Supplementary Data

Reporter gene assay for membrane fusion of extracellular vesicles

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Supplementary Methods

ETTD assay in four cell lines

Human cervical cancer-derived HeLa cells (JCRB Cell Bank), human lung adenocarcinoma A549 cells (RIKEN Cell Bank), human hepatoma cells Huh-7 cells (RIKEN Cell Bank), and HEK293T cells were used as recipient cells. Briefly, 10^4 cells were plated in a 96-well plate, cultured overnight, and transfected with PEI and plasmids encoding TEVp and TRE3G-NlucP. The next day, the cells were treated with PEG-precipitated EVs from either HEK293T or HeLa and cultured for 24 h. Reporter NanoLuc expression level was measured as described in the main text.

Co-transfection with siRNA and plasmid DNA

HEK293T cells were co-transfected with siRNA and plasmid DNA using PEI. The antisense sequences of siRNA against firefly luciferase (siLuc) and TetR (siTetR) were 5'-UCGAAGUACUCAGCGUAAGtt - 3' [1] and 5'- UGAUCUUCCAAUACGCAACtt - 3' [2], respectively (lowercase letters indicate DNA). Briefly, 10^4 cells were plated in a 96-well plate, cultured overnight, and transfected with PEI. PEI was mixed with 100 ng of plasmid and 1 pmol of siRNA. The weight ratio of PEI to pDNA was 4:1. The final concentration of siRNA used was 10 nM. Cells were cultured for 48 h and lysed to measure expression levels. For the quantification of HiBiT-tagged tTA, transfected cells were lysed and mixed with Nano-Glo HiBiT Lytic Detection System (Promega).

EV preparations by ultracentrifugation

One day before the transfection, donor HEK293T cells were seeded in 60 mm-dish and cultured overnight. In the case of serum-free culture condition, the culture medium was replaced with serum-free medium (Advanced-DMEM; Thermo Fischer) just before the transfection. After 4 days of transfection, the supernatant was collected and centrifuged at 1,500 ×g for 5 min to remove cell debris. The clarified supernatant was concentrated by either PEG precipitation (see main text) or ultracentrifugation. Briefly, the supernatant was diluted in PBS and ultracentrifuged (210,000 × g for 70 min at 4ºC) by using CP100MX ultracentrifuge (Hitachi) and P40ST swing rotor (Hitachi). Following the dilution of EV pellet in PBS and another centrifugation, EVs were resuspended in PBS.

Reference

1. Elbashir, S. M., Harborth, J., Weber, K. & Tuschl, T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods San Diego Calif* **26**, 199–213 (2002).
2. Lin, X. *et al.* A robust in vivo positive-readout system for monitoring siRNA delivery to xenograft tumors. *RNA* **17**, 603–612 (2011).

Supplementary Figures

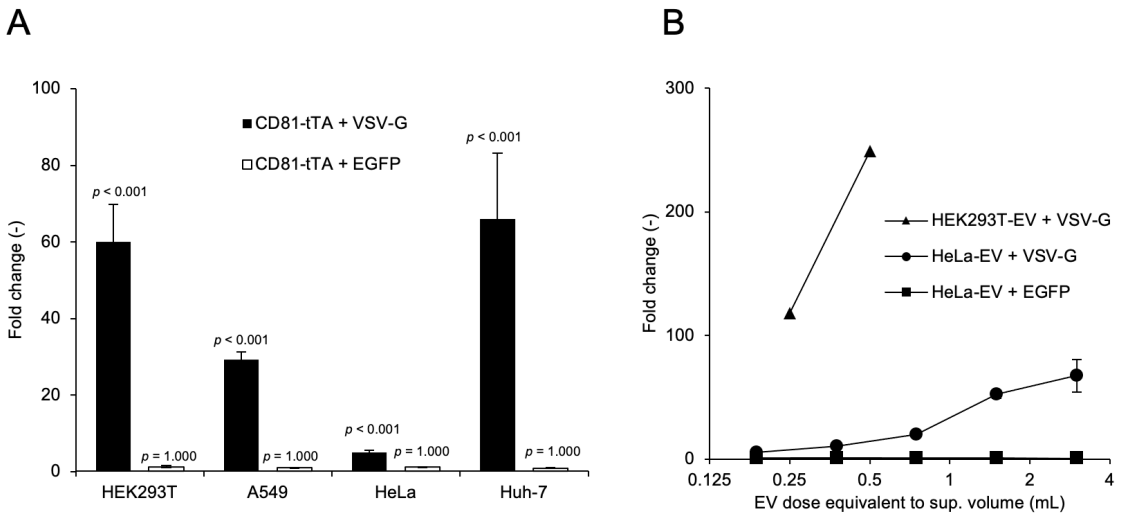

Fig. S1 ETTD assay using four cell lines

(A) Recipient HEK293T, A549, HeLa, and Huh-7 cells transfected with TRE3G-NlucP and TEVp were treated with HEK293T-derived EVs containing tTA-fused CD81 and VSV-G or EGFP.

(B) Recipient HEK293T cells were treated with HeLa-derived concentrated EVs containing tTA-fused CD81 and VSV-G or EGFP. EV dose was expressed as equivalent volume of parental supernatant.

Nluc activity was normalized by the luminescence signal of each non-treatment recipient cell and expressed as fold change. N = 3, mean ± SD. Statistical analysis was performed using one-way ANOVA followed by *post hoc* Dunnett's tests of each treatment group versus non-treatment control.

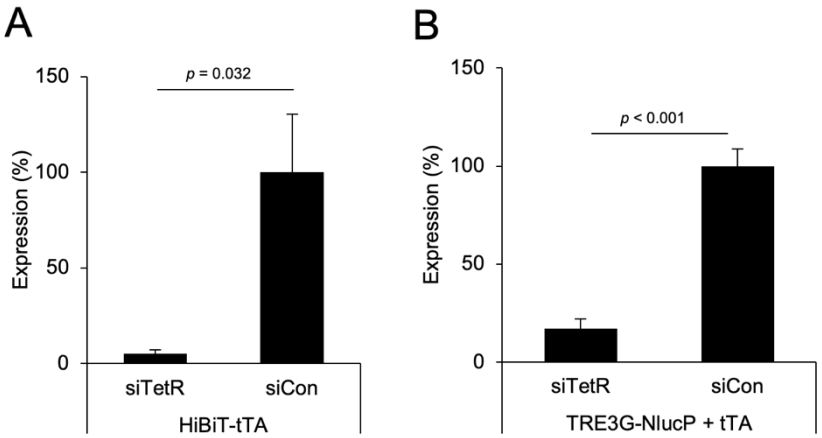

Fig. S2 Knockdown of tTA by siRNA

(A) HEK293T cells were co-transfected with HiBiT-tagged tTA and siTetR (targeting tTA) or siCon (targeting firefly luciferase, negative control) and the expression level of tTA was evaluated by measuring the HiBiT tag. The luminescence signal observed in the siCon sample was set as 100%.

(B) HEK293T cells were transfected with plasmids encoding TRE3G-NlucP and tTA, and either siTetR or siCon. The expression level of NanoLuc was measured after 48 h and the luminescence signal observed in the siCon sample was set as 100%.

N = 3, mean ± SD, Statistical analysis was performed using Student's *t*-test.

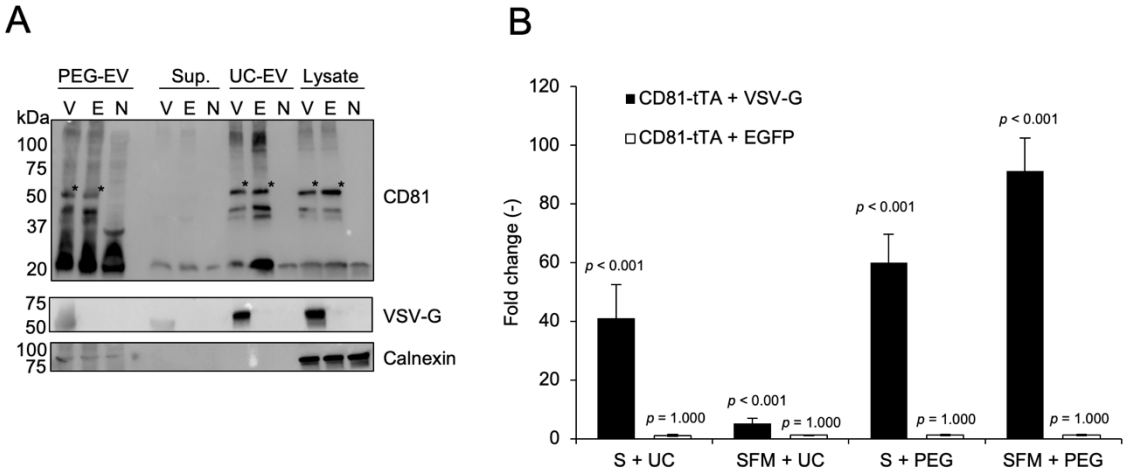

Fig. S3 ETTD assay using four different EV preparations

(A) EVs containing CD81-tTA in the serum-containing medium were concentrated by either PEG precipitation (PEG-EV) or ultracentrifugation (UC-EV) and analyzed by western blotting. Crude supernatant (Sup.) and total cell lysate (Lysate) were also analyzed. Antibodies used to probe the proteins are indicated on the right. Asterisks indicate CD81-tTA. Bands that appeared around 20 kDa are endogenous CD81 and other bands are likely polymerized proteins or degraded proteins. V, CD81-tTA + VSV-G; E, CD81-tTA + EGFP; N, no transfection. The expected mass based on the amino acid sequences were as follows: CD81-tTA, 63.4 kDa; VSV-G, 57.7 kDa; calnexin, 67.6 kDa (observed mass was approximately 90 kDa probably due to post-translational modification).

(B) Recipient HEK293T cells were treated with four EV preparations; serum-containing supernatant and ultracentrifugation (S + UC); serum-free medium supernatant and ultracentrifugation (SFM + UC); serum-containing supernatant and PEG precipitation (S + PEG); serum-free medium supernatant and PEG precipitation (SFM + PEG). Donor cells expressed CD81-tTA with VSV-G or EGFP. The applied volume of concentrated EVs was 10 μL for all preparations, which is corresponding to the 250 μL of the original conditioned medium.

N = 3, mean ± SD. Statistical analysis was performed using one-way ANOVA followed by *post hoc* Dunnett's tests of each treatment group versus non-treatment control.