cells

Anti-Human CD9 Fab Fragment Antibody Blocks the Extracellular Vesicle-Mediated Increase in Malignancy of Colon Cancer Cells

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Supplementary Figures

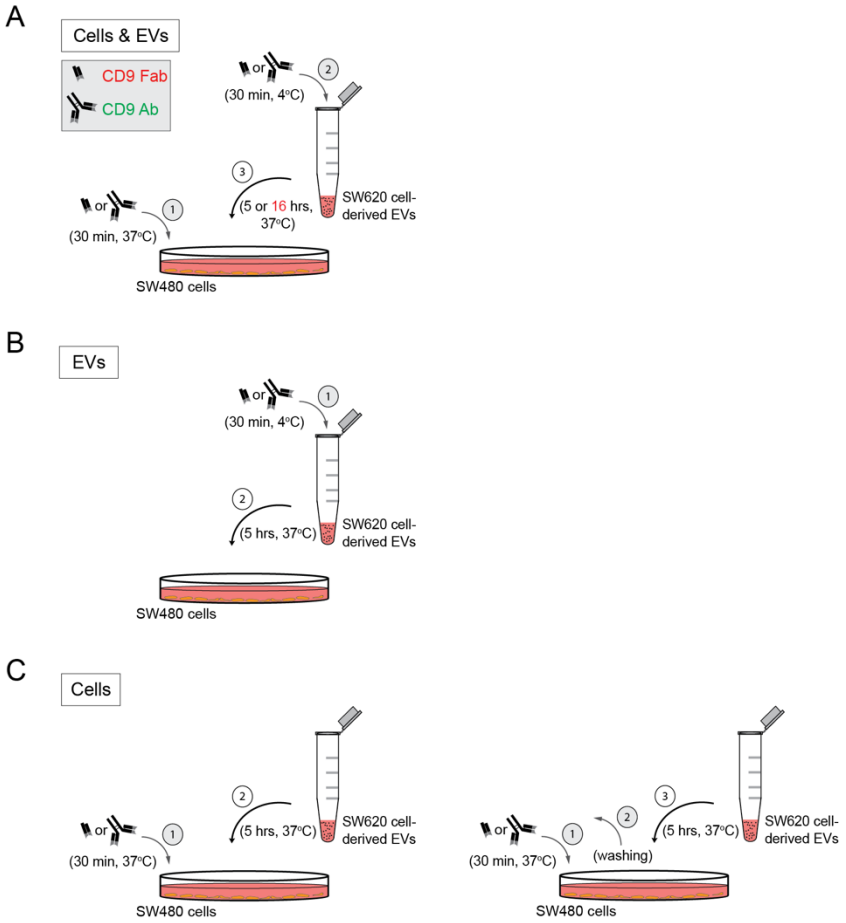

Figure S1. Schematic representation of the three main protocols used to study the impact of SW620 cell-derived on SW480 cells. **(A-C**) SW480 cells and SW620-cell-derived EVs were pre-incubated with either CD9 Fab or divalent Ab for 30 min at 37 °C or 4 °C, respectively, prior their co-incubation at 37 °C for 5 (or 16) h in the presence of Abs. This is referred to as protocol #1 (**A**). As alternatives, EVs or cells alone were pre-incubated with CD9 Fab or divalent CD9 Ab and then added to cells and EVs, respectively. These are referred to as protocol #2 (**B**) and protocol #3 (**C**), respectively. Note that in all conditions, Abs were not removed before co-incubation of cells and EVs, resulting in different Ab concentrations during the cell-EV incubation. As a control, Abs were omitted. Alternatively, Abs were removed from the cells after the 30-min pre-incubation and before the addition of EVs, which were not pre-incubated with Abs. This variant of protocol #3 is referred to as protocol #3' (**C**). Various experimental steps are numbered.

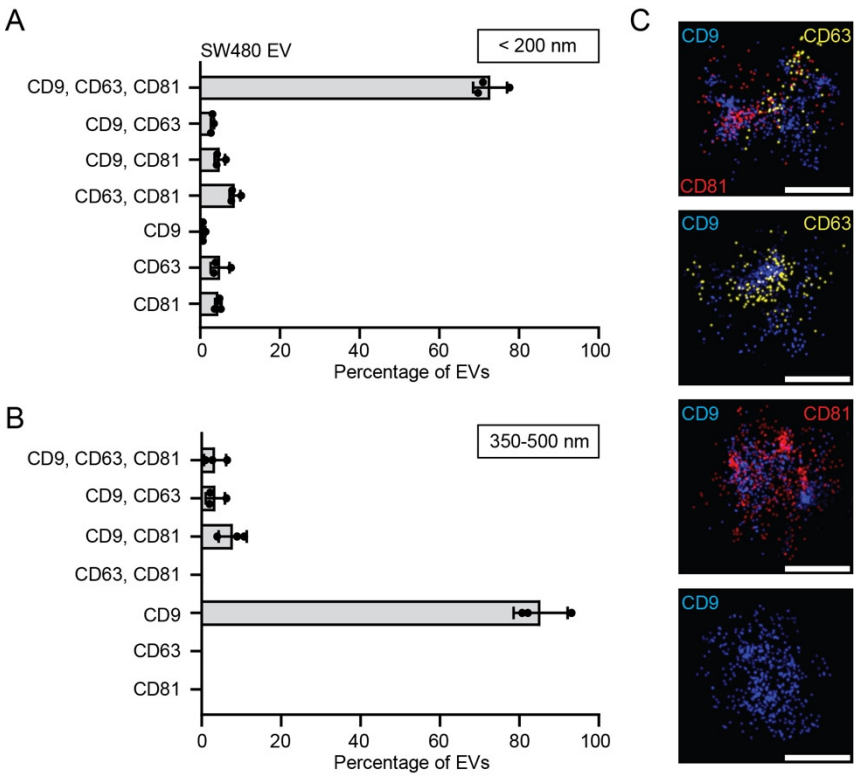

Figure S2. Characterization of small and large EVs released by SW480 cells. (**A-C**) Small (<200 nm, **A**) and large (350 to 500 nm, **B**) EVs derived from SW480 cells were imaged after immunolabeling of three tetraspanins as indicated using dSTORM, and the percentage of single, double and triple positive EVs was quantified. Means ± S.D. and individual values for three experiments are shown (n > 5000 (**A**) or 100 (**B**) EVs per experiment). Representative images of large EVs immunolabeled for CD9, CD63, and CD81 and pseudo-colored as indicated are shown (**C**). Note that the majority of large EVs are positive only for CD9. Scale bars, 200 nm.

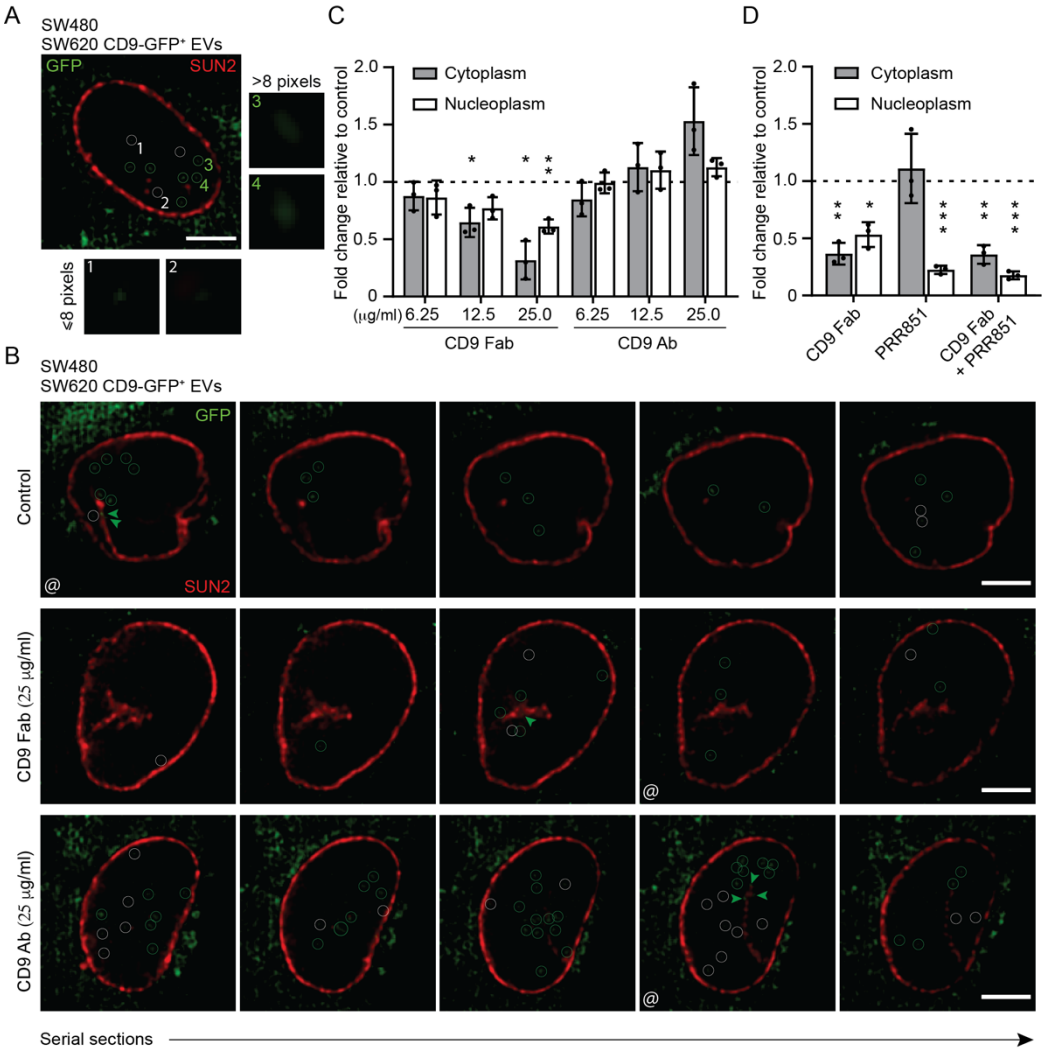

Figure S3. Quantification EV-derived CD9-GFP signal in the nucleoplasm of recipient SW480 cells. (**A**, **B**) SW480 cells exposed to fluorescent EVs (1×10^9 particles) derived from CD9-GFP$^+$ SW620 cells were immunolabeled for SUN2 and analyzed by CLSM. To measure GFP fluorescence in the nucleoplasm, a serial x-y optical section through the cells was collected (20 sections, and one (**A**) or five (**B**) are displayed as examples), and each individual section (0.45 µm thick) was evaluated by analyzing ROIs delimitated by SUN2$^+$ inner nuclear membrane labeling using the <<analyze function>> in Fiji. Because faint (auto)fluorescent signals equal to or less than 8 pixels (see enlargements 1 and 2, white circle) can be detected in cells not exposed to CD9-GFP$^+$ EVs (data not shown), the threshold was set at a level greater than 8 pixels (#3 and 4, green circle) (**A**). Examples of serial sections of SW480 cells exposed to anti-CD9 Fab and divalent Ab or not (Control) as indicated before their 5-hour-co-incubation with CD9-GFP$^+$ EVs, which themselves were exposed or not to CD9 Abs (**B**, for detail see Figure 3C). Arrowheads indicate CD9-GFP signal in nuclear envelope invaginations. Note that the images indicated by @ are also shown in Figure 3C. (**C**) The fold change of cytoplasmic and nuclear signals of CD9-GFP$^+$ EVs of cells treated with anti-CD9 Fab or divalent Ab (original data are from Figure 3) relative to the control (dashed line) is shown. (**D**) SW480 cells pre-treated with PRR851 (10 µM) or without in the presence or absence of anti-CD9 Fab (25 µg/ml) for 30 min were incubated with EVs derived from CD9-GFP$^+$ SW620 cells for 5 h. The fold change relative to the control (i.e., without Ab and drug, dashed line) was then measured as above. Means ± S.D. and individual values for each experiment are shown ($n = 3$). p values correspond to *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Scale bars, 5 µm.

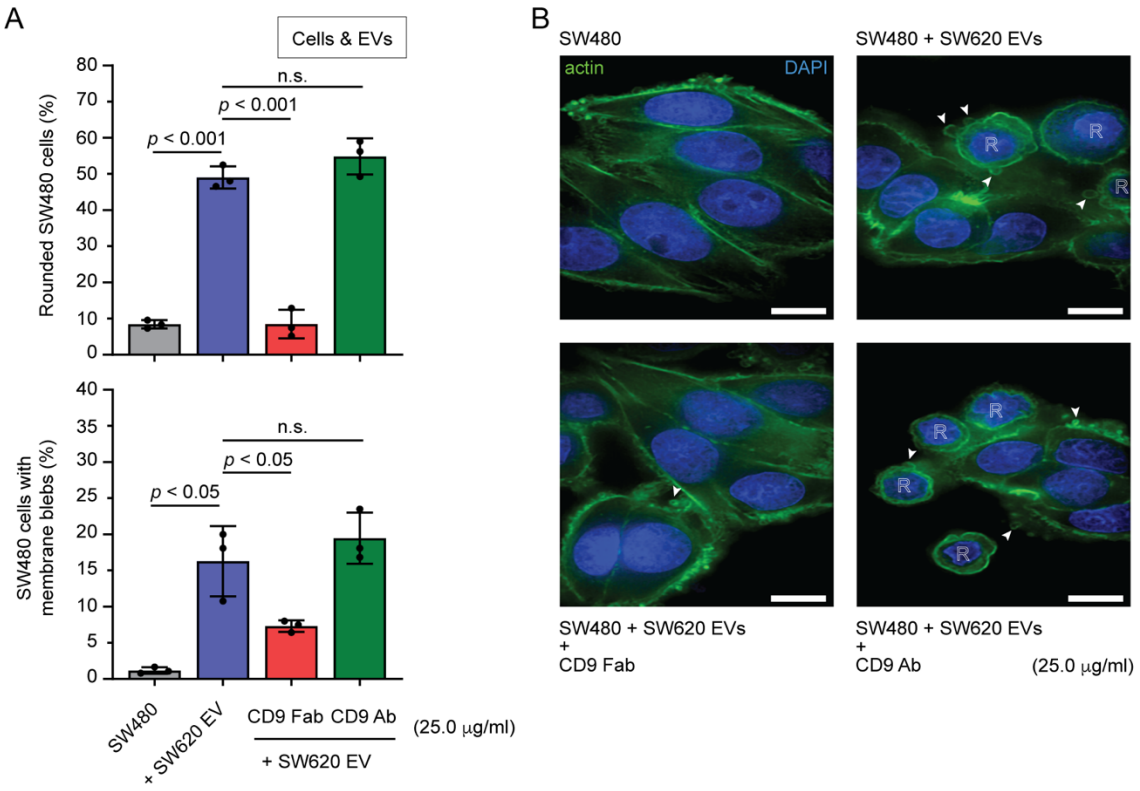

Figure S4. Effects of CD9 Fab and divalent Ab on the pro-metastatic morphological alterations of SW480 cells exposed to SW620 cell-derived EVs. (**A, B**) SW480 cells and SW620 cell-derived EVs (1×10^9 particles) were individually pre-incubated for 30 min with 25 μg/ml of anti-CD9 Fab or divalent Ab as indicated before their co-incubation for 16 h in the presence of Abs as described for protocol #1. As negative and positive controls, cells were not exposed (SW480) or were exposed to EVs (+ SW620 EV) in the absence of Ab, respectively. Afterward, fixed cells were stained with DAPI and fluorochrome-conjugated phalloidin to label nuclei and actin filaments, respectively, before observation by CLSM. The percentage of cells with rounded morphology (top panel) or membrane blebs (bottom panel) was quantified (**A**). Means ± S.D. and individual values for each experiment are shown ($n = 3$). At least 100 cells were evaluated per experiments. p values are indicated. n.s., not significant. Representative images were displayed for each condition (**B**). Single sections are presented. Rounded cell morphology and membrane blebs induced by EVs are indicated by R letter or arrowhead, respectively. Scale bars, 10 μm.

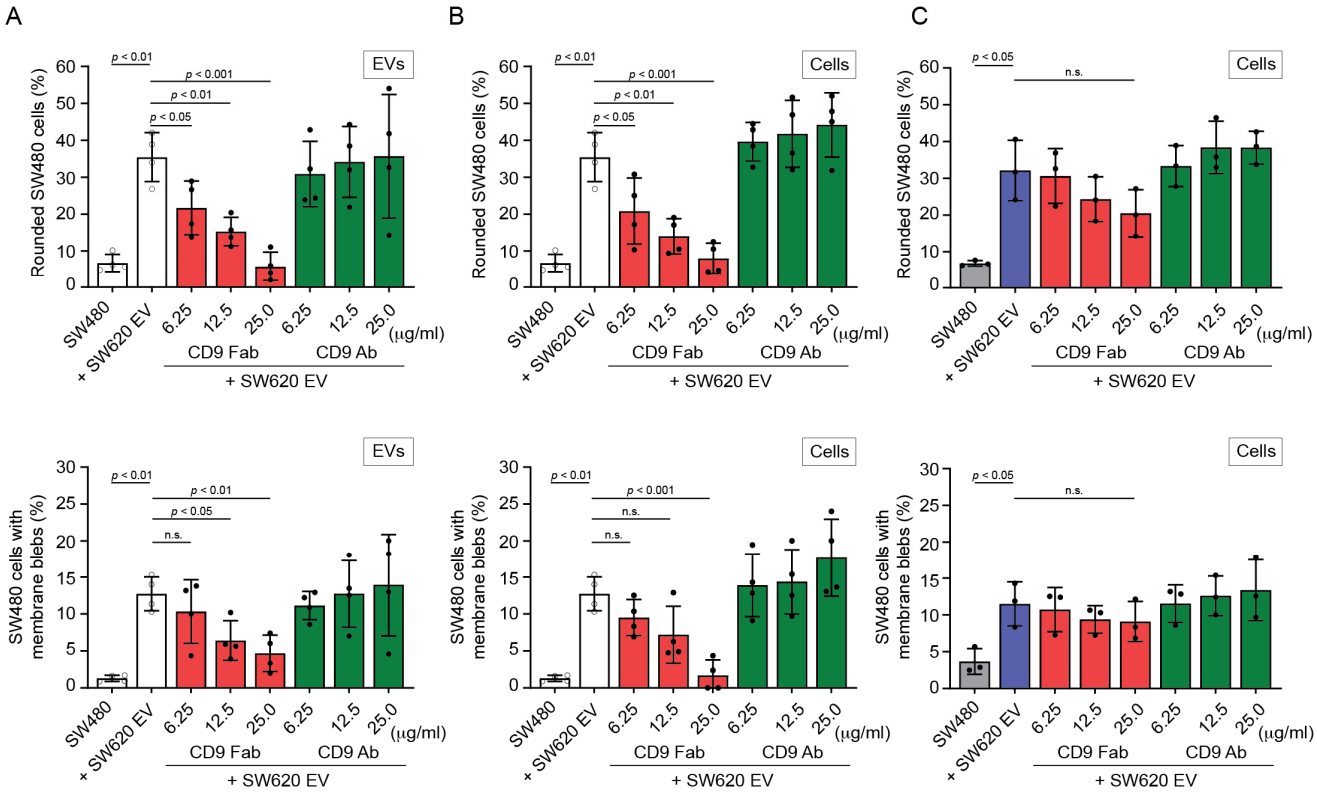

Figure S5. Effects of different concentrations of CD9 Fab and divalent Ab on the pro-metastatic morphological alterations of SW480 cells exposed to SW620 cell-derived EVs. (**A-C**) SW620 cell-derived EVs (**A**, 1 × 10^9 particles) or SW480 cells (B) were pre-incubated for 30 min with different concentrations of anti-CD9 Fab (red) or divalent (green) Ab as indicated prior to their co-incubation for 5 h with cells (**A**) or EVs (**B**), which were not exposed to Abs as described for protocols #2 and #3, respectively. In both protocols, Abs added for the pre-incubation were not removed. As negative and positive controls, cells were not exposed (SW480, grey) or were exposed to EVs in the absence of Ab (+ SW620 EV, blue). Controls (**A**, **B**, white) are displayed for comparison (for detail see Figure 4). Alternatively, Abs were removed from cells prior to the addition of EVs according to protocol #3′ (**C**). All cells were fixed and stained with DAPI and fluorochrome-conjugated phalloidin to label nuclei and actin filaments, respectively, before observation by CLSM. The percentage of cells harboring a rounded morphology (top panels) or membrane blebs (bottom panels) were assessed and graphed. The target of the pre-treatment with Abs is indicated (**A**, EVs; **B** and **C**, Cells). Means ± S.D. and individual values for each experiment are shown (n = 3-4). At least 100 cells were evaluated per experiments. p values are indicated. n.s., not significant.