Supplementary Material

Supplementary methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained and maintained as described before (Desch et al., 2016). Primary Human brain endothelial cells (HBMECs; ScienCell , USA) were grown in a 10 µg/ml Fibronectin (Sigma-Aldrich) coated surface in Endothelial Cell Medium (ECM) supplemented with 5% FBS (ScienCell), 1% endothelial cell growth supplements and 1% penicillin/streptomycin solution (ScienCell). The murine brain endothelial cell line (bEnd3; ATCC Genuine Cultures® CRL-2299™, USA) was grown in a 0.5% Gelatin coated surface in Dulbecco's Modified Eagle's Medium (DMEM) (Merck) supplemented with 10% FBS. The murine melanoma cell line Ret was maintained in RPMI-medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids, 1% glutamic acid and 1% penicillin/streptomycin.

Plasmatic VWF concentration

Plasmatic VWF concentration was calculated by ELISA using a polyclonal rabbit anti-VWF antibody (Dako, Copenhagen, Denmark) and a polyclonal rabbit peroxidase-labeled anti-human VWF antibody (Dako, Copenhagen, Denmark). Human Plasma was used to create a standard curve with a defined content of VWF.

Plasmatic ADAMTS13 activity

ADAMTS13 activity was analyzed in the plasma of patients using a commercial screening assay kit following the instructions of the manufacturer (Technoclone GmbH).

Isolation of platelets and erythrocytes

Platelets and erythrocytes were isolated from freshly drown citrate blood of human donors or mice according to the approval of the local ethics committee. Platelet-rich plasma (PRP) was

obtained by centrifugation (120 g, 15 min, RT) of citrated blood. PRP was then transferred into 1:1 washing buffer (103 mM NaCl, 5 mM KCl, 3 mM NaH_2PO_4* H_20, 5 mM HEPES, 5.5 mM Glucose) with a pH of 6.5 and supplemented with 1 U/ml of Apyrase (Sigma-Aldrich). After a second centrifugation step (120 g, 15 min, RT) the resulting platelet pellet was resuspended in 5% BSA supplemented washing buffer with a physiological pH of 7.4.

To provide a physiological haematocrit for the perfusion assays, erythrocytes from citrate blood were also isolated. The erythrocyte pellet was transferred into PBS (1:1) and centrifuged at 800 g for 10 minutes. The supernatant and buffy coat was aspirated and the same procedure was repeated. The erythrocytes were washed with HEPES (1:1) and centrifuged at 800 g for 10 minutes. The resulting supernatant was aspirated obtaining a pure pellet of erythrocytes.

Electric cell–substrate impedance sensing (Supplement)

Transendothelial electrical resistance was measured with electric cell-substrate impedance sensing (ECIS). ECs were seeded into gelatine-coated 8-well ECIS slides (8W1E PET; Applied BioPhysics Inc., NY, USA) at a concentration of $1x10^5$ cells/ well. Impedance at a frequency of 4,000 Hz was measured every 48 seconds (ECIS-zeta system; Applied BioPhysics Inc., NY, USA) while cells were continuously maintained in a humidified atmosphere at 37 °C and 5% CO_2. The influence of resting platelet or the supernatant of platelets activated with Collagen type I (50 µg/ml) with or without preincubation with tinzaparin (100 IU/ml) and bevacizumab (0.65 mg/mL) on transendothelial electrical resistance was analyzed.

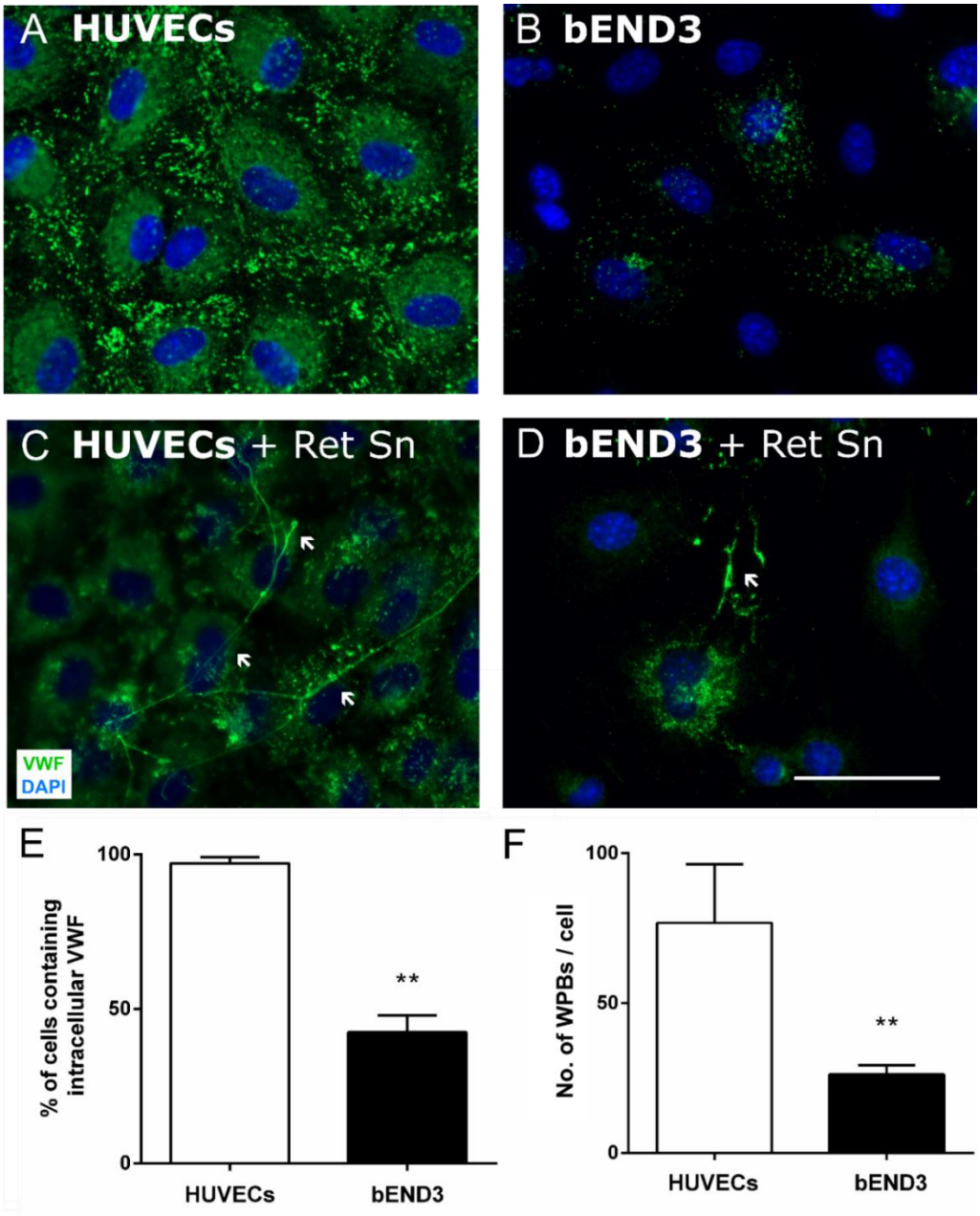
Quantitative real-time PCR (qRT-PCR)

RNA from HBMECs was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The cDNA was synthesized from 1 µg of total RNA per sample using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). To determine the mRNA transcript level from cDNA, quantitative real-time polymerase chain reaction (RT-qPCR) was performed using the QuantiFast SYBR Green PCR Kit (Qiagen, Germany) and specific primers to VWF, PAR-1, VEGFR-1, VEGFR-2, p-selectin and β-actin for normalization.

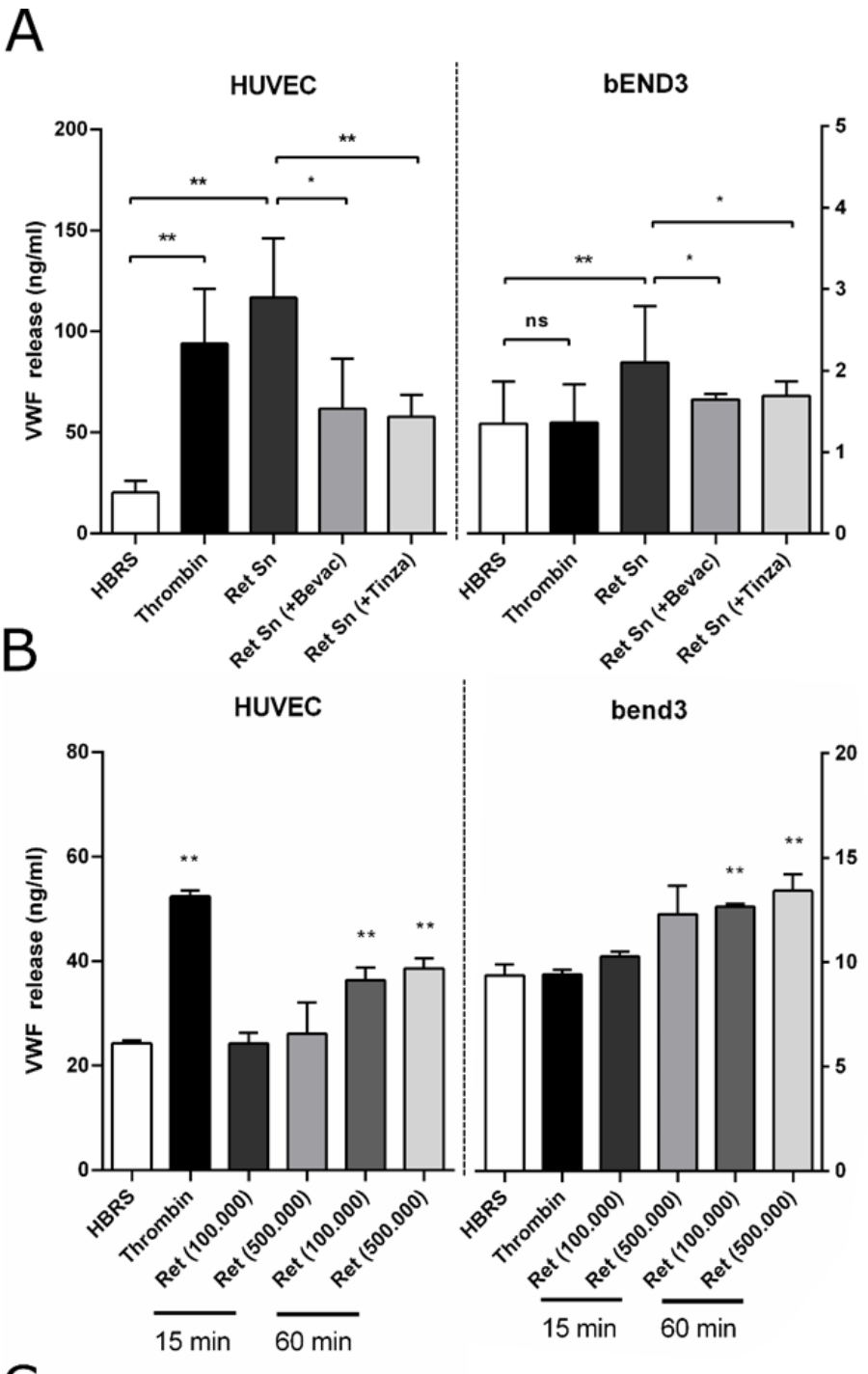
Supplemental Table 1. Primers used:

Targeted gene	Sequence	
VWF	Forward	5'-TGGTGCAGGATTACTGCGGC-3'
	Reverse	5'-GCTTTGCCCAGCAGCAGAAT-3'
PAR-1	Forward	5'-CCTGCTTCAGTCTGTGCGG-3'
	Reverse	5'-CTGGTCAAATATCCGGAGGCA-3'
VEGFR-1	Forward	5'-GCAAAGCCACAACCAGAAG-3'
	Reverse	5'-ACGTTCAGATGGTGGCCAAT-3'
VEGFR-2	Forward	5'-CGTGTCTTTGTGGTGCACTG-3'
	Reverse	5'-GGTTTCCTGTGATCGTGGGT-3'
P-selectin	Forward	5'-CGTGGAATGCTTGGCTTCTG-3'
	Reverse	5'-TGAGCGGATGAACACAGTCC-3'
ß-Actin	Forward	5'-AGAAAATCTGGCACCACACC-3'
	Reverse	5'-CCATCTCTTGCTCGAAGTCC-3'

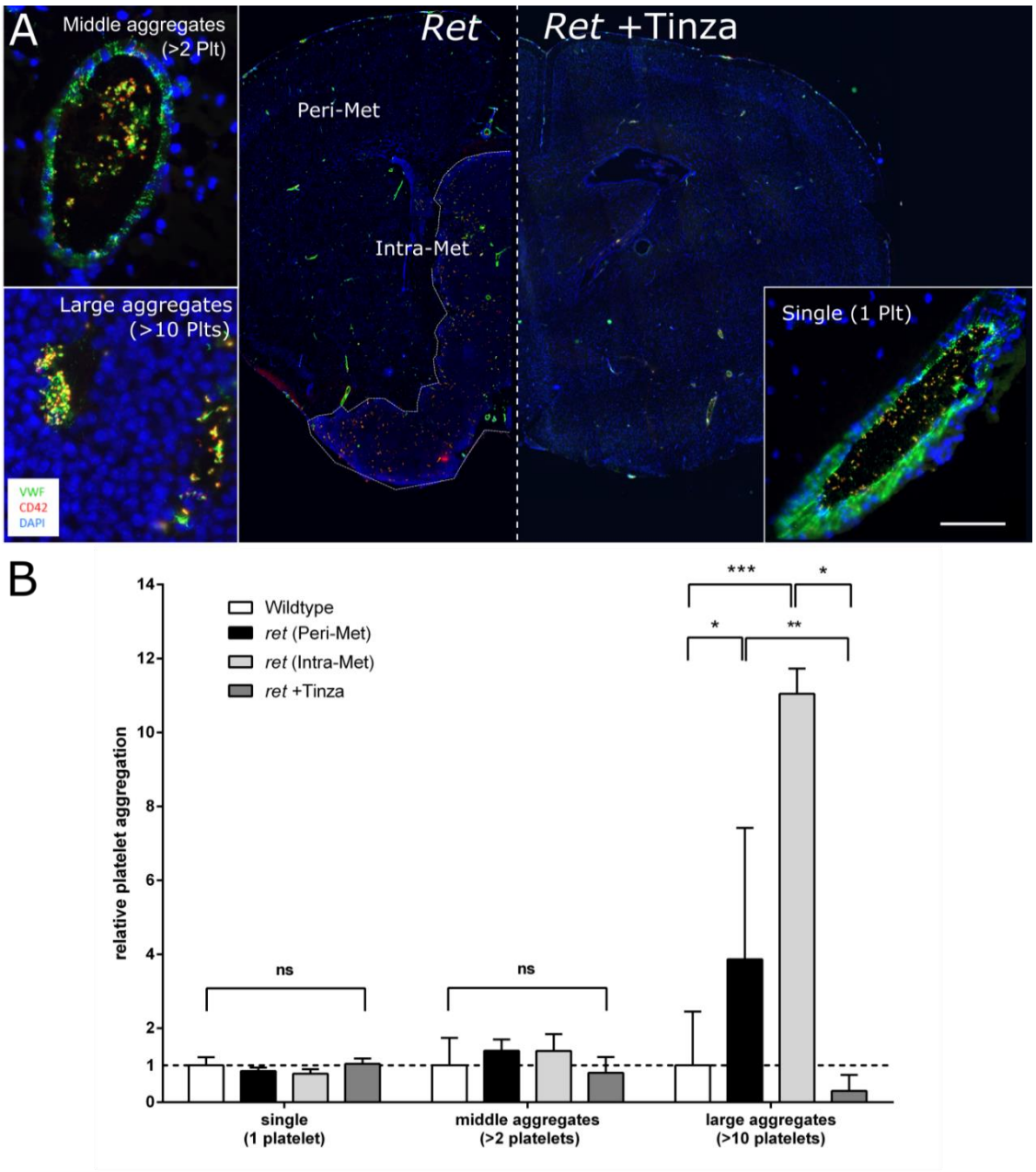
Supplementary Figures



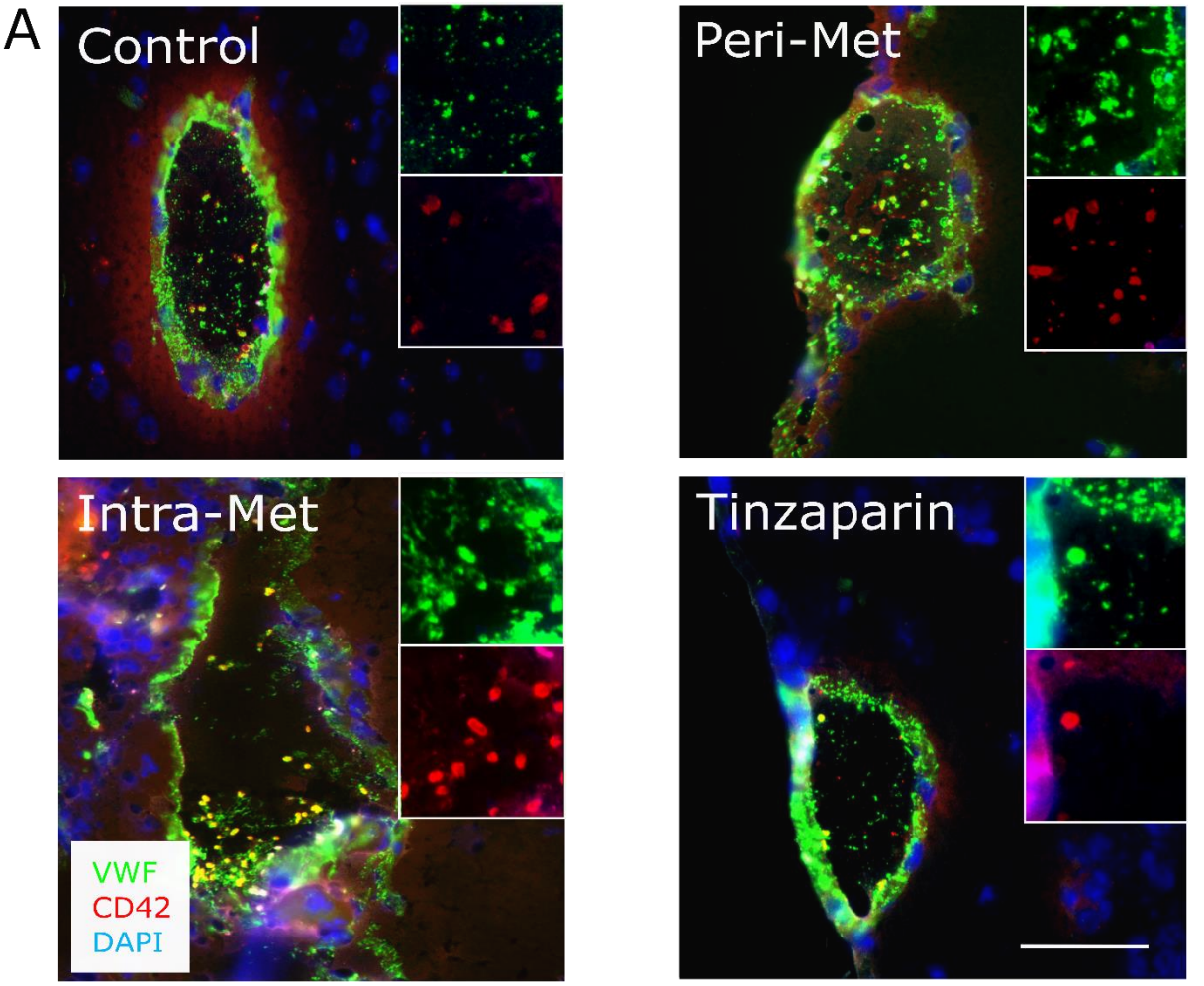
Supplementary Figure 1 Immunofluorescence staining reveals a heterogeneous distribution of Von Willebrand factor (VWF) in mouse brain endothelial cells. (A and B) Confluent quiescent human umbilical vein endothelial cells (HUVECs) and murine brain endothelial cell line (bEND3) were stained for VWF (green) and DAPI (blue) for nuclei. (C and D) Incubation with the supernatant of Ret melanoma cells (Ret Sn) induced the release of VWF and the formation of luminal VWF fibers (arrows). (E and F) The number of cells containing intracellular VWF and the number of VWF storage intracellular granules (WPBs) per cell were quantified in HUVECs and bEND3 (n = 50 cells/group from 3 independent experiments); *, $P < 0.05$, **, $P < 0.01$ (Student *t* test). Scale bar: 50 µm. Data are presented as mean ± SD. Scale bar: 50 µm.

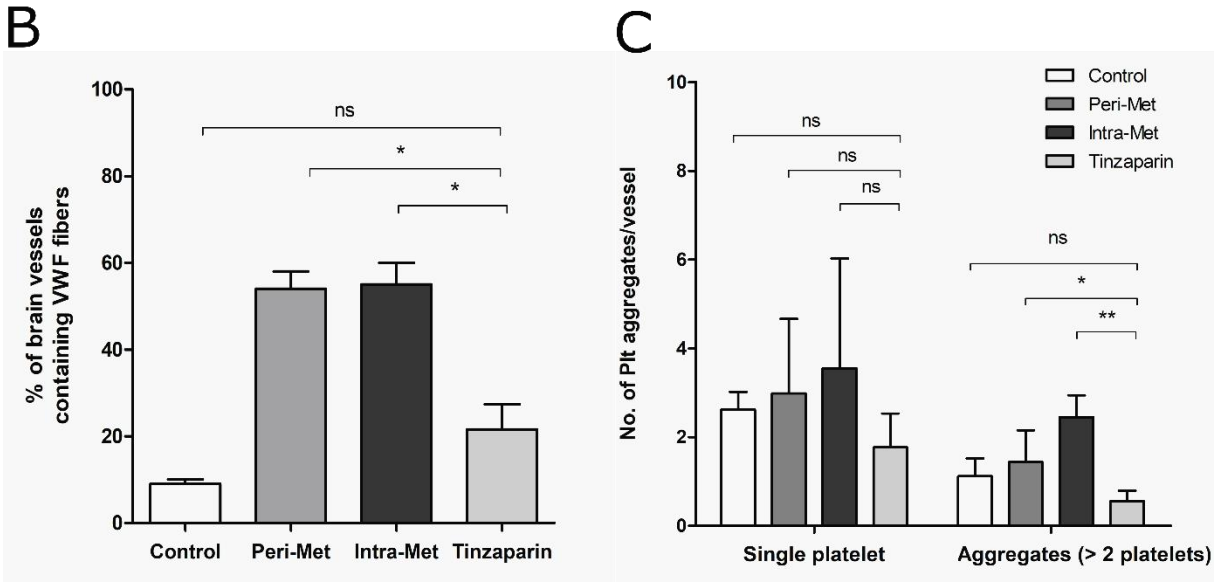

A
HUVEC
bEND3
VWF release (ng/ml)
HBRS
Thrombin
Ret Sn
Ret Sn (+Bevac)
Ret Sn (+Tinza)
ns
B
HUVEC
bend3
VWF release (ng/ml)
Ret (100.000)
Ret (500.000)
15 min
60 min
C
HUVECs
bEND3
HBMECs
Relative VWF release
(normalized to HBMECs - HBRS)
Trap-6
(10ng/ml)
Trap-6
(50ng/ml)

Supplementary Figure 2 Activation of cerebral endothelial cells results in a minor secretion of VWF. (A) HUVECs and murine brain endothelial cells bEND3 were stimulated for 15 minutes with HEPES-buffered Ringer Solution (HBRS) as control, Thrombin (0.5 IU/ml) and Ret melanoma supernatant (Ret Sn), with or without the anti-VEGF antibody Bevacizumab (Bevac; 0.65 mg/ml) or Tinzaparin (Tinza; 100 IU/ml). The concentration of VWF in cell supernatants was analyzed by ELISA (n = 6 of 2 independent experiments). (B) VWF release was measured in the supernatant of HUVECs and bEND3 after incubation with Ret cells for 15 or 60 minutes (100,00 or 500,000 Ret cells; n = 3). (C) HUVECs, bEND3 and human brain microvascular endothelial cells (HBMECs) were stimulated for 15 minutes with HBRS (Control), Thrombin (0.5 IU/ml) and different concentrations of thrombin receptor activator peptide 6 (TRAP-6) (10 and 50 pg/ml). Bars show the relative differences of secreted VWF by the different cells in comparison to HBMECs treated with HBRS (n = 6-9 of 3 independent experiments); ns, not significant, *, $P < 0.05$, **, $P < 0.01$ vs Control (Student *t* test). Data are presented as mean ± SD.

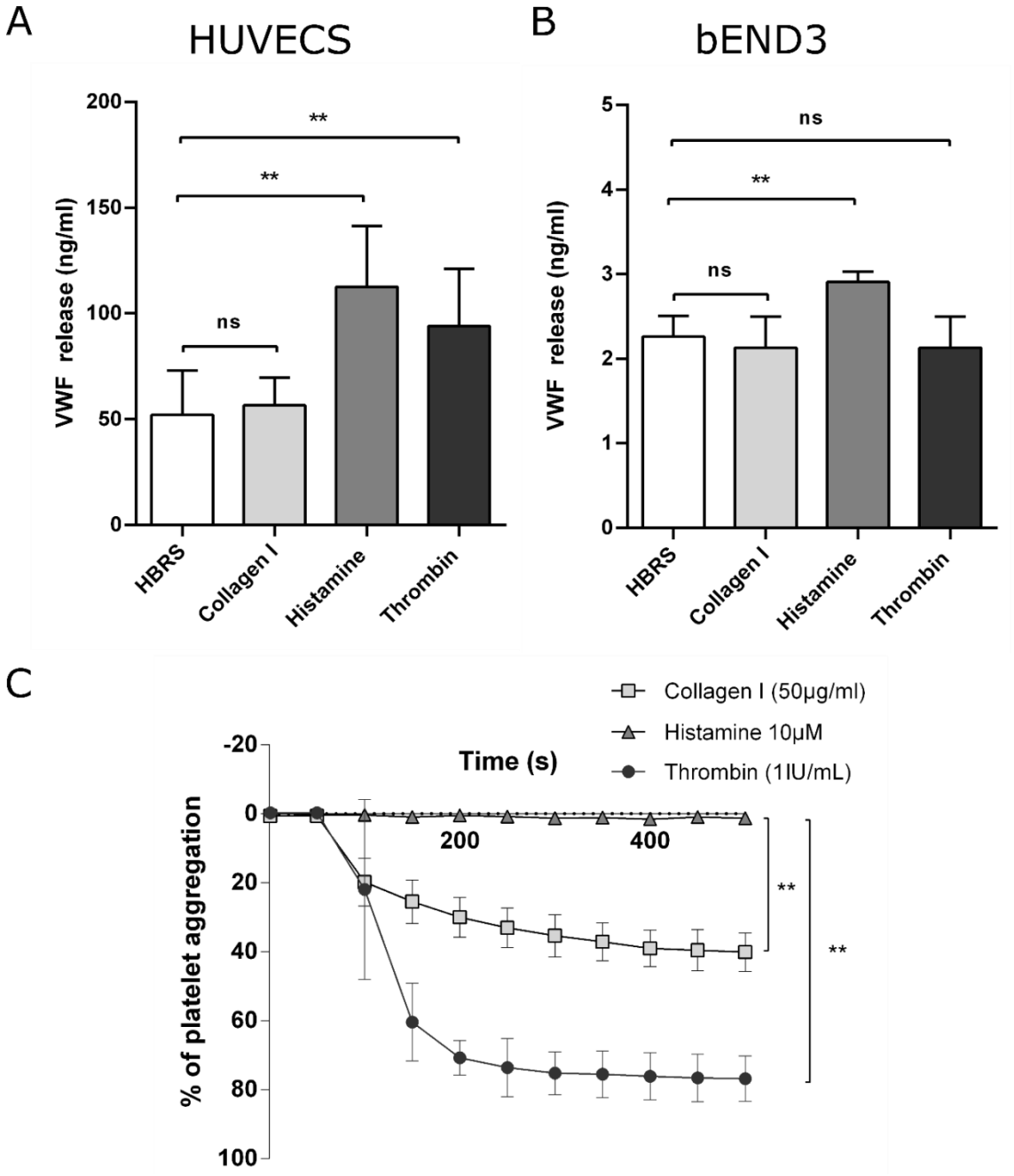


Supplementary Figure 3 Tinzaparin inhibits the formation of large platelet aggregates *in vivo.* (A) Brain sections of wild type and *ret* mice, treated with NaCl or Tinzaparin (Tinza) (0.6 IU/g) were stained for VWF (green), CD42 (red) and nuclei (blue) with DAPI. Shown are images of two different brain sections. On the left, a brain section from a *ret* mouse treated with NaCl showing a macroscopic metastasis and on the right, a brain section from a *ret* mouse treated with Tinzaparin. The corresponding magnifications show the distribution of platelets and VWF in brain vessels from the two treated groups. (B) The number and size of intraluminal platelet aggregates was calculated in each group of mice (n = 4-6 brains per group). The corresponding quantification shows the impact of Tinzaparin on the formation of platelet aggregates, grouped by their sizes and normalized to the results in the wild type group; ns, not significant, *, $P < 0.05$, **, $P < 0.01$ (One-way Anova). Data are presented as mean ± SD. Scale bar: 50 µm.

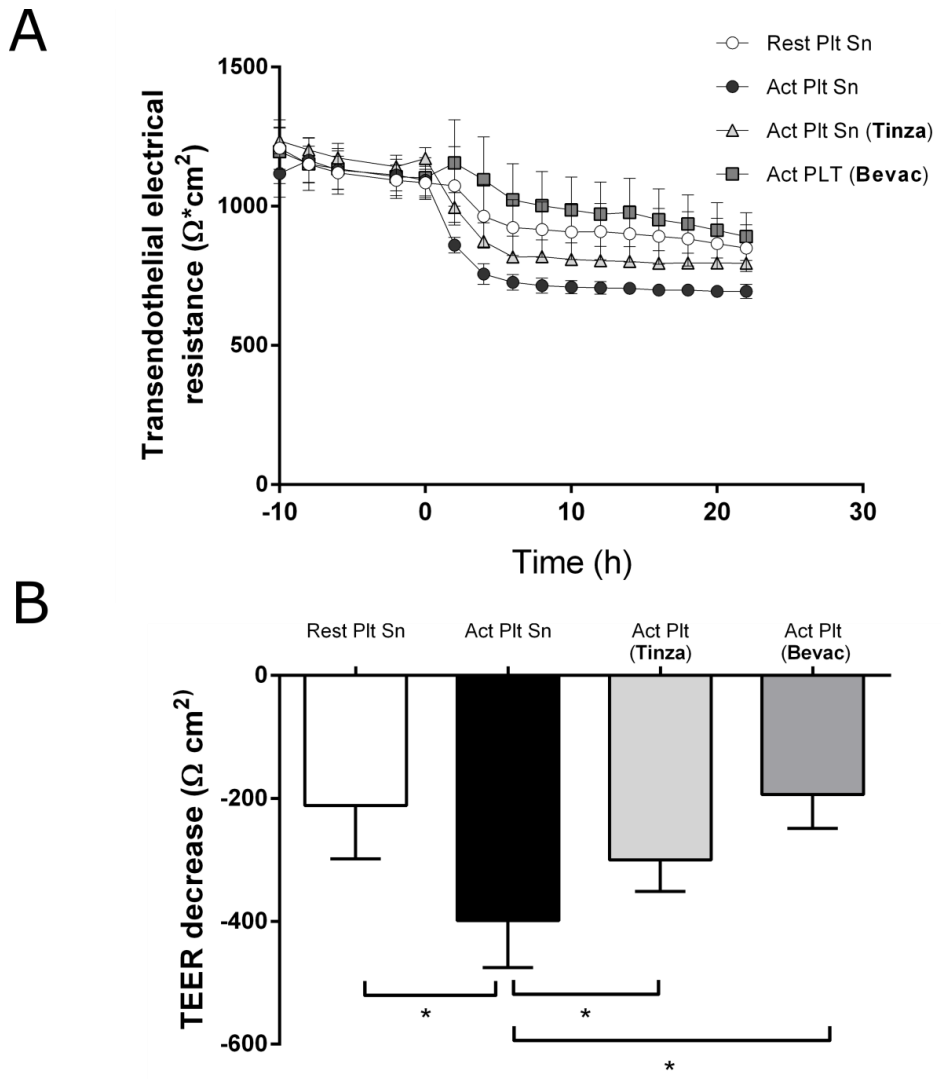

A
Control
Peri-Met
Intra-Met
Tinzaparin
VWF
CD42
DAPI


B
% of brain vessels containing VWF fibers
100
80
60
40
20
0
ns
*
*
Control
Peri-Met
Intra-Met
Tinzaparin
C
No. of Plt aggregates/vessel
10
8
6
4
2
0
ns
ns
ns
ns
*
**
Control
Peri-Met
Intra-Met
Tinzaparin
Single platelet
Aggregates (> 2 platelets)

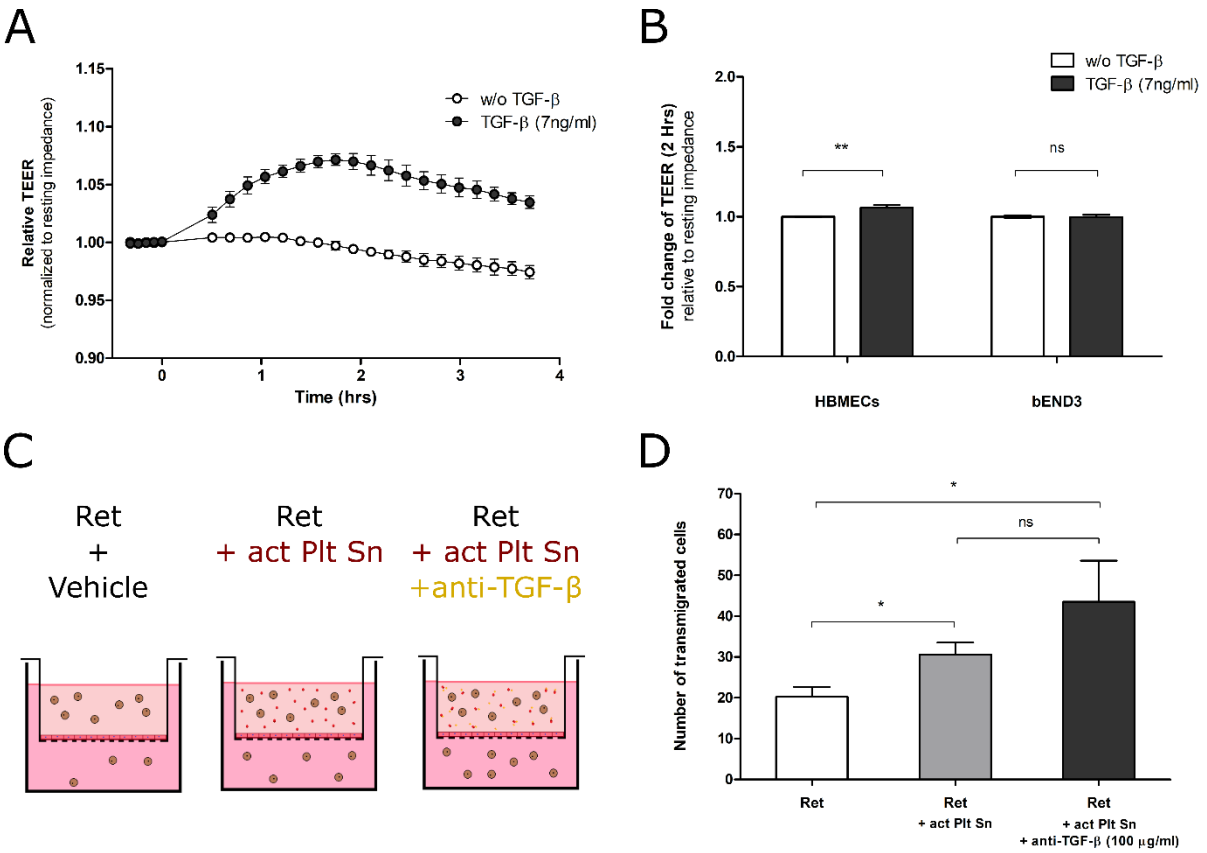
Supplementary figure 4 Impact of systemic anticoagulation on intracranial hypercoagulation. Human A2058 melanoma cells were injected into the left heart ventricle of NMRI-nu/nu mice followed by anticoagulant treatment with Tinzaparin (0.6 IU/g). Brains were removed and analyzed 28 days post tumor cell injection. Brains were grouped as follows: brains from non-injected NMRI-nu/nu mice (Control), brains from injected NMRI-nu/nu mice with macroscopic metastases, which were subdivided in brain perimetastatic tissue (Peri-Met) and brain metastatic tissue (Intra-Met), and brains from injected NMRI-nu/nu mice treated with Tinzaparin (Tinzaparin). (A) Brain sections were stained for VWF (green), platelet marker CD42 (red) and DAPI (blue) for nuclei. (B) The formation of luminal VWF fibers was analyzed in each group and the corresponding quantification shows the percentage of cerebral vessels containing luminal VWF fibers in each group (n = 2-5 animals per group). (C) The area of single platelets was measured to estimate the mean number of single platelets and platelet aggregates (more than 2 platelets together) per vessel in each group (n = 2-5 brains per group); ns, not significant, *, $P< 0.05$, **, $P<0.01$ (Student *t* test). Data are presented as mean ± SD. Scale bar: 50 µm.



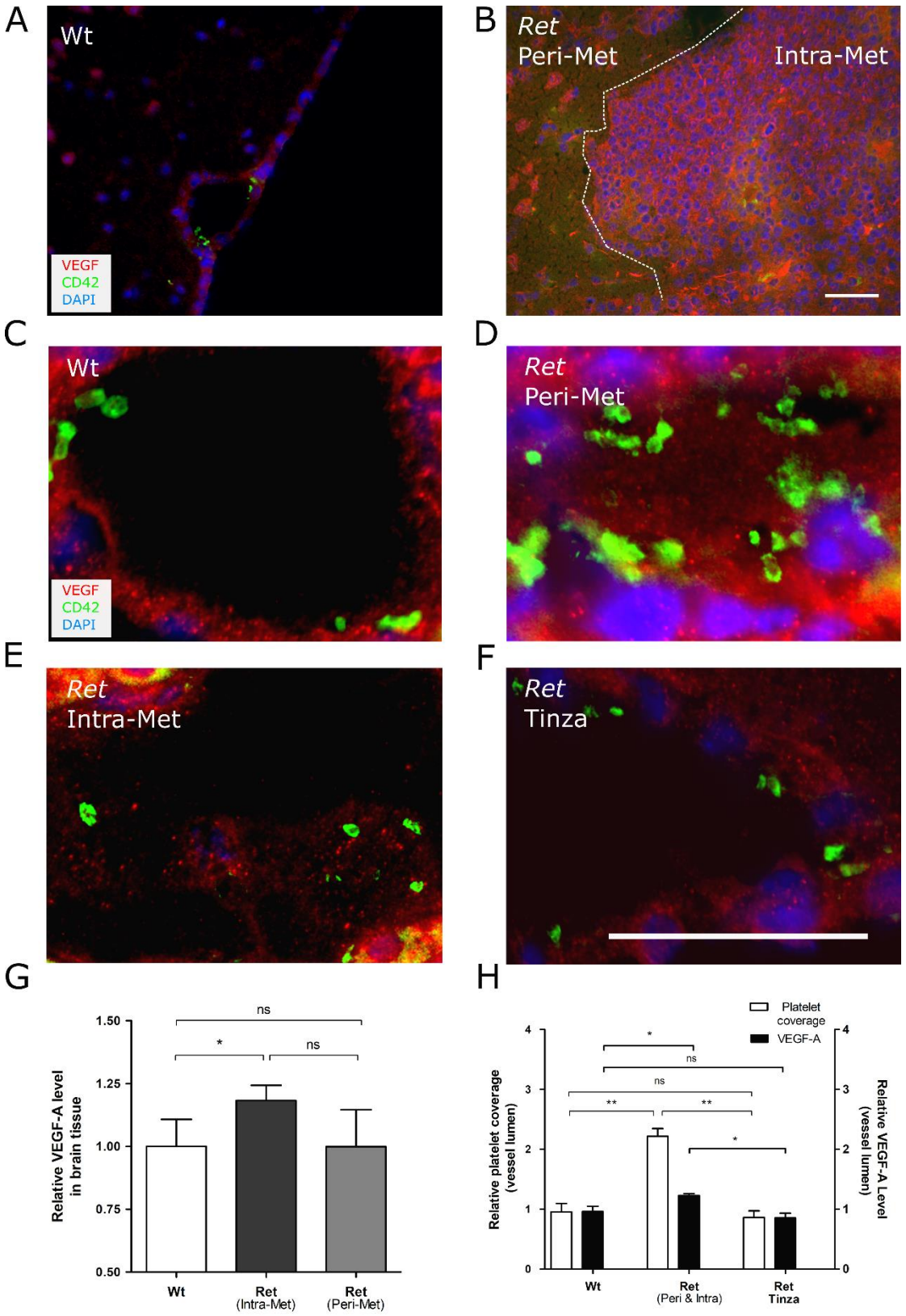
Supplementary Figure 5 Activation of ECs and platelets requires different agonists**.** (A and B) HUVECs and bEND3 were separately stimulated with HBRS (Control), Collagen I (50 µg/ml), Histamine (100µM) and Thrombin (0.5 IU/ml) for 15 minutes and the supernatants were analyzed by ELISA for VWF release (n = 3 independent experiments); ns, no significant, **, *P*<0.01 (Student *t* test). (C) To test the impact of different agonist in platelet activation, stirred platelets were incubated with Collagen Type I (50µg/ml), Histamine (100µM) and Thrombin (0.5 IU/ml) and platelet aggregation was monitored for 500 seconds (s) by LTA. (n = 4 independent experiments); **, P < 0.01 (F-test). Data are presented as mean ± SD.



Supplemental Figure 6 Supernatant of activated platelets disrupts the endothelial barrier. (A) HBMEC monolayers were incubated with the supernatants of resting platelets (Rest Plt Sn) or with the supernatant of Thrombin (0.5 IU/ml)-activated platelets (Act Plt Sn), with or without a preincubation with Tinzaparin (100 IU/ml; Act Plt Sn (Tinza)) or Bevacizumab (0.65 mg/ml; Act Plt Sn (Bevac)). Transendothelial electrical resistance (TEER) was used to evaluate endothelial integrity (n = 6 of 3 independent experiment per group). (B) Bars show the absolute decrease of TEER in each group after incubation with platelet releasates. *, $P< 0.05$ (Student *t* test). Data are presented as mean ± SD.



Supplementary figure 7 Impact of transforming growth factor *β* (TGF-*β*) on tumor cell transmigration. (A) HBMEC monolayers were incubated in presence of TGF-*β* (7 ng/ml). TEER was measured for 5 hours (hrs) after addition of TGF- *β*. Results were normalized to impedance values measured prior to TGF-*β* addition (n = 4 of 2 independent experiments). (B) Bars show the changes in TEER in HBMECs and bEND3, when the differences were at the maximum, 2 hours after the addition of TGF-*β* (n = 4 of 2 independent experiment per group); ns, not-significant. C, schematic diagram of the tumor cell transendothelial migration assay: Ret melanoma cells were coincubated with HBMEC monolayers for 8 hours, alone or supplemented with the supernatant of activated platelet (act Plt Sn). An anti-TGF-*β* antibody (100 µg/ml) was added to test the impact of platelet-derived TGF-*β* on tumor cell transmigration. Then, upper chambers were removed and transmigrated tumor cells were counted after 24 hours. (D) The corresponding quantification shows the number of transmigrated Ret cells in each condition (n= 4-5 independent experiments per condition). *, *P*< 0.05, **, *P*<0.01 (Student *t* test). Data are presented as mean ± SD.


A
Wt
VEGF
CD42
DAPI
B
Ret
Peri-Met
Intra-Met
C
Wt
VEGF
CD42
DAPI
D
Ret
Peri-Met
E
Ret
Intra-Met
F
Ret
Tinza
G
Relative VEGF-A level in brain tissue
1.50
1.25
1.00
0.75
0.50
ns
*
ns
Wt
Ret (Intra-Met)
Ret (Peri-Met)
H
Platelet coverage
VEGF-A
Relative platelet coverage (vessel lumen)
Relative VEGF-A Level (vessel lumen)
Wt
Ret (Peri & Intra)
Ret Tinza

Supplementary Figure 8 Immunofluorescence staining reveals a heterogeneous distribution of VEGF-A in brain tissue during metastasis. Brain sections of wild type (Wt) and *ret* mice were stained for CD42 (green), VEGF-A (red) and DAPI (blue) for nuclei. Representative images show the differences in VEGF-A fluorescence intensity between Wt brain tissue (A) and metastatic *ret* brain (Peri- and Intra-Met tissue). (G) The quantification shows the relative fold change in the fluorescence intensity of VEGF-A in the distinct tissues normalized to the levels observed in Wt mice (n = 2-5 animals per group). (C-F) The distribution of VEGF-A was also analyzed in the lumen of brain vessels of each group and in *ret* mice treated with Tinzaparin (Tinza), the corresponding magnifications show the differences of luminal VEGF-A among the different groups. (H) Bars show the relative platelet coverage (white) and the relative fold change of luminal VEGF-A fluorescence intensity (black) in brain tissue from *ret* mice (Peri- and Intra-Met) and brain tissue from *ret* mice treated with Tinzaparin, shown results were normalized to values observed in Wt brains (n = 2-5 animals per group); ns, not significant, *, $P < 0.05$, **, $P < 0.01$, (Student *t* test). Data are presented as mean ± SD. Scale bars: 50 µm.

Videos

Video 1

The impact of platelet-derived VWF on thrombus formation was analyzed in real time by using microfluidic devices. A confluent monolayer of the murine bEND3 cells (blue) was perfused with Wt mouse platelets (red). In a first step, endothelial-derived VWF secretion was induced by histamine (100 µM). In a second step, platelet activation and subsequent aggregation was induced by addition collagen type I (50 µg/ml). For the visualization of VWF strings anti-VWF-FITC (green) antibody (1:100) was added (n = 4 experiments). Scale bars 50 µm.

Video 2

The impact of platelet-derived VWF on thrombus formation was analyzed in real time by using microfluidic devices. A confluent monolayer of the murine bEND3 cells (blue) was perfused with VWF -/- mouse platelets (red). In a first step, endothelial-derived VWF secretion was induced by histamine (100 µM). In a second step, platelet activation and subsequent aggregation was induced by addition collagen type I (50 µg/ml). For the visualization of VWF strings anti-VWF-FITC (green) antibody (1:100) was added (n = 6 experiments). Scale bars 50 µm.