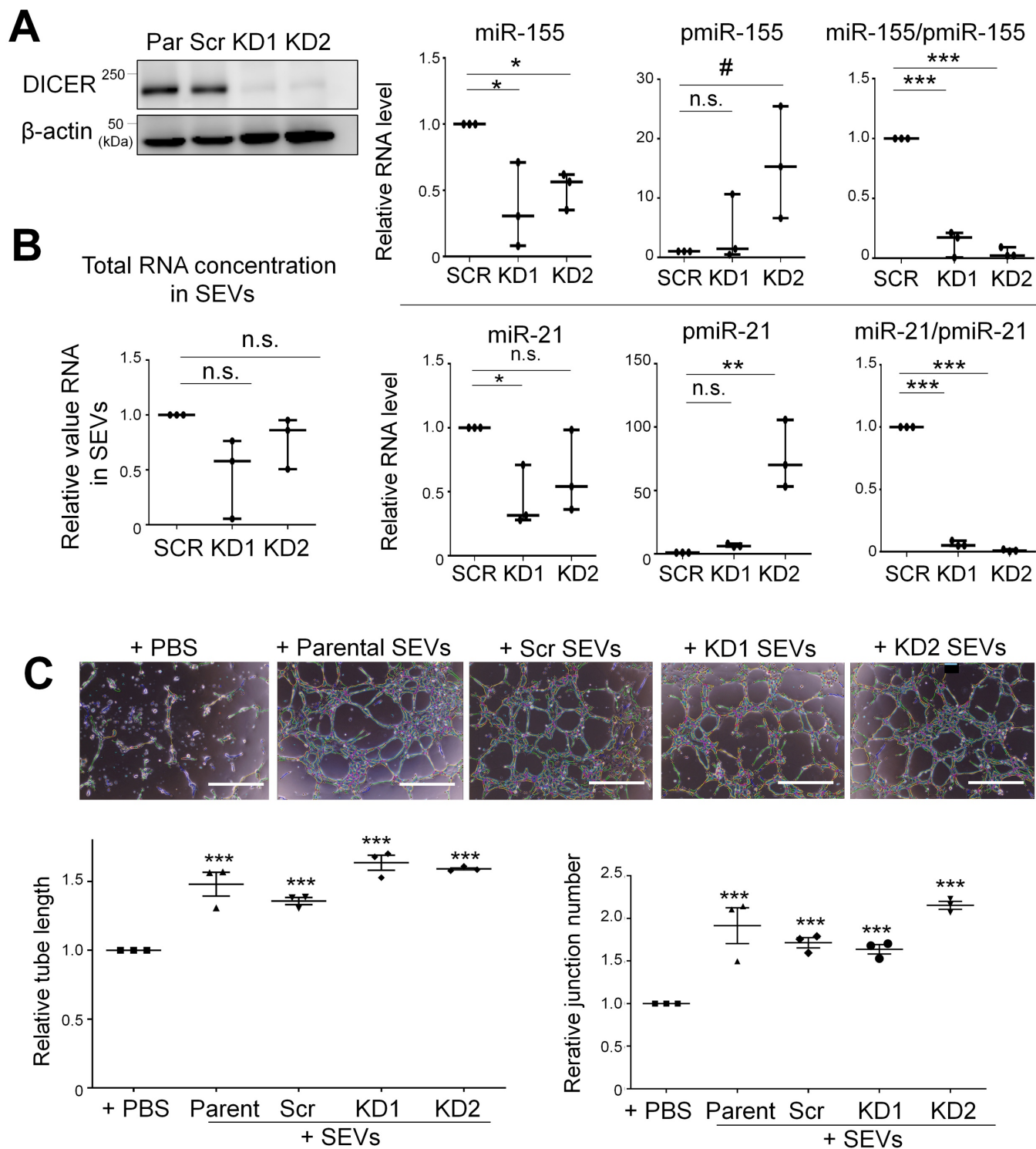


Supplementary Figure 4. EPHB2-ephrin-B interactions mediate binding and uptake of SEVs. SEVs labeled with Dil (“+SEVs”, red), SEVs pre-blocked with 1 $\mu\text{g/ml}$ Fc-ephrin-B2 (“+SEVs+Fc-ephrin-B2”) or control Dil labeling reactions (“Control”, Dil only carried through the labeling reaction without SEVs) were incubated with HUVECs for 12 h before fixation and staining for filamentous actin with Alexa-488-phalloidin (green). (Top) Representative images. Scale bar: 20 μm . (Bottom) Plot of Dil-labeled area / cell. Mean $\pm$ SE. * $P < 0.05$, *** $P < 0.001$.



Supplementary Figure 5

(A) (Top) Representative images of H&E stained Matrigel plug tissue from control (+PBS) and +SEVs conditions. Blood vessels are outlined with arrowheads and are evident in the +SEVs conditions. (Bottom) Representative images of anti-CD31 (green) and anti-Hemoglobin (red) staining. Scale bar = 50 μ m. (B) Top: Representative images and quantitation of CD31-staining (black) in tongue tumors. Scale bar = 100 μ m. (middle) Quantitative analysis of tumor volume and blood vessel density in each group. Box and whisker plots show median and 25th-75th %ile. Tukey-Kramer method was used for statistical analysis. 1 group: n = 5. *, $P < 0.05$. n.s. not significant. (Bottom) Representative Western blot of EPHB2 in parental, scrambled control (SCR), empty vector control (Cont), EPHB2-KD (KD1 and KD2), and EPHB2-overexpressed (EPHN2OE) Detroit562 whole cell lysates and SEVs. β -actin serve as loading controls. (C) Frequency of lymph node metastasis. Chi-square test was performed for statistical analysis. SCR VS KD: $P = 0.361$. SCR VS OE: $P = 0.439$, and KD VS OE: $P = 0.09$.



Supplementary Figure 6. miRNAs do not appear to be critical HNSCC SEV cargoes driving angiogenesis.

(A) Representative Western blot analysis of parental (Par), scrambled control (Scr), and DICER-knockdown (KD1, KD2) whole cell lysates from OSC19 cells. (B) (Left) Relative total RNA concentration in Scr and DICER-KD SEVs. (Middle): Quantitative RT-PCR analysis showing the effect of Dicer-KD on the expression levels of candidate mature (miRs) and pre-miRNAs (pmiRs), as indicated. (Right): Mature miRNA/ pre-miRNA ratios in indicated SEVs. Bar graphs show mean $\pm$ SE. (C) (Top) Representative images of HUVEC cells cultured with PBS (control), or 5×10^7 SEVs purified from parental, scrambled shRNA, or DICER-KD OSC19 cells. Scale bar = 500 μ m. (Bottom) Quantification of relative total tube length and junction number for indicated groups. Dot plots represent mean $\pm$ SE. Dunnett method was used for statistical analysis using the average of ≥ 3 technical replicates per condition for each n value from ≥ 3 independent experiments. #, $P = 0.0504$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary method

Dil-labeled SEV uptake experiment

For labeling EVs, 20 µg of EVs were incubated with Dil (5 µg / ml) in 1 ml PBS for 10 min. The labeled EVs were centrifuged at 100,000 x g for 3 hrs, resuspended in 3 ml PBS and recentrifuged at 100,000 x g for 3 hrs. Finally the pellet was resuspended in PBS (50 µl). A control reaction consisting of Dil without EVs was carried through the same labeling and purification steps. HUVECs (5×10^4) with Opti-MEM medium containing 10% EBM-2 medium were cultured with stained EVs (5×10^7) on coverslips coated with poly-D-lysine in 24 well plates for 12 hours. After that, HUVECs were fixed in 4% paraformaldehyde in PBS for 5 min, and filamentous actin was stained with Alexa Fluor 488-conjugated phalloidin. Epifluorescence images of stained HUVEC cells cultured with Dil-labeled SEVs or control reaction were acquired with a Nikon Eclipse TE2000E microscope using a x40/0.60-N.A. Plan Fluor lens.

Additional Supplementary Files

Supplementary Table 1. Excel spreadsheet with proteomics data from OSC19 SEV samples.

Supplementary Table 2. Excel spreadsheet with the proteomics data from Detroit 562 SEV samples.

Supplementary Table 3. Excel spreadsheet with hallmark angiogenesis genes.