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Supplemental information

CAD increases the long noncoding RNA *PUNISHER* in small extracellular vesicles and regulates endothelial cell function via vesicular shuttling

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Primers for Taqman RT-qPCR

Taqman RT-qPCR primers: *MALAT1* (Hs01910177_s1, Thermo Fisher Scientific); *GAS5* (Hs05021116_g1, Thermo Fisher Scientific); *H19* (Hs00399294_g1, Thermo Fisher Scientific); *PUNISHER* (Hs01096080_s1, Thermo Fisher Scientific); *VEGFA* (Hs00900055_m1, Thermo Fisher Scientific); *hnRNPK* (Hs03989611_gH, Thermo Fisher Scientific), and *GAPDH* (Hs02758991_g1, Thermo Fisher Scientific).

Antibodies for western blotting

Primary antibodies used: anti-CD81 (1:1000; Santa Cruz, #sc-166029); anti-CD9 (1:1000; Cell Signaling Technology, #13403); anti-Syntenin1 (1:1000; Abcam, #ab133267); anti-albumin (1:2000; Abcam, #ab10241); anti-β-Actin (1:2500; Sigma-Aldrich, #A1978); anti-hnRNPK (1:1000; Abcam, #ab39975); anti-VEGFA (1:1000; Novus Biologicals, #NB100-2381); anti-Histone H3 (1:1000; Abcam, #1791). Secondary antibodies used: anti-Rabbit IgG (1:1000; Sigma-Aldrich, #A9169) or anti-Mouse IgG (1:3000; Sigma-Aldrich, #A9044).

siRNAs for EC knockdown

All siRNAs used in these studies were purchased from Thermo Fisher Scientific: *PUNISHER* siRNA (Assay ID: n272074, Thermo Fisher Scientific), *hnRNPK* siRNA (Assay ID: s6737, Thermo Fisher Scientific), or control siRNA (#AM4611, Thermo Fisher Scientific).

Proliferation assay by fluorescence microscopy

Bromodeoxyuridine (BrdU; 10 μM stock solution, BD Biosciences, #550891) was added to the cell medium and cultured for 6 hours. ECs were fixed and denatured, followed by the detection of BrdU incorporation using rat anti-BrdU antibody (Abcam, #ab6326) and anti-rat-Cy3 (Jackson ImmunoResearch, #712-165-150) secondary antibody. Nuclei were counterstained with DAPI

(Vector laboratories, #H-1500-10). A Zeiss Axiovert 200M microscope and ZEN 2.3 pro software were used to take images.

Spheroid sprouting assays

Spheroid assays were performed as previously described[1]. *In vitro* angiogenesis was quantified by measuring the cumulative length of all sprouts of each spheroid or the maximal distance of the migrated cells using digital imaging analysis software (AxioVision Rel. 4.8, Carl Zeiss). 10 spheroids were analyzed for each experiment.

Nanoparticle tracking analysis

Size and concentration distribution of plasma sEVs and endothelial sEVs were performed by using nanoparticle tracking analysis (NTA) with a Nanosight NS 300 (Malvern Instruments, UK). Each sample was recorded 5 times for 60 seconds at a speed level of 20. The analysis was performed by setting a detection threshold of 6. PBS was used to perform a background measurement, in order to confirm the absence of residual particles. The NTA software (version 3.1 Build 3.1.46) was used to record and analyze the samples.

Absolute RT-qPCR analysis of *PUNISHER* expression

The absolute expression of *PUNISHER* was determined by using a standard-curve method with a plasmid containing the *PUNISHER* sequence, GenScript, vector name: pUC57, length: 201bp. The plasmid sequence is shown here:

5'-*ACGGCGGCCCACAGCTGGCGGCCCAGCGGCTCCTCCGAGGTGCTCAGCGGCGCCAG*
GAACAGTAGCTGCTCGTACTTGGCGCGAATCCACGACTCGCGCTCCTCCCTGCAAGACC
AGGGATCAACGGAAAAGGCTCTAGGGACCCCCAGCCAGGACTTCTGCCCCTACCCACGG
GACCGTCTCAGGTTCGCACACCCTCAG-3'

Subcellular fractionation of RNA

Fractionation of nuclear and cytoplasmic RNA was carried out with the cytoplasmic and nuclear RNA purification kit (Norgen Biotek, #21000), strictly following the manufacturer's protocol. RNA quality and concentration were assessed using a NanoDrop 2000 (Thermo Fisher Scientific). To isolate nuclear and cytoplasmic RNAs, ECs were washed once with 1xPBS and detached from the dish. After two washes with ice-cold PBS, pellets were resuspended in 200 µl of Buffer A (10 mM Tris pH=8; 140 mM NaCl; 1.5 mM $MgCl_2$; and 0.5% Nonidet P-40) and incubated on ice for 5 min, with gentle flicking of the tube every 90 sec. Following incubation, the suspension was centrifuged at 1000xg at 4°C for 3 min. The supernatant (containing the cytoplasmic fraction) was collected and loaded unto spin columns, according to manufacturer's protocol. The cell pellet was washed twice with Buffer A and resuspended in Buffer B (Buffer A + 1% Tween-40; 0.5% deoxycholate). After centrifugation at 1000xg at 4°C for 3 min, the supernatant was discarded. The pellet (containing nuclei) was resuspended in Buffer B and processed in order to obtain nuclear fractions.

Subcellular fractionation of protein

Protein extraction from nucleoplasm and cytoplasm was conducted with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, #78833) by following the manufacturer's protocol. ECs were detached with a solution of trypsin-EDTA and then centrifuged at 500×g for 5 minutes. The cells were resuspended in PBS, counted, and then 8×10^6 cells were centrifuged at 500×g for 3 minutes. The supernatant was discarded and the cell pellets were dried. The dried pellet was resuspended with 500 µl ice-cold Cytoplasmic Extraction Reagent I (CER I), then the tube was incubated on ice for 10 minutes. Following this,

27.5 µl ice-cold Cytoplasmic Extraction Reagent II (CER II) was added and incubated on ice for 1 minute. The tube was then centrifuged at 16,000×g for 5 minutes. The supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube and stored at -80°C until it was used. Finally, the nuclear pellet was resuspended in ice-cold Nuclear Extraction Reagent (NER). The sample was vortexed for 15 seconds every 10 minutes, for a total of 40 minutes. The pellet was centrifuged at 16,000×g for 10 minutes. The supernatant (nuclear extract) was transferred to a pre-chilled tube and then stored at -80°C until it was used.

Immunocytochemical staining

Immunocytochemistry of ECs (ECs) was performed by using anti-hnRNPK antibody (Abcam, #ab39975) and Phalloidin–Atto 594 (Sigma-Aldrich, #51927-10NMOL). 3×10^4 ECs per well were grown in a 4-well chamber slide (Milipore, #PEZGS0416). Twenty-four hours after seeding, cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at RT. Fixed cells were washed with PBS and incubated with 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at RT for permeabilization of the cell membranes. After three washing steps with PBS, cells were incubated with the blocking solution (0.25% Triton X-100; 1% bovine serum albumin (BSA) in PBS) for 1 hour at RT. Subsequently, cells were incubated with anti-hnRNPK antibody (1:500) in the blocking solution overnight at 4°C. After extensive washing with PBS, the cells were incubated with Alexa Fluor-488 conjugated secondary antibody (Thermo Fisher Scientific) and Phalloidin–Atto 594 (Sigma-Aldrich, 51927-10NMOL) for 60 min at RT. After washing with PBS, DAPI (Sigma-Aldrich) staining was applied and the chamber slide was mounted by using ProLong Gold Antifade (Invitrogen, P36941). Images were taken with a Zeiss Axio Observer inverted microscope and analyzed with the ZEN 2.3 pro software.

Scratch-wound assay

A scratch wound was made in the middle of the culture dish with a sterile p10 pipette tip. Cell migration was observed by capturing bright-field images of the dish at different time points (0, 4, 8, and 12 hours). The tendency of cells to migrate towards the scratch was measured by using digital image analysis software (AxioVision Rel.4.8, Carl Zeiss). The rate of migration was calculated by quantifying the total distance covered by the cells from the edge of the scratch toward the center of the scratch.

Boyden chamber assay

1×10^5 ECs were seeded onto the upper compartment of a Boyden chamber (BD Falcon) with trans-well polycarbonate inserts (8.0 μm pore size) and the cells were allowed to migrate for 4 hours. Following this incubation, cells on the upper side of the insert were scraped off with a cotton swab. The inserts were fixed with 4% paraformaldehyde following staining with DAPI (Vector laboratories, #H-1500-10). Cell migration was quantified by counting cells from three randomly selected fields from each well.

EXOCET exosome quantitation assay

The exosome concentration was measured by using an EXOCET Exosome Quantitation Kit (System Biosciences, EXOCET96A-1), according to the manufacturer's protocol. Briefly, sEVs were first lysed with exosome lysis buffer, and then the esterase activity of cholesteryl ester transfer protein (CETP) was measured with an Infinite M200 Microplate reader (Tecan) at a wavelength of 405 nm.

Table S1. Representative samples of RNA purity

Sample	Concentration (ng/μl)	260/280	260/230
Plasma-sEV-1	38.3	1.86	1.18
Plasma-sEV-2	19.5	1.95	1.93
Plasma-sEV-3	19.3	1.92	1.18
Plasma-sEV-4	15.4	1.93	1.33
Plasma-sEV-5	24.3	1.99	1.66
HCAEC-1	203.8	1.86	1.45
HCAEC-2	208.1	1.85	1.13
HCAEC-3	214.7	1.84	1.58
HCAEC-4	199.4	1.86	1.31
HCAEC-5	165.8	1.86	1.25
HCAEC-sEV-1	67.9	1.91	0.97
HCAEC-sEV-2	46.5	1.88	0.99
HCAEC-sEV-3	67.9	1.91	0.85
HCAEC-sEV-4	60.7	1.91	0.97
HCAEC-sEV-5	50	1.87	0.93

260/280. ratio of absorption at 260 nm to 280 nm; 260/230. ratio of absorption at 260 nm to 230 nm; HCAEC. human coronary artery endothelial cell; sEV. small extracellular vesicles.

Table S2. Function and clinical relevance of selected lncRNAs in atherosclerosis

lncRNAs	Function in Atherosclerosis	Clinical relevance	Reference
MALAT1	Promotes angiogenesis, regulates EC function	MI (pheripheral blood)	42-43
GAS5	EC activation, EC proliferation, SMC phenotypic changes	CAD, Biomarker of CAD (plasma)	44-45
AGAP2-AS1 (PUNISHER)	Promotes angiogenesis, regulates EC function	Unknown	46
H19	Promotes angiogenesis, regulates EC function	CHD (serum and whole blood)	47-48

MALAT1. metastasis-associated lung adenocarcinoma transcript1; GAS5. growth arrest-specific transcript 5; PUNISHER. AGAP2 antisense RNA 1; H19. imprinted maternally expressed transcript; CAD. coronary artery disease; CHD. coronary heart disease; MI. myocardial infarction; EC. endothelial cell; SMC. smooth muscle cell.

Table S3. Association of the level of PUNISHER with baseline characteristics

	Exp(B) (95% CI)	**P value**
Age	1.071 (0.979 to 1.171)	0.068
Male sex	0.340 (0.340 to 3.446)	0.361
Arterial hypertension	0.929 (0.140 to 6.166)	0.940
Hyperlipoproteinemia	0.979 (0.181 to 5.296)	0.980
HDL	0.953 (0.881 to 1.031)	0.234
Type I diabetes	1.746 (0.106 to 28.975)	0.697
Type II diabetes	10.954 (0.386 to 311.071)	0.161
Family history	1.417 (0.190 to 10.574)	0.734
Smoking	0.435 (0.067 to 2.806)	0.381
Angiotensin Converting Enzyme inhibitors (ACEI)	0.061 (0.002 to 2.381)	0.135
Angiotensin receptor blockers (ARB)	0.035 (0.001 to 1692)	0.090
Beta blockers	4.373 (0.322 to 59.398)	0.268
Calcium channel blockers (CCB)	2.144 (0.238 to 19.330)	0.497
Diuretics	0.780 (0.127 to 4.793)	0.789
Statins	1.457 (0.078 to 27.284)	0.801
Nitrates	9.259 (0.309 to 277.466)	0.200
Clopidogrel	0.550 (0.087 to 3.485)	0.525
Aspirin	0.371 (0.021 to 6.630)	0.501

The coefficient of the continuous variables was relative to 1-U differences. Binary logistic regression according to the median of PUNISHER level; CAD. coronary artery disease; Exp(B). exponentiation of the B coefficient; HDL. high-density lipoprotein.

Table S4. The prediction of PUNISHER–protein interaction partners

#	Protein ID	RNA ID	Z-score	Discriminative Power (%)	Interaction Strength (%)	Domain	Motif
1	LN28B_HUMAN	NR_027032.1.HOM_1_1428-1533	- 0.29	35	98	yes	yes
2	LN28B_HUMAN	NR_027032.1.HOM_1_1422-1533	- 0.13	50	100	yes	yes
3	LN28B_HUMAN	NR_027032.1.HOM_1_1420-1533	- 0.23	40	99	yes	yes
4	LN28B_HUMAN	NR_027032.1.HOM_1_1404-1533	- 0.34	32	97	yes	yes
5	LN28B_HUMAN	NR_027032.1.HOM_1_1411-1533	- 0.37	28	96	yes	yes
6	HNRNPK_HUMAN	NR_027032.1.HOM_1_1404-1533	- 0.44	22	94	yes	yes
7	HNRNPK_HUMAN	NR_027032.1.HOM_1_1428-1533	- 0.62	17	43	yes	yes
8	HNRNPK_HUMAN	NR_027032.1.HOM_1_1422-1533	- 0.58	17	65	yes	yes
9	HNRNPK_HUMAN	NR_027032.1.HOM_1_1411-1533	- 0.48	22	90	yes	yes
10	HNRNPK_HUMAN	NR_027032.1.HOM_1_1399-1533	- 0.42	24	95	yes	yes
11	SRSF2_HUMAN	NR_027032.1.HOM_1_1428-1533	- 0.68	14	21	yes	yes
12	HNRNPK_HUMAN	NR_027032.1.HOM_1_1420-1533	- 0.59	17	60	yes	yes
13	PCBP2_HUMAN	NR_027032.1.HOM_1_1404-1533	- 0.64	17	32	yes	yes
14	PCBP2_HUMAN	NR_027032.1.HOM_1_1399-1533	- 0.48	22	69	yes	yes
15	SRSF2_HUMAN	NR_027032.1.HOM_1_1422-1533	- 0.67	14	22	yes	yes
16	SRSF2_HUMAN	NR_027032.1.HOM_1_1420-1533	- 0.64	14	31	yes	yes
17	PCBP2_HUMAN	NR_027032.1.HOM_1_1411-1533	- 0.57	17	52	yes	yes
18	SRSF2_HUMAN	NR_027032.1.HOM_1_1404-1533	- 0.68	14	19	yes	yes
19	PCBP2_HUMAN	NR_027032.1.HOM_1_1388-1533	- 0.48	22	66	yes	yes
20	PCBP2_HUMAN	NR_027032.1.HOM_1_1420-1533	- 0.71	14	24	yes	yes
21	PCBP2_HUMAN	NR_027032.1.HOM_1_1411-1533	- 0.62	17	39	yes	yes
22	PCBP2_HUMAN	NR_027032.1.HOM_1_1404-1533	- 0.74	14	15	yes	yes
23	SRSF2_HUMAN	NR_027032.1.HOM_1_1411-1533	- 0.62	17	37	yes	yes
24	LN28B_HUMAN	NR_027032.1.HOM_1_1399-1533	- 0.56	17	57	yes	yes
25	PCBP2_HUMAN	NR_027032.1.HOM_1_1420-1533	- 0.74	14	15	yes	yes
26	PCBP2_HUMAN	NR_027032.1.HOM_1_1422-1533	- 0.84	14	7	yes	yes
27	PCBP2_HUMAN	NR_027032.1.HOM_1_1388-1533	- 0.53	20	49	yes	yes
28	PCBP2_HUMAN	NR_027032.1.HOM_1_1339-1533	- 0.01	59	92	yes	yes
29	PCBP2_HUMAN	NR_027032.1.HOM_1_1335-1533	- 0.06	56	85	yes	yes
30	HNRNPK_HUMAN	NR_027032.1.HOM_1_1388-1533	- 0.5	20	83	yes	yes

31	PCBP2_HUMAN	NR_027032.1.HOM_1_1428-1533	- 0.84	14	6	yes	yes
32	PCBP2_HUMAN	NR_027032.1.HOM_1_1422-1533	- 0.86	10	4	yes	yes
33	PCBP2_HUMAN	NR_027032.1.HOM_1_1380-1533	- 0.48	22	67	yes	yes
34	PCBP2_HUMAN	NR_027032.1.HOM_1_1428-1533	- 0.83	14	9	yes	yes
35	PCBP2_HUMAN	NR_027032.1.HOM_1_1399-1533	- 0.59	17	38	yes	yes
36	PCBP2_HUMAN	NR_027032.1.HOM_1_1374-1533	- 0.36	28	77	yes	yes
37	PCBP2_HUMAN	NR_027032.1.HOM_1_1365-1533	- 0.3	33	82	yes	yes
38	PCBP2_HUMAN	NR_027032.1.HOM_1_1345-1533	- 0.09	54	89	yes	yes
39	HNRNPK_HUMAN	NR_027032.1.HOM_1_1380-1533	- 0.48	20	86	yes	yes
40	PCBP2_HUMAN	NR_027032.1.HOM_1_1374-1533	- 0.4	26	81	yes	yes
41	LN28B_HUMAN	NR_027032.1.HOM_1_1388-1533	- 0.63	17	42	yes	yes
42	PCBP2_HUMAN	NR_027032.1.HOM_1_1380-1533	- 0.57	17	40	yes	yes
43	PCBP2_HUMAN	NR_027032.1.HOM_1_1363-1533	- 0.36	28	66	yes	yes
44	PCBP2_HUMAN	NR_027032.1.HOM_1_1357-1533	- 0.44	22	51	yes	yes
45	PCBP2_HUMAN	NR_027032.1.HOM_1_1348-1533	- 0.27	35	68	yes	yes
46	PCBP2_HUMAN	NR_027032.1.HOM_1_1399-1533	- 0.64	14	22	yes	yes
47	PCBP2_HUMAN	NR_027032.1.HOM_1_1388-1533	- 0.64	14	25	yes	yes
48	PCBP2_HUMAN	NR_027032.1.HOM_1_14365-1533	- 0.45	22	68	yes	yes
49	PCBP2_HUMAN	NR_027032.1.HOM_1_1380-1533	- 0.69	14	16	yes	yes
50	PCBP2_HUMAN	NR_027032.1.HOM_1_1363-1533	- 0.58	17	36	yes	yes
51	PCBP2_HUMAN	NR_027032.1.HOM_1_1357-1533	- 0.68	14	20	yes	yes
52	SRSF2_HUMAN	NR_027032.1.HOM_1_1374-1533	- 0.6	17	45	yes	yes
53	SRSF2_HUMAN	NR_027032.1.HOM_1_1357-1533	- 0.67	14	18	yes	yes
54	HNRNPK_HUMAN	NR_027032.1.HOM_1_1374-1533	- 0.57	17	62	yes	yes
55	SRSF2_HUMAN	NR_027032.1.HOM_1_1365-1533	- 0.58	17	42	yes	yes
56	SRSF2_HUMAN	NR_027032.1.HOM_1_1363-1533	- 0.62	17	30	yes	yes
57	PCBP2_HUMAN	NR_027032.1.HOM_1_1345-1533	- 0.43	24	60	yes	yes

The ranking (#) of PUNISHER interaction proteins (predicted by catRAPID omics algorithm)

Table S5. Expression of human functional long noncoding RNAs

Table S6. The prediction of RNA–protein interaction based on interaction score by RNAInter database

Figure S1

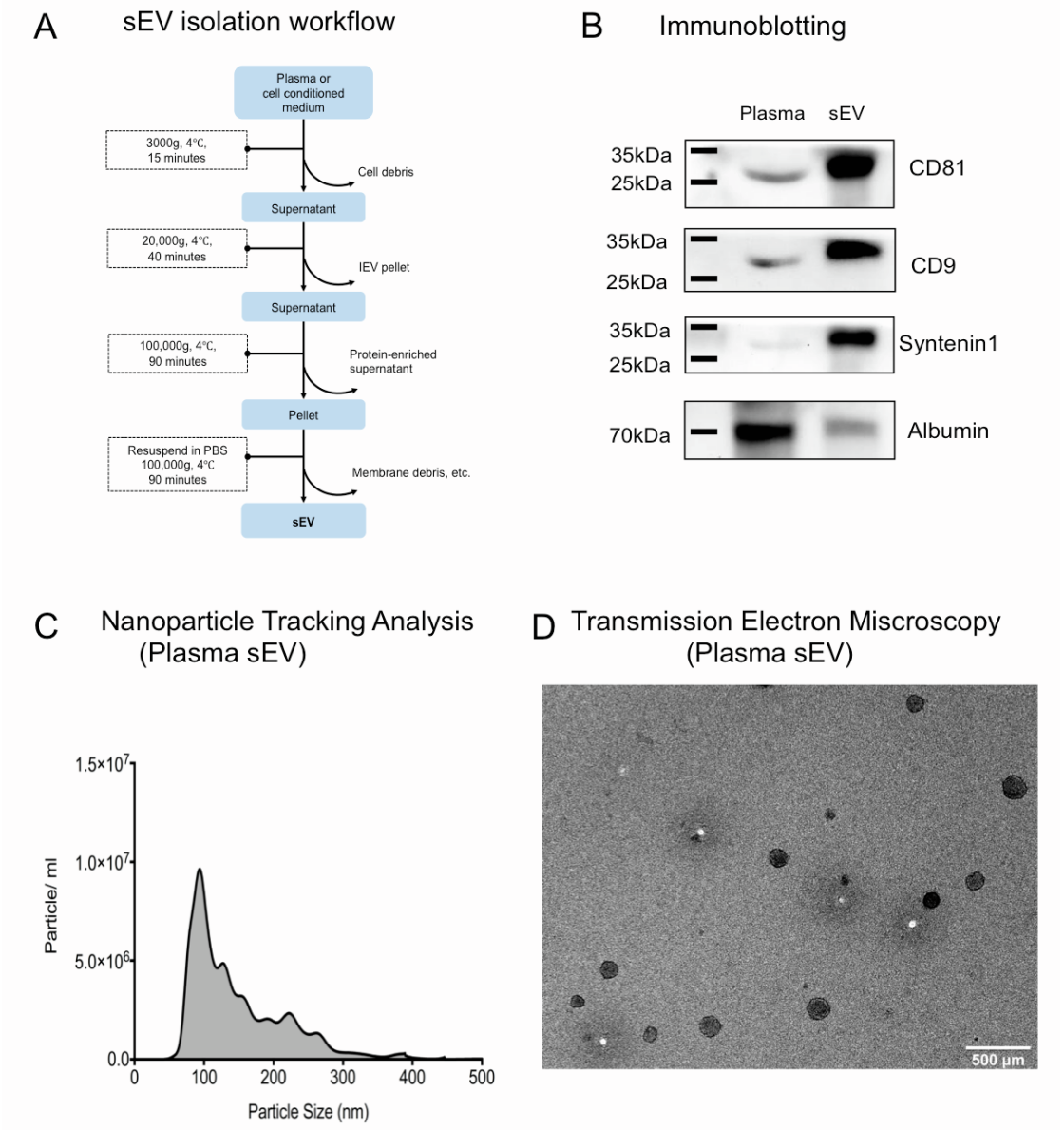

Figure S1. Plasma sEV identification

(A) Workflow for the isolation of sEV from plasma. (B) Western-blot analysis of the expression of the sEV markers, CD81, CD9, and Syntenin1 in plasma as well as in sEV. Albumin is used as a control protein. (C) Nanoparticle tracking analysis (NTA) was used to determine the size and concentration of sEV in the plasma of patients. (D) Transmission electron microscopic (TEM) image of pelleted sEV (diameter ~30–150 nm) derived from the plasma of patients. sEV, small extracellular vesicles.

Figure S2

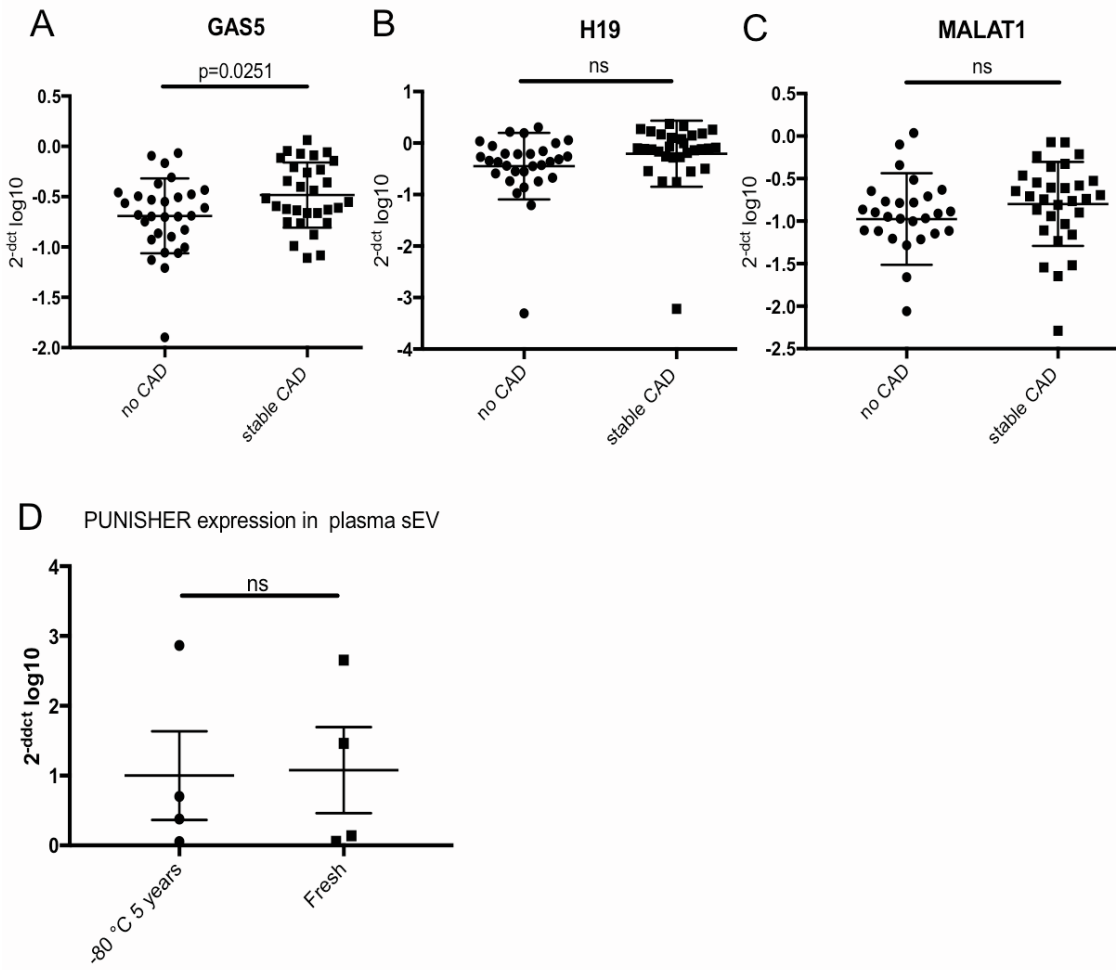

Figure S2. Analysis of lncRNA expression in circulating sEV from patients with or without CAD

(A-C) In the validation phase, *GAS5*, *H19*, and *MALAT1* were quantified in isolated circulating sEV from non-CAD and stable CAD patients by RT-qPCR. Values were normalized to *GAPDH* (n=30, Student t-test). (D) *PUNISHER* expression was quantified in sEV under different plasma storage conditions (ns: not significant, n=4, by Student t-test). *MALAT1*, metastasis associated lung adenocarcinoma transcript 1; CAD, coronary artery disease; sEV, small extracellular vesicles.

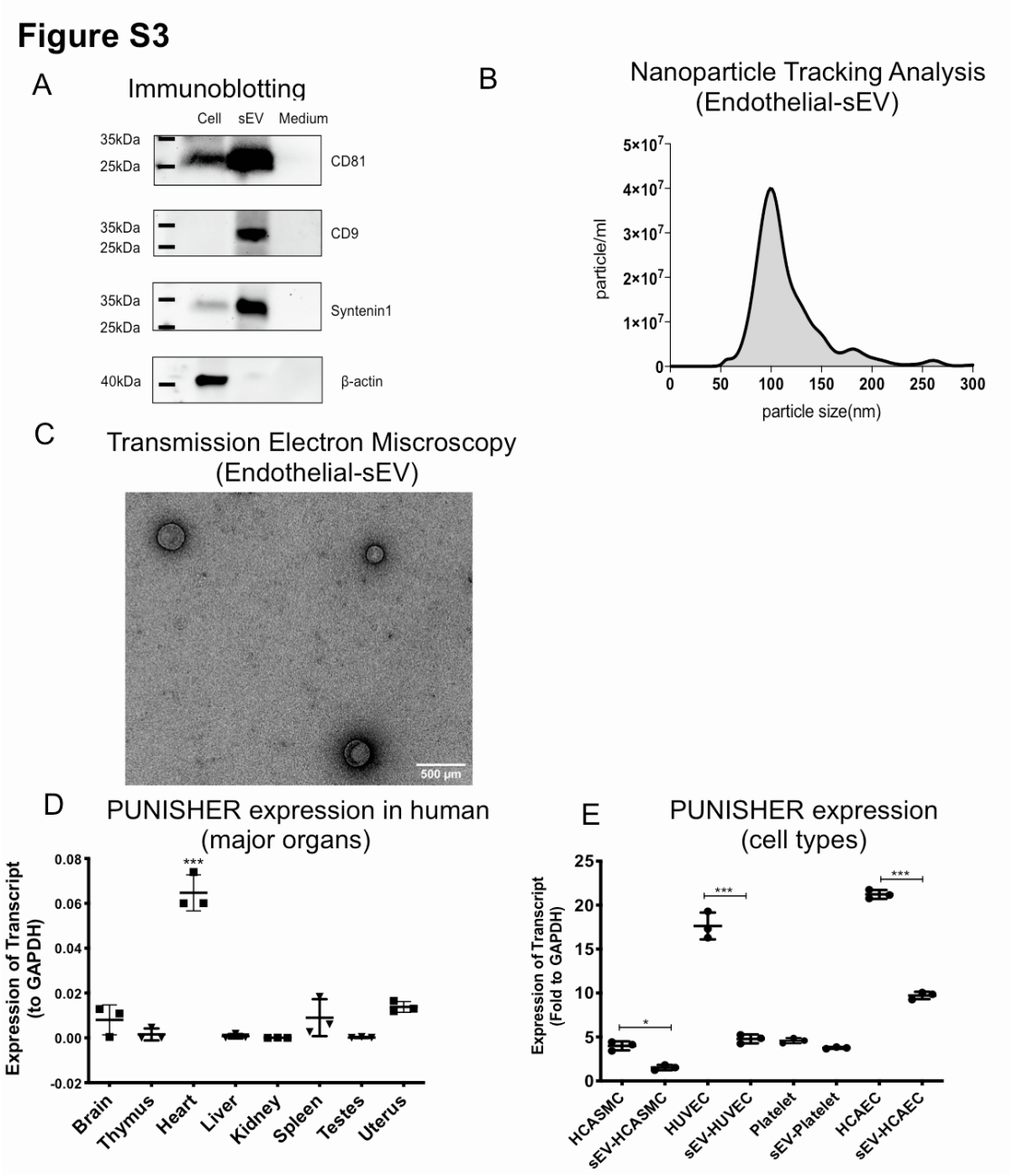

Figure S3. Endothelial-sEV identification

(A) Western blotting of the expression of the sEV markers (CD81, CD9, and Syntenin1) from lysed ECs, sEV, and conditioned-cell medium. β-Actin acts as a marker for the cell lysate. (B) Nanoparticle tracking analysis (NTA) was used to examine the size and concentration of sEV *in vitro*. (C) Transmission electron microscopic (TEM) image of pelleted sEVs (diameter ~30–150 nm) derived from ECs. (D) Expression profiling of *PUNISHER* RNA in 10 major human tissues. Results from RT-PCR by using cDNA of 10 different commercially available tissues (major organs) from humans. Purified RNA was purchased from commercial vendors as follows: Human Total RNA Master Panel II

(Clonetech, #636643) (LOT NUMBER 1202050A); human heart (Amsbio, #R1234122-50, Lot No. A804058). *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used to normalize the data. (E) Expression of *PUNISHER* in different cell types and their corresponding sEV. (*p<0.05, ***p<0.001, n=3, by Student t-test). sEV, small extracellular vesicles; EC, endothelial cells; HCASMC, human coronary artery smooth muscle cells; HUVEC, human umbilical cord endothelial cells; HCAEC, human coronary artery endothelial cells.

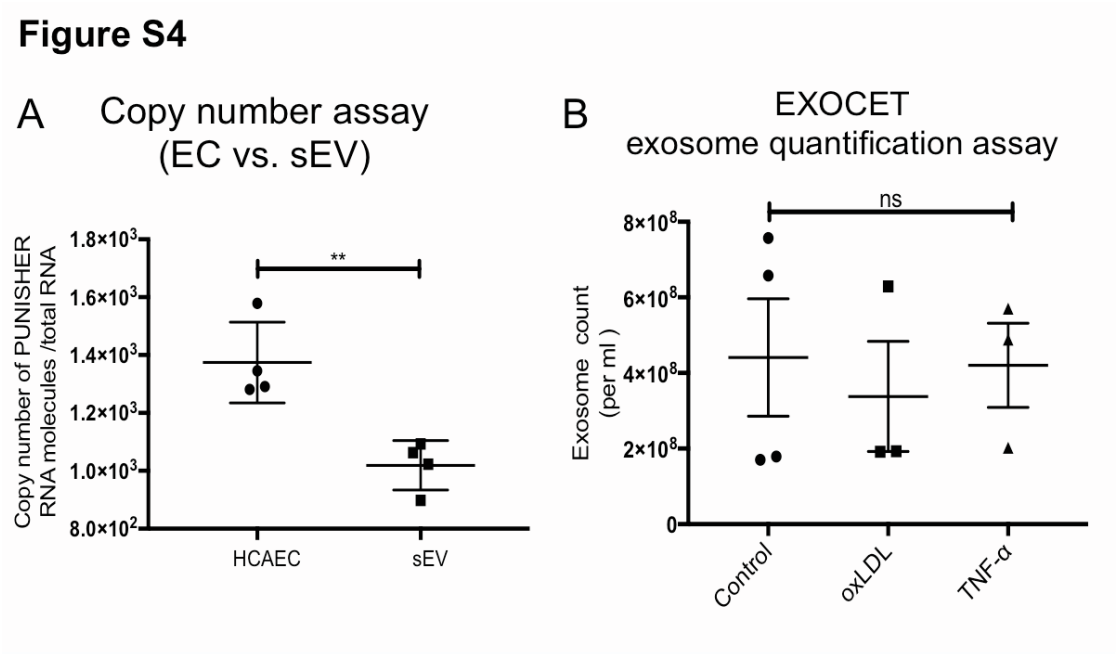

Figure S4. Exosome quantitation assay

(A) Absolute PCR analysis (copy number assay) of *PUNISHER* in HCAECs and the corresponding sEV. (n=4, by Student t-test). (B) EXOCET exosome quantitation assay was used to determine the exosome concentration. Parent ECs were stimulated with oxLDL, TNF-α, or vehicle, then sEV were isolated from the parent cells and quantified (n=3, by 1-way ANOVA with Bonferroni's multiple comparisons test). sEV, small extracellular vesicles; ECs, endothelial cells; oxLDL, oxidized low-density lipoprotein; TNF-α, tumor necrosis factor alpha.

Figure S5

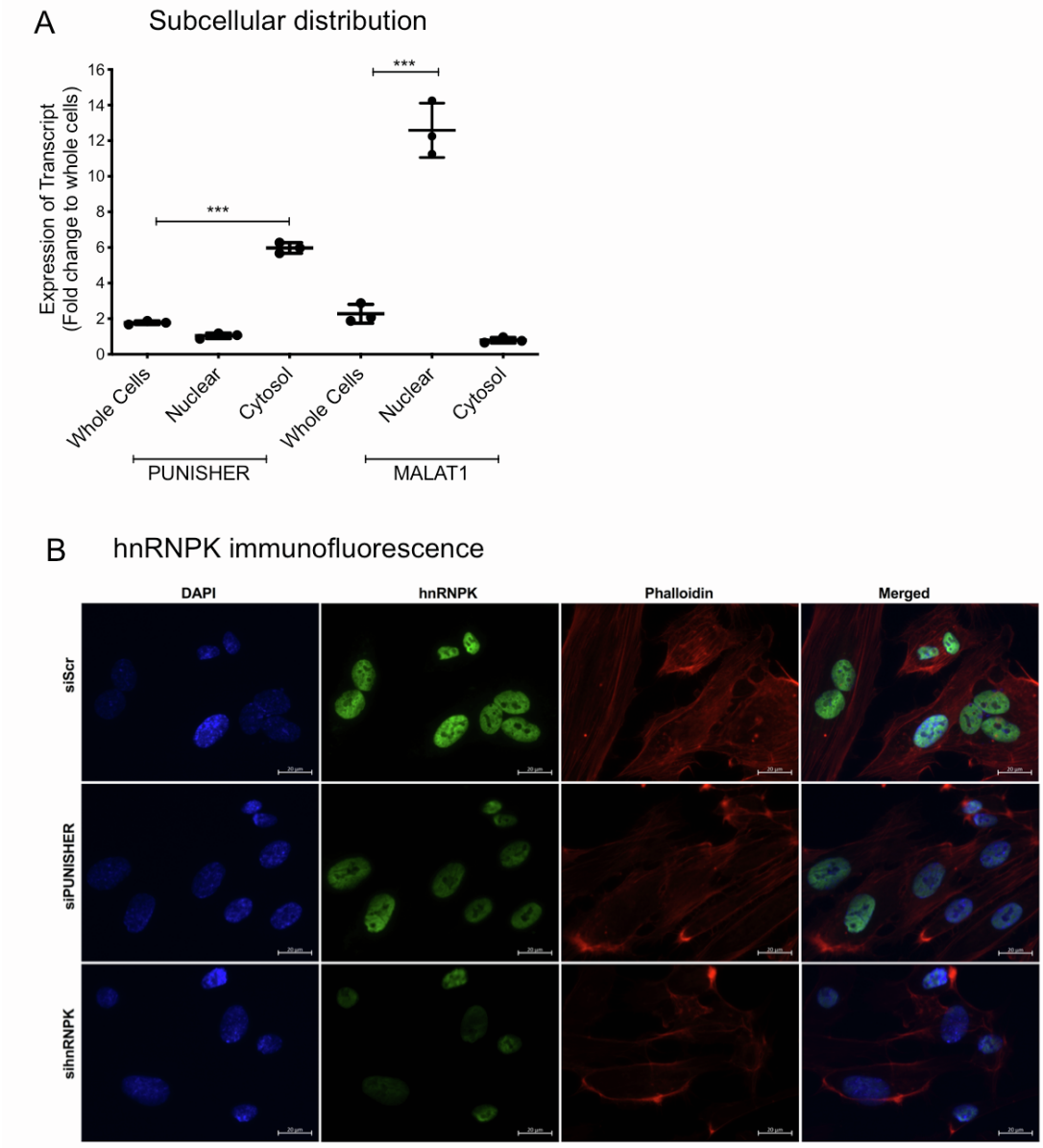

Figure S5. Subcellular localization of PUNISHER and hnRNPK protein knockdown

(A) Shown is the subcellular localization of *PUNISHER* to the cytosol and nucleus, which was quantified using RT-qPCR via RNA fractionation. Interestingly, *PUNISHER* is highly enriched in the cytosol compared to the nuclear fraction of endothelial cells (HUVECs). One of the most-studied lncRNAs *MALAT1* demonstrates a predominantly nuclear localization, which was used here as a control (***p<0.001, n=4, by Student t-test). lncRNA, long noncoding RNA. (B) Immunocytochemistry of hnRNPK (green), nuclear counterstaining with DAPI (blue), and F-actin staining with Phalloidin (red) in ECs confirmed the reduced of expression of hnRNPK (63×). Upper left panel DAPI mono

staining, middle (left) Phalloidin mono staining, middle (right) hnRNPK mono staining, right panel overlay. Scale bar = 10 μm.

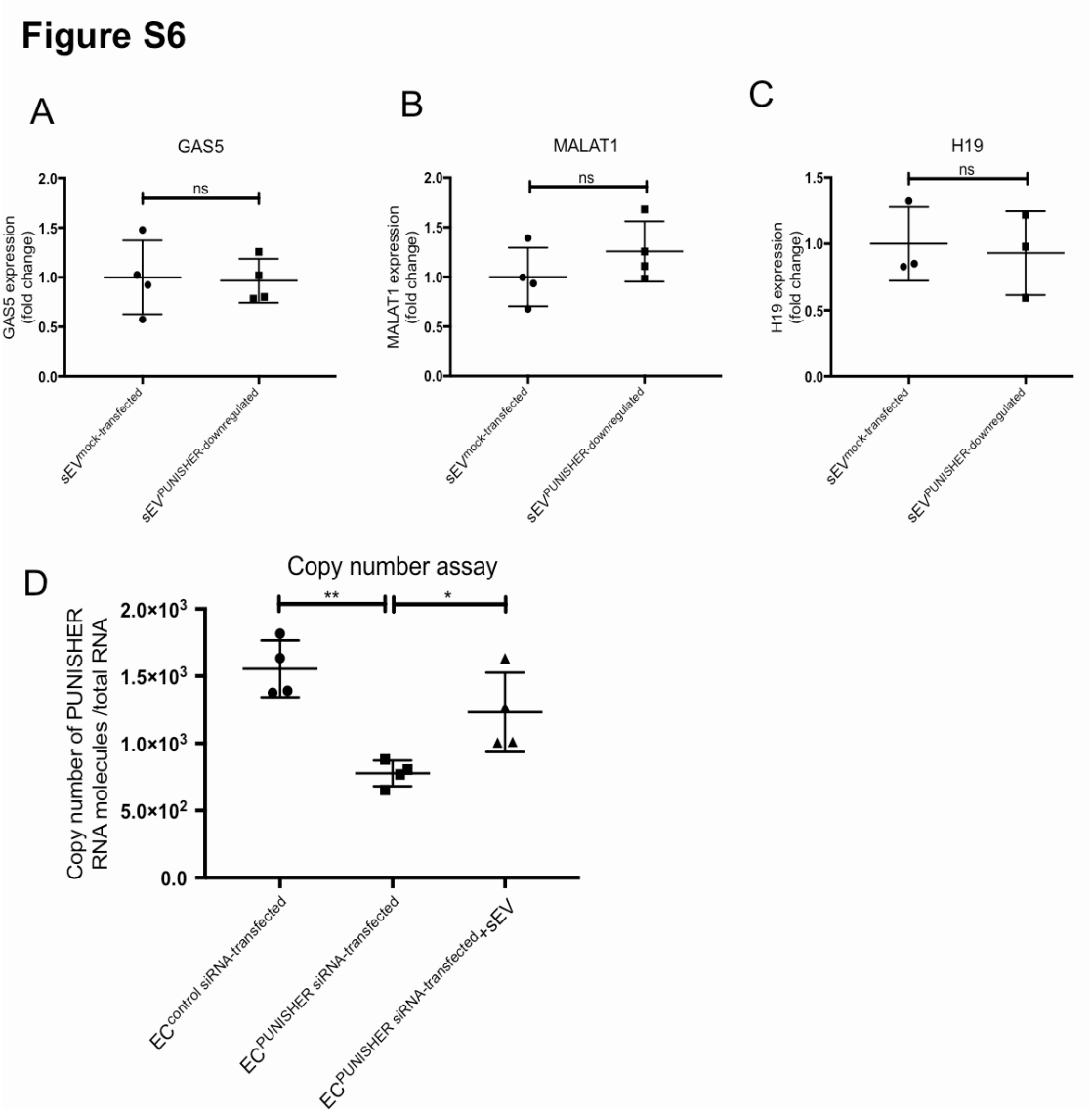

Figure S6. lncRNA expression in sEVs

(A-C) $sEV^{PUNISHER\text{-}downregulated}$ and $sEV^{mock\text{-}transfected}$ were separately derived from the corresponding parent ECs. *GAS5*, *MALAT1*, and *H19* were analyzed in $sEV^{PUNISHER\text{-}downregulated}$ and $sEV^{mock\text{-}transfected}$ by RT-qPCR, *GAPDH* was used as an endogenous control (n=4, by Student t-test). (D) Copy number analysis of *PUNISHER* transcripts in *PUNISHER*-downregulated recipient ECs by RT-qPCR (n=4, by Student t-test). sEV, small extracellular vesicles.

Figure S7

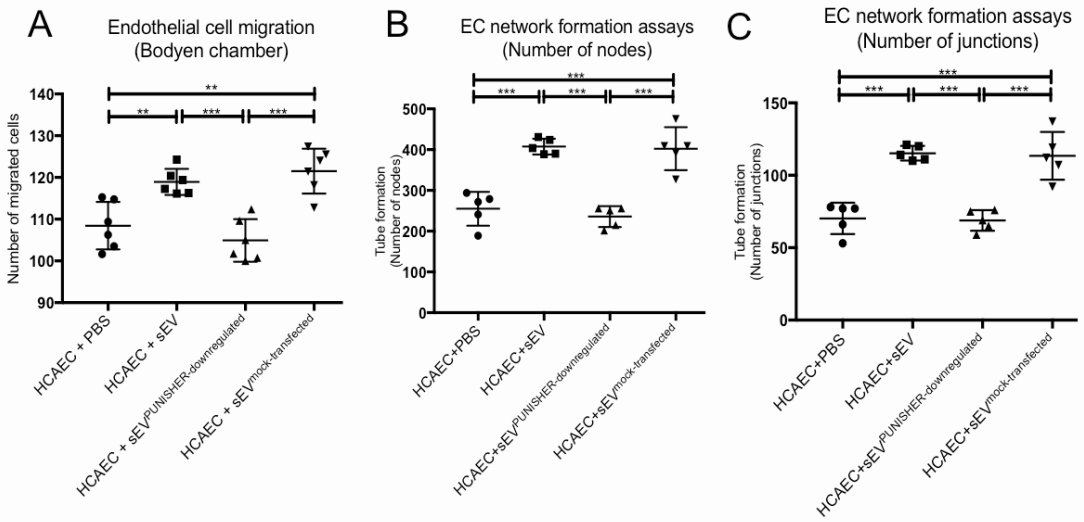

Figure S7. Endothelial sEV-incorporated *PUNISHER* regulates the angiogenic function of target ECs

$sEV^{PUNISHER\text{-}downregulated}$ and $sEV^{mock\text{-}transfected}$ were separately derived from parent EC. ECs in basal media were co-incubated with sEV, $sEV^{PUNISHER\text{-}downregulated}$, $sEV^{mock\text{-}transfected}$, or vehicle. (A) A Boyden chamber migration assay was performed on target ECs. Data are presented as the number of migrated cells (**p<0.01, ***p<0.001, n=6, by 1-way ANOVA with Bonferroni multiple comparisons test). (B-C) ECs in basal media were co-incubated with sEV, $sEV^{PUNISHER\text{-}downregulated}$, $sEV^{mock\text{-}transfected}$, or vehicle. Network formation assays were performed with ECs. Capillary tubes were imaged with an immunofluorescence microscope. The number of nodes and junctions were measured and quantitated by using ImageJ image-analysis software ((B) ***p<0.001, n=5, by 1-way ANOVA with Bonferroni multiple comparisons test). ((C)***p<0.001, n=6, by 1-way ANOVA with Bonferroni multiple comparisons test). sEV, small extracellular vesicles; ECs, endothelial cells.

Figure S8

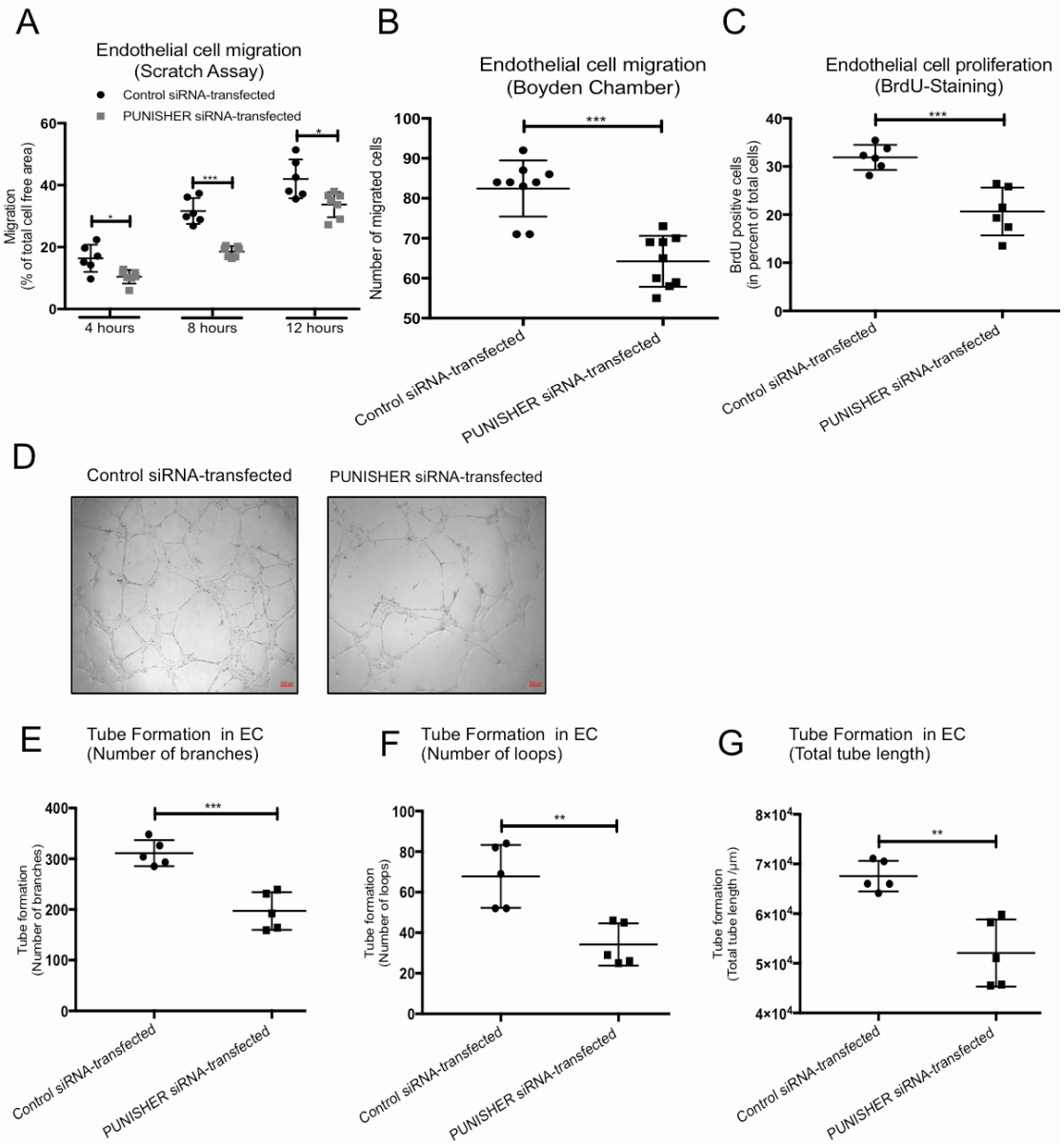

Figure S8. *PUNISHER* promotes angiogenesis in ECs

ECs were transfected with *PUNISHER* siRNA or control siRNA. (A) A scratch–wound assay was performed on donor ECs. Quantitative analysis of the migration of the cells was measured as a percentage of the total cell-free area (*p<0.05, ***p<0.001, n=6–7, by Student t-test). (B) A Boyden chamber assay was performed on ECs. Data are presented as the numbers of migrated cells (***p<0.001, n=9, by Student t-test). (C) BrdU incorporation was determined by immunofluorescence. The percentage of BrdU-positive cells was compared with the total number of cells (***p<0.001, n=6, by Student t-test). (D-G) Network formation assays in ECs. Capillary tubes were imaged with an

immunofluorescence microscope. The number of nodes, number of junctions, and total tube length were measured and quantitated by using ImageJ image-analysis software (**p<0.01, ***p<0.001, n=5, by Student t-test). Scale bar = 200 μm. ECs, endothelial cells.

Figure S9

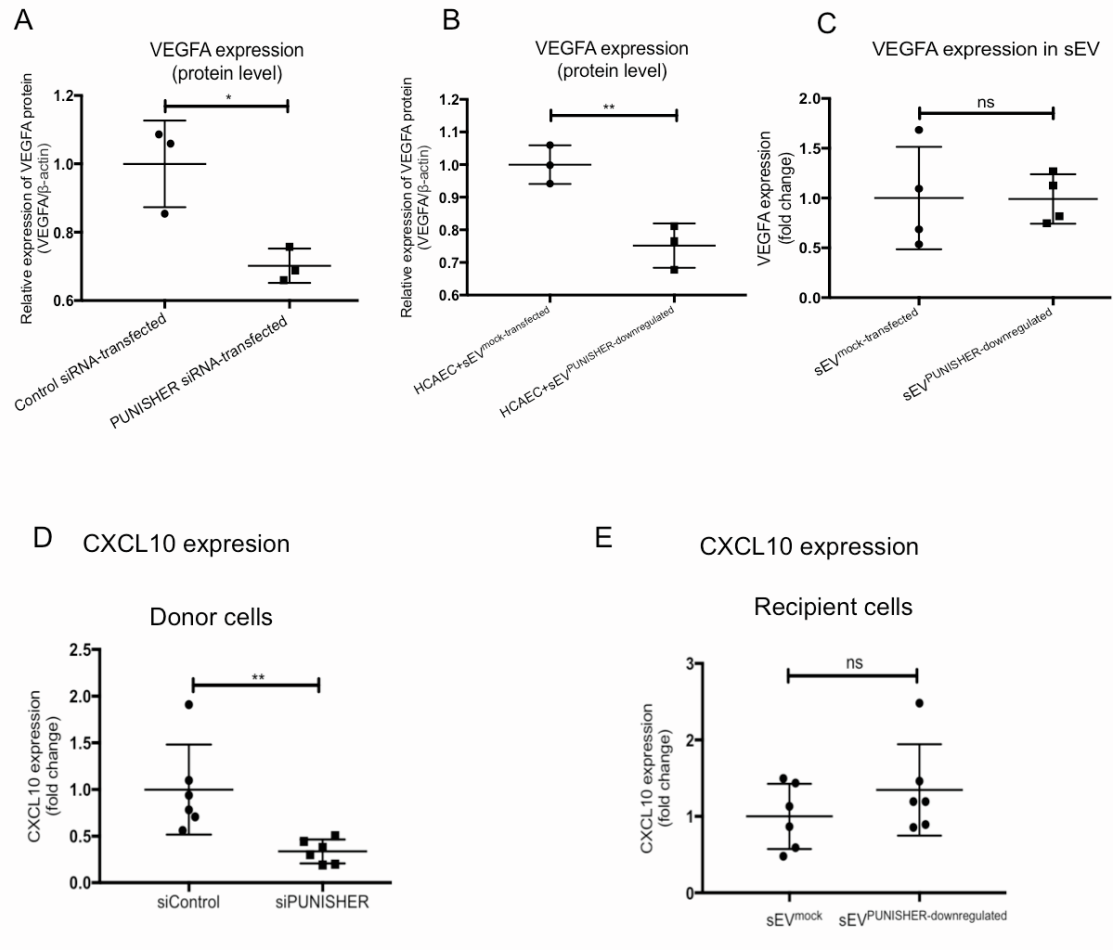

Figure S9. *PUNISHER* controls VEGFA mRNA and protein synthesis

(A-C) VEGFA protein levels in donor or recipient ECs were quantified from western blots. (*p<0.5, **p<0.01, n=3, by Student t-test). (D-E) *CXCL10* expression was analyzed in donor cells after siScr and siPUNISHER treatment and in recipient cells after $sEV^{PUNISHER\text{-}downregulated}$ and $sEV^{mock\text{-}transfected}$ by RT-qPCR. *GAPDH* was used as an endogenous control (**p<0.01, n=6, by Student t-test). sEV, small extracellular vesicles; ECs, endothelial cells; VEGFA, vascular endothelial growth factor A; CXCL10, C-X-C motif chemokine ligand 10.

Figure S10

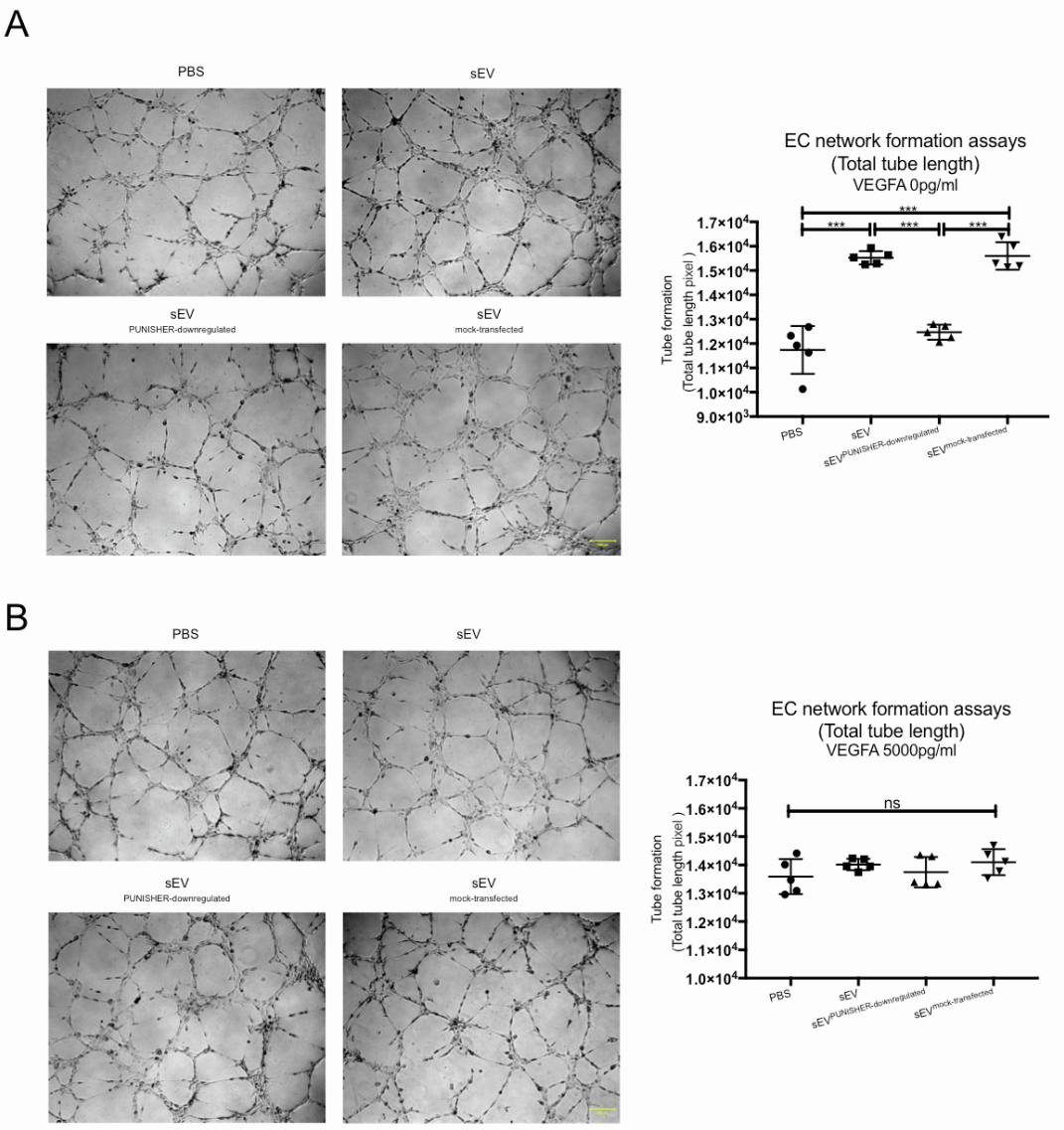

Figure S10. Effects of *PUNISHER* can be rescued by exogeneous VEGFA addition

ECs were transfected with *PUNISHER* siRNA or control siRNA, and the corresponding sEV were used to treat the recipient cells. (A-B) Network formation assays in ECs. Capillary tubes were imaged with an immunofluorescence microscope. Different concentrations of exogenous supplementation of VEGFA (0 pg/ml (A) or 5000 pg/ml (B)) were used. The number of nodes, number of junctions, and total tube length were measured and quantitated by using ImageJ image-analysis software (***p<0.001, n=5, by Student t-test). Scale bar = 200 μm. ECs, endothelial cells; VEGFA, vascular endothelial growth factor A.