Supplemental Data

Factor VIIa induces extracellular vesicles from the endothelium: A potential mechanism for its hemostatic effect

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Materials and Methods

Reagents

Human recombinant FVIIa used in current studies was essentially similar to that of commercially available rFVIIa (NovoSeven) and was provided by the Late Walter Kisiel, University of New Mexico, Albuquerque (NM). FVIIai was prepared by incubating rFVIIa with a molar excess of a peptidyl inhibitor, D-Phe-L-Phe-L-Arg chloromethyl ketone, and then removing the excessive inhibitor as described earlier.[1] FVa was from Hematologic Technologies (Essex Junction, VT); prothrombin, FX, and FXa were from Enzyme Research Laboratories (South Bend, IN). Annexin V was from eBioscience, Inc (San Diego, CA), and hirudin was from MilliporeSigma (Burlington, MA). FXa substrate [CS-11 (65)] was purchased from Biophen (France), whereas the thrombin substrate (Chromozyme TH) was from Millipore Sigma. Mouse monoclonal antibodies against human EPCR (JRK-1494/blocking mAb and JRK-1500/non-blocking mAb) were prepared as described earlier.[2] Preparation and characterization of monospecific polyclonal antibodies against human TF were described earlier.[3] Monoclonal antibodies against human PAR1 (ATAP2 and WEDE-15), PAR2 (SAM11), PAR4, human and mouse VE-Cadherin, mouse CD14 were purchased from Santa Cruz Biotechnology (Dallas, TX). Mouse CD41 antibody was obtained from eBioscience, Inc. All secondary antibodies were from Cell Signaling Technology (Danvers, MA). LyseBio solution was from HORIBA, and trypsin and DMEM were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA).

Cells

Human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) were obtained from Lonza (Walkersville, MD). Murine brain endothelial cell line bEnd.3 was obtained from American Type Culture Collection (ATCC). Primary murine brain endothelial cells were isolated as described in our recent publication.[4]

Mice

Wild-type C57BL/6J (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in-house. The generation of EPCR-deficient mice (*procr*$^{-/-}$, denoted as EPCR-KO) and EPCR-overexpressing mice (Tie2-EPCR, denoted as EPCR-OX) was described in earlier publications.[5,6] Generation and characterization of mouse strains carrying a homozygous point mutation at the canonical cleavage site of PAR1 at amino acid residue R41 (PAR1-R41Q) or noncanonical cleavage site of PAR1 at amino acid residue R46 (PAR1-R46Q) were described recently.[7] These mice were used to demonstrate that APC-induced cytoprotective signaling requires the cleavage of PAR1 at the R46 site,[7] whereas FVIIa-induced anti-inflammatory and barrier protective effects require the cleavage of PAR1 at the R41 site.[4] Generation of FVIII$^{-/-}$ mice in the C57BL/6J genetic background were described earlier.[8] Eight to 12-week old mice, both sexes weighing between 24 to 30 g, were used in experiments. All the animal studies were approved by the Institutional Animal Care and Use Committee and conducted according to the animal welfare guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

Isolation of EVs

EVs from cells cultured *in vitro* were isolated from culture supernatants by a procedure described earlier with a few minor modifications.[9] Briefly, supernatants were subjected to centrifugation at 1,000 x g for 10 min to pellet cell debris and apoptotic bodies. The pellet was discarded, and the supernatant was re-centrifuged at 21,000 x g for 1 h to sediment EVs. The EVs were washed twice with HBSS by suspending the EVs into the original volume and subjecting the suspension to centrifugation at 21,000 x g for 1 h. The EVs were suspended in the HBSS w/o phenol red (Lonza) in one-fifth of the original volume for further analysis. All the above procedures were performed at 4°C. The EVs isolated by the above procedure were generally referred to as microvesicles or microparticles in most of the earlier publications in the field.[10-12] To isolate exosomes, after sedimenting microvesicles, the supernatant was centrifuged at 100,000 x g for 1 h, and the pellet was suspended in HBSS.

For isolation of EVs from mice blood, approximately one ml of the blood was collected from the mouse via submandibular vein puncture into citrate anticoagulant buffer. Platelet-depleted plasma was prepared by two consecutive centrifugation steps at 3,000 X g for 15 min. The supernatant was centrifuged then at 21,000 x g for 1 h to pellet the EVs. The EVs were washed twice with HBSS and resuspended in HBSS.

Labeling of cells with PKH67 dye

The PKH67 dye (Sigma) was added to confluent monolayers of HUVEC cultured on glass coverslips or in 6-well culture dishes at a concentration of 20 mM and incubated for 30 min at 37°C. At the end of 30 min, the supernatant was removed, and cells were washed with HEPES-buffered saline (1X HBSS, Lonza). To check the labeling, PHK67-labeled cells on coverslips were fixed with 4% paraformaldehyde (PFA) for 30 min, followed by washing and counterstaining with DAPI (1 mg/ml; 1:1000; Sigma) for 30 min. Coverslips were mounted on glass slides with fluoro-gel and viewed under a confocal immunofluorescence microscope equipped with an inverted microscope (LSM 510 Meta confocal system equipped with Axio Observer Z1, Carl Zeiss).

To generate PKH67-labeled EVs, first endothelial cells were labeled with PHK67 dye, as described above. The labeled cells were treated with a control vehicle or FVIIa (100 nM) for 24 h, and the EVs from cell supernatants were isolated as described above.

Quantification and analysis of EVs

EVs isolated from PHK67 dye-labeled cells were quantified by measuring their fluorescence, alter correcting autofluorescence, using a multi-mode microplate reader (Cytation 5, Bio-Tek). EVs isolated from unlabeled cells were analyzed by nanoparticle tracking analysis (NTA) using NanoSight NS300 (Malvern Panalytical, Westborough, MA). The number of EVs and their diameter was measured using the following parameters: camera level, 14 (NTA 3.0 levels), temperature, 21-23°C, and detection threshold, 5. The isolated EVs were lysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, subjected to immunoblotting, and probed for EPCR, VE-cadherin, and other cell-specific markers.

Silencing of EPCR, PAR1, PAR2, or PAR4

HUVEC were transfected with siRNA specific for EPCR (5'- GUG GAC GGC GAU GUU AAU UAC TT-3'; 200 nM), PAR1 (5'-AGA UUA GUC UCC AUC AAU-3' and 5'-AGG CUA CUA UGC CUA CUA C-3'; 100 nM), PAR2 (5'-CCU CAU AAC AUU AAA CAG GTT-3'; 100 nM) (Eurofins MWG Operon Louisville, KY or Sigma), or PAR4 (5'-UCA CUA GCG GAG GUC ACU UUG-3' and 5'-CAA AGU GAC CUC CGC UAG UGA-3'; 100 nM) (Sigma). As controls, scrambled nucleotide of the sequences mentioned above or non-specific siRNA (5'-GAU UAU GUC CGG UUA UGU AUU -3') were used. The transfection was carried out using Lipofectamine RNAIMAX (Invitrogen) or X-treme GENE siRNA transfection (Sigma) reagent in a serum-free medium. Eight hours following transfection, the serum-free medium was replaced with EGM-2 medium, and cells were cultured

for 48 h before they were used in the experiment. The extent of knock-down of the target gene was determined by immunoblot analysis.

Blocking of EPCR, TF, and PAR1 on endothelial cells by antibody treatment

To investigate the role of EPCR, TF, and PAR1 on FVIIa-induced EVs generation, monolayers of HUVEC were treated with EPCR blocking mAb (JRK 1494; 100 μg/ml), rabbit polyclonal antibody against human TF (10 μg/ml) or PAR1 mAb (ATAP2, 25 μg/ml) for 1 h at room temperature before treating them with FVIIa. As controls, cells were treated with EPCR non-blocking mAb (JRK 1500; 100 μg/ml) or control IgG (100 μg/ml).

FXa generation assay

EVs, suspended in buffer B (10 mM HEPES, 0.15 M NaCl, pH 7.5 buffer containing 4.0 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, and 1 mg/ml BSA) were incubated with FVIIa (10 nM) for 5 min, followed by the substrate, factor X (175 nM). At the end of the 2 h activation period, the reaction was terminated by taking an aliquot into TBS buffer containing 25 mM EDTA and 1 mg/ml BSA. To assess phospholipid- and TF-dependency of FX activation, EVs were incubated with either annexin V (400 nM) or TF neutralizing antibodies (polyclonal anti-human TF IgG or 1H1 mAb, 5 μg/ml), or control IgG (5 μg/ml) for 1 h before the addition of FVIIa. The amount of FXa generated was assessed in a chromogenic assay using Chromogenix S2765 as a substrate, as described earlier.[13,14] A similar assay was used to determine cell surface-associated FX activation.

Prothrombinase assay

To determine the thrombin generation potential of EVs, they were incubated with FVa (10 nM) and FXa (1.0 nM) for 5 min, followed by the addition of prothrombin (1.4 μM). At the end of the 1 h activation period, the reaction was terminated with EDTA, and the amount of thrombin generated was determined in a chromogenic assay using Chromozyme TH as a substrate.[13]

Protein C activation assay

Protein C (0.2 μM) was added to 200 μl of EVs suspended in buffer B, and protein C activation was initiated by the addition of thrombin (10 nM). After 30 min, the reaction was terminated by adding antithrombin (1.66 mg/ml) and heparin (1.4 units/ml).[2] APC activity was measured using a chromogenic substrate (S-2366).

Annexin V staining of intact endothelial cells

To investigate the externalization of PS on the endothelial cell surface following the exposure to FVIIa, HUVEC grown on glass coverslips were treated with control vehicle or FVIIa for 5 h. Then, the cells were incubated with FITC-conjugated annexin V (5 µl of annexin V conjugate in 100 µl of 1X annexin V-binding buffer; Invitrogen; Cat. No.: A13201) for 1 h. After 1 h, the cell monolayers were washed thrice with HBSS, fixed with 4% *p*-formaldehyde for 30 min. The cells were imaged using confocal microscopy (LSM510 Meta, Zeiss).

MTT cell viability assay

HUVEC were grown in a 96-well plate to confluency. Cells were deprived of serum for 1 h and then treated with a control vehicle, FVIIa (100 nM), or TNF-α (40 ng/ml) for 24 h. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml. After incubating cells with MTT for 4 h at 37°C, the supernatant was removed, and the insoluble formazan crystals were solubilized in DMSO (100 µl) for 30 min. The resulting colored solution was quantified by measuring absorbance at 570 nm.

Determination of apoptosis by PARP1 cleavage

HUVEC monolayers grown in a 12-well plate were serum-starved for 1 h and then treated with a control vehicle, FVIIa (100 nM), or TNF-α (40 ng/ml). After 4 h, cells were lysed and subjected to immunoblot analysis to probe with anti-PARP1 antibodies (Cell Signaling Technologies) to determine PARP1 cleavage.

vWF exocytosis

HUVEC grown in a 6-well plate were treated with a control vehicle, FVIIa (100 nM), or Ca^{2+} Ionophore (10 µM; positive control) for 30 min. EVs from the overlying medium were sedimented by centrifugation at 21,000 x g for 1 h, and proteins in the supernatant were precipitated with 10% TCA. Both EVs and supernatant fractions were subjected to immunoblot analysis to probe for vWF.

Cell surface expression of P-selectin

HUVEC, cultured in a 96-well plate (for cell surface ELISA) or on glass coverslips (for immunostaining), were treated with a control vehicle, FVIIa (100 nM), thrombin (1 U/ml; positive control) for 5 min. After 5 min, cells were with 4% *p*-formaldehyde and blocked with BSA. Fixed cells were incubated with a P-selectin antibody overnight at 4°C. After washing cells thrice with PBS, HRP-conjugated secondary antibody (for cell surface ELISA) or Alexa-flour 488-conjugated secondary antibody was added to the cells. After 2 h incubation at room temperature, the cells were washed, and the TMB solution was added to determine

cell surface expression of P-selectin in ELISA. For immunostaining, cells were stained with murine anti-P-selectin antibody, followed by AF488-conjugated donkey anti-murine IgG. Nuclei were stained with DAPI. Immunofluorescence of cells was analyzed by confocal microscopy.

Determination of plasma fibrinogen levels

Plasma fibrinogen levels eas determined by using the Clauss assay method.[15] Briefly, plasma samples were diluted (5X) in buffer B. One hundred µl of diluted plasma was incubated with 100 µl of thrombin (100 nM), and the clot formation was measured by measuring the change in the absorbance at 405 nm at different intervals (5 to 15 min). In parallel, a standard curve was constructed using a range of known concentrations of fibrinogen. Fibrinogen levels in unknown plasma samples were deduced from the standard curve.

Determining the accumulation of EVs at the wound site

PKH67-labeled murine bEnd.3 cells were treated with control vehicle or FVIIa (100 nM) for 24 h and EVs were isolated from the culture supernatants and quantified by NTA. Equal numbers (1.5×10^9 EVs/mouse) of control or FVIIa-generated EVs were administered to $FVIII^{-/-}$ mice via the tail vein injections. Following EVs, mice were injected with rFVIIa (0.25 mg/ml). and immediately subjected to the saphenous vein incision. Blood was collected from both the submandibular and the incision site. Fifty µl of the blood sample was subjected to measurement of PKH67 fluorescence intensity of the EVs by spectrofluorimeter (Cytation 5 Multi-Mode Reader, Bio-Tek). After 5 min, the injured site was dissected, fixed, and processed for sectioning. Sections were stained for fibrin using antibodies against murine fibrin (Millipore), and nuclei were stained with DAPI and analyzed by confocal microscopy.

Isolation of platelets from human blood

Platelets were isolated from human blood as described in our early publication.[16] Briefly, venous blood was drawn from healthy volunteers after their consent into acid citrate-dextrose anticoagulant solution (1:6). The human subject protocol was approved by the Institutional Review Board at the University of Texas Health Science Center at Tyler, TX. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 200 x g for 20 min at room temperature. Prostaglandin (E1 1 µmol/L was added to the PRP to prevent platelet activation. Platelets from the PRP were sedimented by centrifugation at 900 x g for 10 min at room temperature. The platelet pellet was resuspended in platelet resuspension buffer (Tyrode's buffer with 12 mmol/L $NaHCO_3$, 5 mmol/L dextrose, 2 mmol/L $MgCl_2$, and 0.3% BSA) containing 0.1 U/mL of apyrase. After resting the platelets for 30 min at room temperature, they were sedimented by

centrifugation at 900 x g for 10 min at room temperature, resuspended in the resuspension buffer, and used in experiments.

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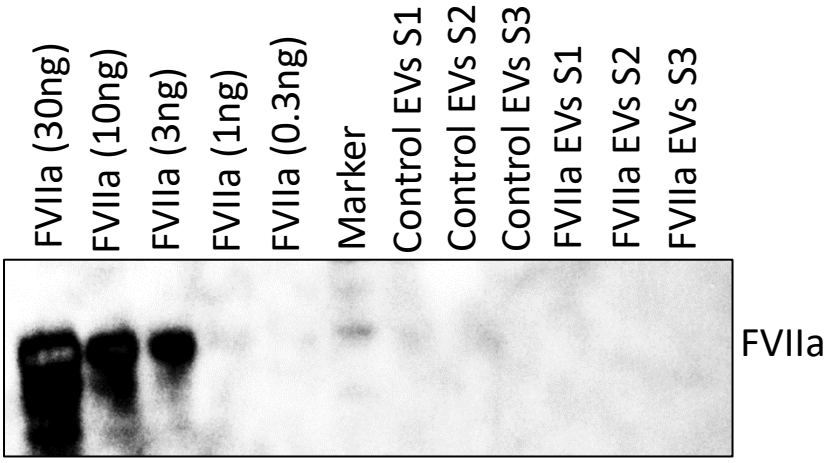
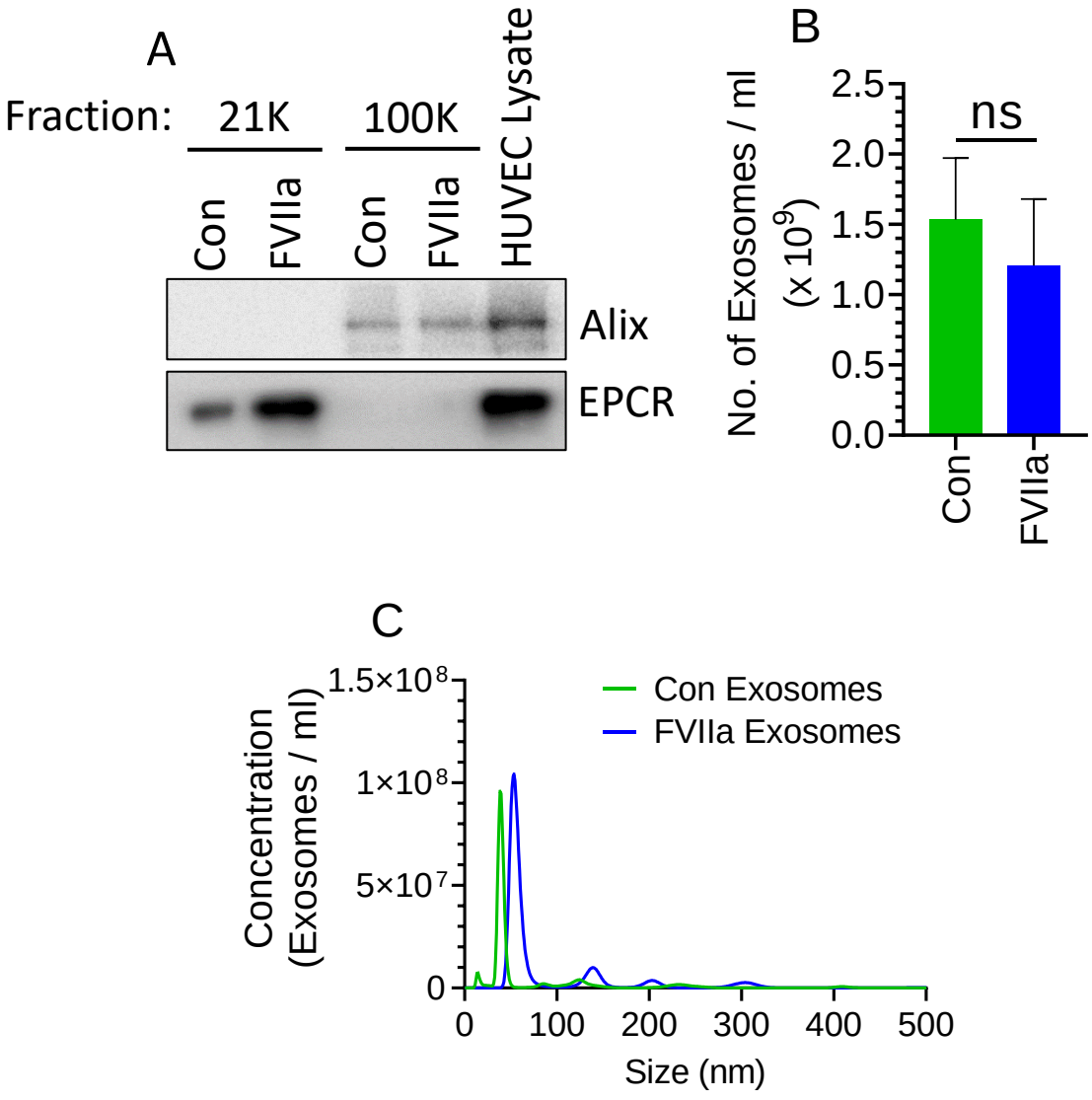
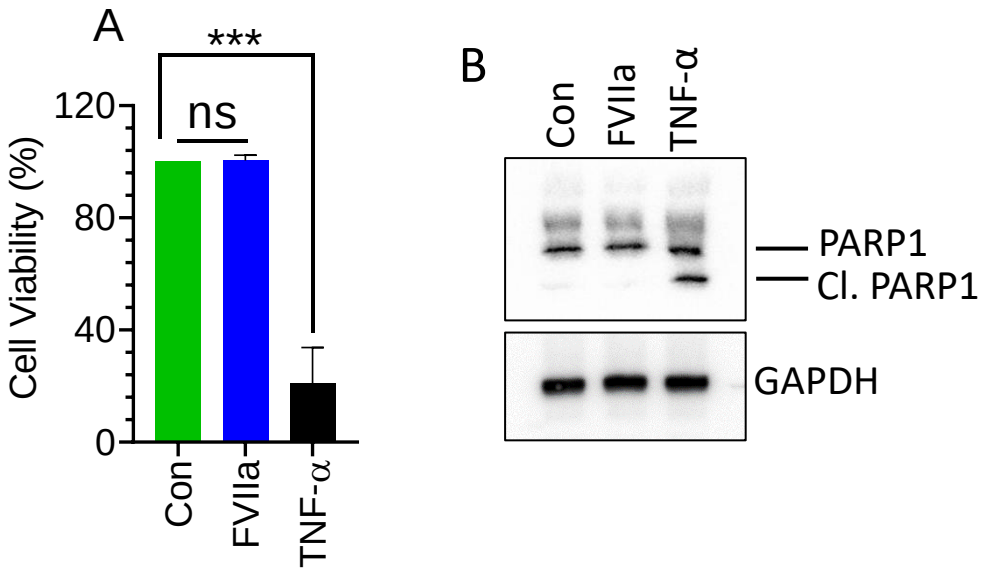
Supplemental Figure 1. Endothelial EVs do not bear detectable FVIIa. HUVEC monolayers were serum-starved for 1 h and then treated with a control vehicle or FVIIa (100 nM). EVs, isolated from the supernatant after 24 h, were subjected to immunoblot analysis to detect the presence of FVIIa. Different concentrations of FVIIa (0.3 ng to 30 ng) were used as a standard and positive control. Control EVs and FVIIa-generated EVs from three different experiments were subjected to immunoblot analysis.



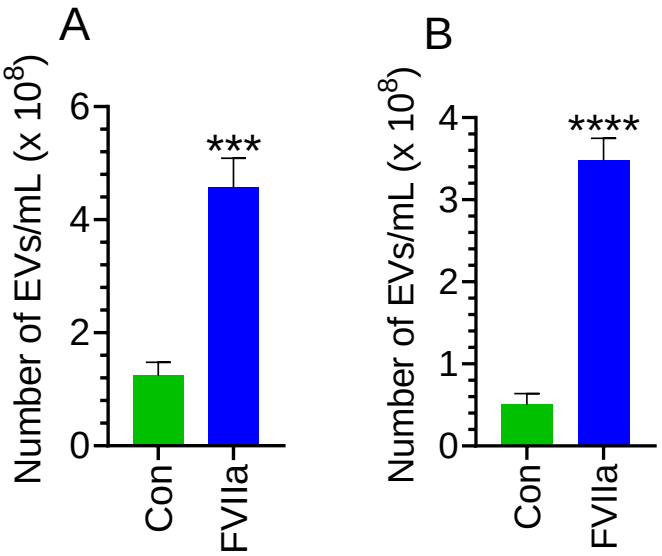
Supplemental Figure 2. FVIIa treatment does not induce exosomes generation from endothelial cells. HUVEC were treated with a control vehicle (Con) or FVIIa (100 nM). After 24 h, EVs from the conditioned media were first separated by centrifugation at 21,000 x g for 1 h. After collecting the EVs pellet, the supernatant was again centrifuged at 100,000 x g for 1 h to sediment the exosomes. Both EV and exosome fractions were subjected to western blot analysis and probed for Alix (exosome marker) or EPCR (A). Exosome numbers were quantified (B), and size distribution was characterized (C) by nanoparticle tracking analysis (NTA) using Nano-Sight. Data presented here are mean ± SEM from three independent experiments. ns, not statistically significantly different.



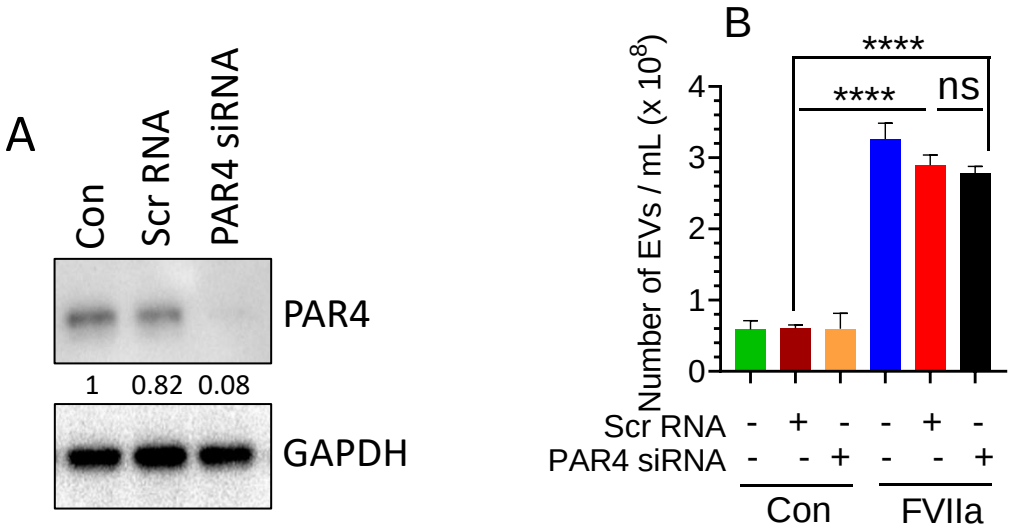
Supplemental Figure 3. FVIIa treatment does not induce apoptosis of endothelial cells. Confluent monolayers of HUVEC were serum-starved for 1 h and then treated with a control vehicle (Con), FVIIa (100 nM), or TNF-α (40 ng/ml; positive control). (A) Cell viability was monitored after 24 h of treatment with control vehicle, FVIIa, or TNF-α in MTT assay. (B) After 4 h of treatment, cell extracts were harvested and subjected to immunoblot analysis for PARP1 and GAPDH (loading control). ***, $p<0.001$; ns, not statistically significantly different.



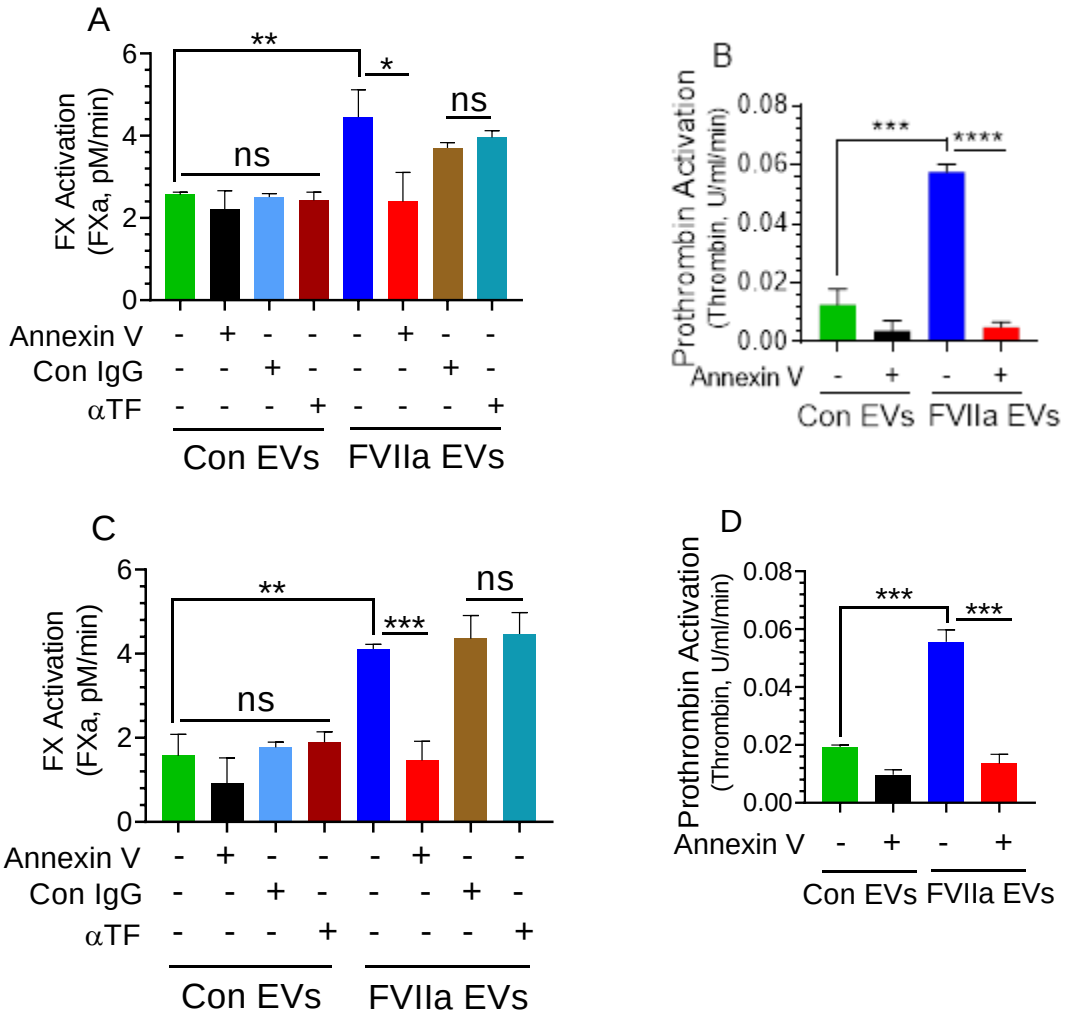
Supplemental Figure 4. FVIIa induces EVs generation from endothelial cells from different vascular beds. (A) Human aortic endothelial cells (HAEC) and (B) murine brain endothelial cells (bEnd.3) were serum-starved for 1 h and then treated with control vehicle (Con) or FVIIa (100 nM). After 24 h, EVs were isolated from the conditioned media and quantified by nanoparticle tracking analysis using Nano-Sight. ***, p<0.001; ****, p<0.0001.



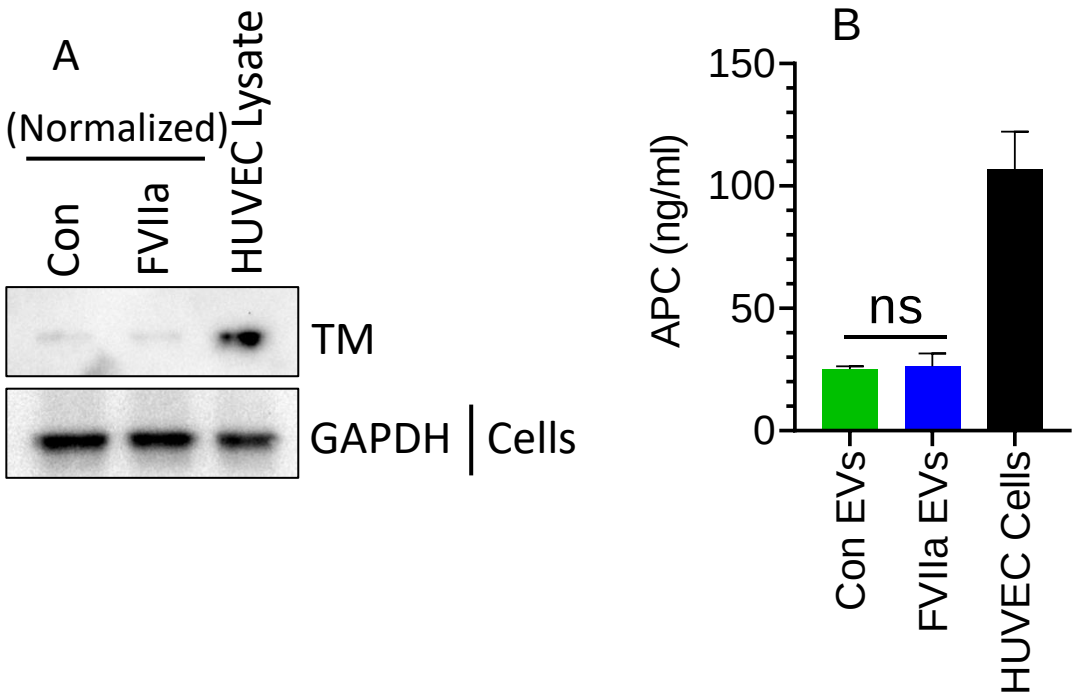
Supplemental Figure 5. FVIIa-mediated release of EVs from HUVEC was independent of PAR4 activation. HUVEC were transfected with scrambled RNA (Scr RNA; 100 nM) or PAR4-specific siRNA (100 nM). After 48 h, cell lysates were harvested and probed for PAR4 by immunoblotting (A). Control and transfected HUVEC were serum-starved for 1 h and then treated with control vehicle (Con) or FVIIa (100 nM) for 24 h. EVs, isolated from the conditioned media, were quantified by Nano-Sight (B). ****, $p<0.0001$; ns, not statistically significantly different.



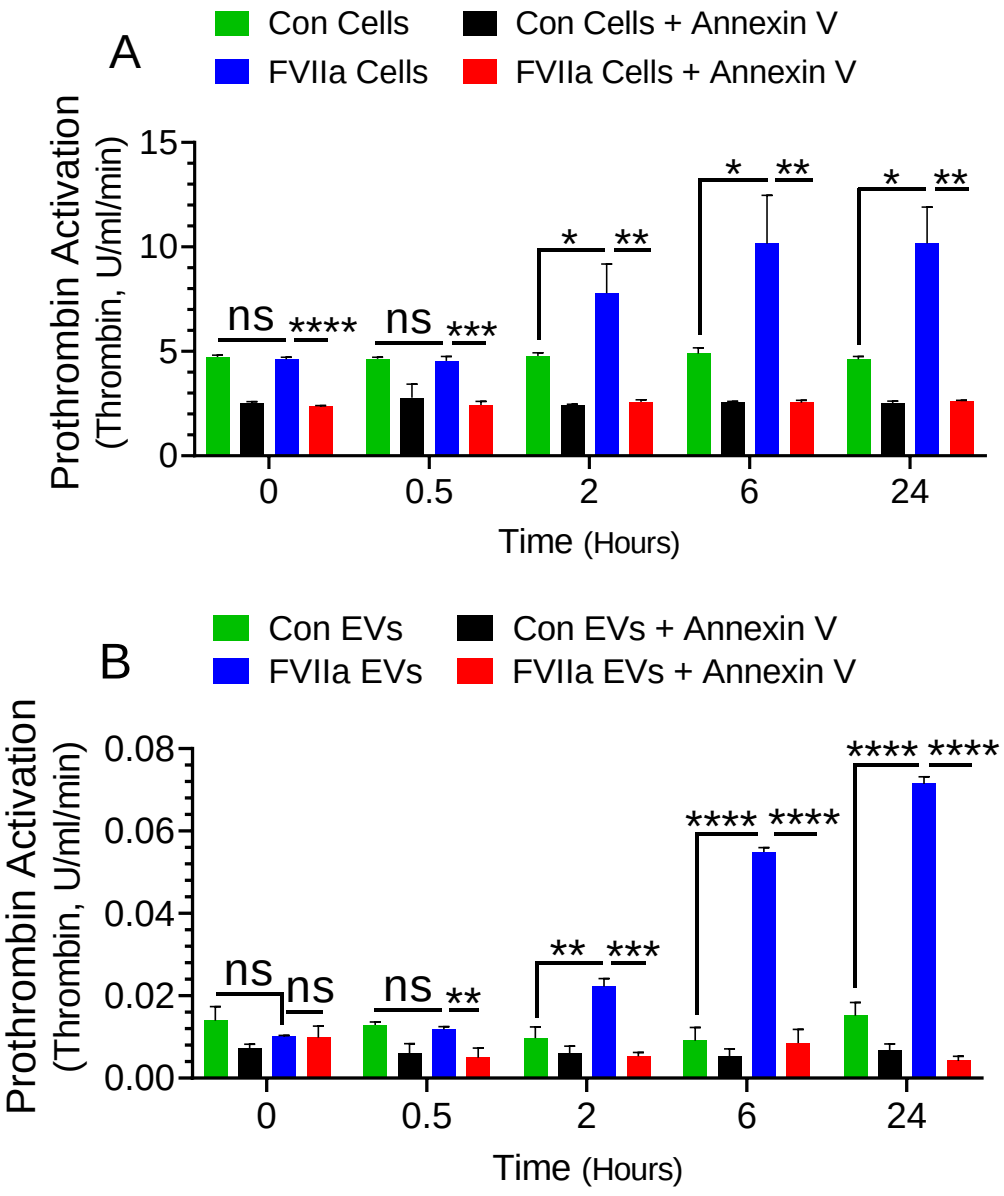
Supplemental Figure 6. FVIIa generated EVs from human aortic endothelial cells (HAEC) and murine brain endothelial cells (bEnd.3) exhibit higher PS-dependent procoagulant activity compared to EVs generated in basal conditions (Con EVs). (A, C) Equal numbers of EVs (1 x 10^8/ml) isolated from control vehicle- or FVIIa-treated HAEC (A) or bEnd.3 cells (C) were incubated with either annexin V (400 nM), control IgG (10 µg/ml), or TF neutralizing antibodies (10 µg/ml) for 1 h. After that, FVIIa (10 nM) was added to the EVs suspension, and 5 min later, FX (175 nM) was added. The rate of FX activation was measured in a chromogenic assay**.** (B, D) Equal numbers of EVs (1 x 10^8/ml) isolated from control vehicle- or FVIIa-treated HAEC (B) or bEnd.3 cells (C) were incubated with control vehicle (-) or annexin V (400 nM) for 1 h. EVs' ability to support prothrombin activation was measured by adding FVa (10 nM) and FXa (1.0 nM), followed by the substrate prothrombin (1.4 µM), and measuring thrombin generated in the reaction mixture in a chromogenic assay. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not statistically significantly different.



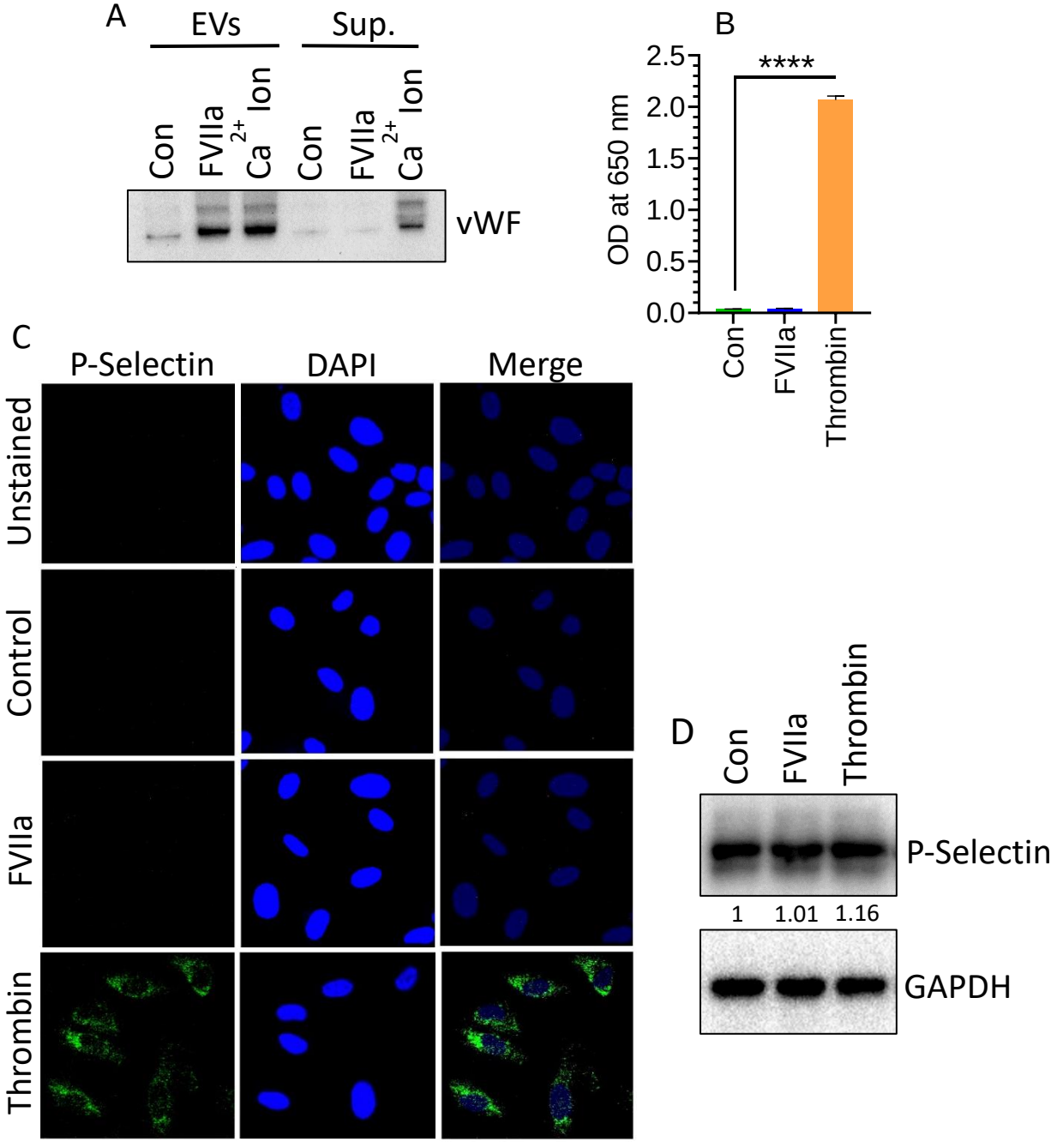
Supplemental Figure 7. FVIIa-generated EVs do not enhance protein C activation. (A) Equal numbers of control- and FVIIa-derived HUVEC EVs were subjected to immunoblot analysis to probe for thrombomodulin (TM). An endothelial cell lysate was used as a positive control. (B) Equal numbers of EVs (1 x10^8) were suspended in 200 µl of buffer B, and protein C (0.2 µM) was added to the EVs suspension. Protein C activation was initiated by the addition of thrombin (10 nM). After 30 min, the reaction was terminated by adding antithrombin (1.66 mg/ml) and heparin (1.4 units/ml). APC activity was measured using a chromogenic substrate. Intact HUVEC cells were used as control. ns, not statistically significantly different.



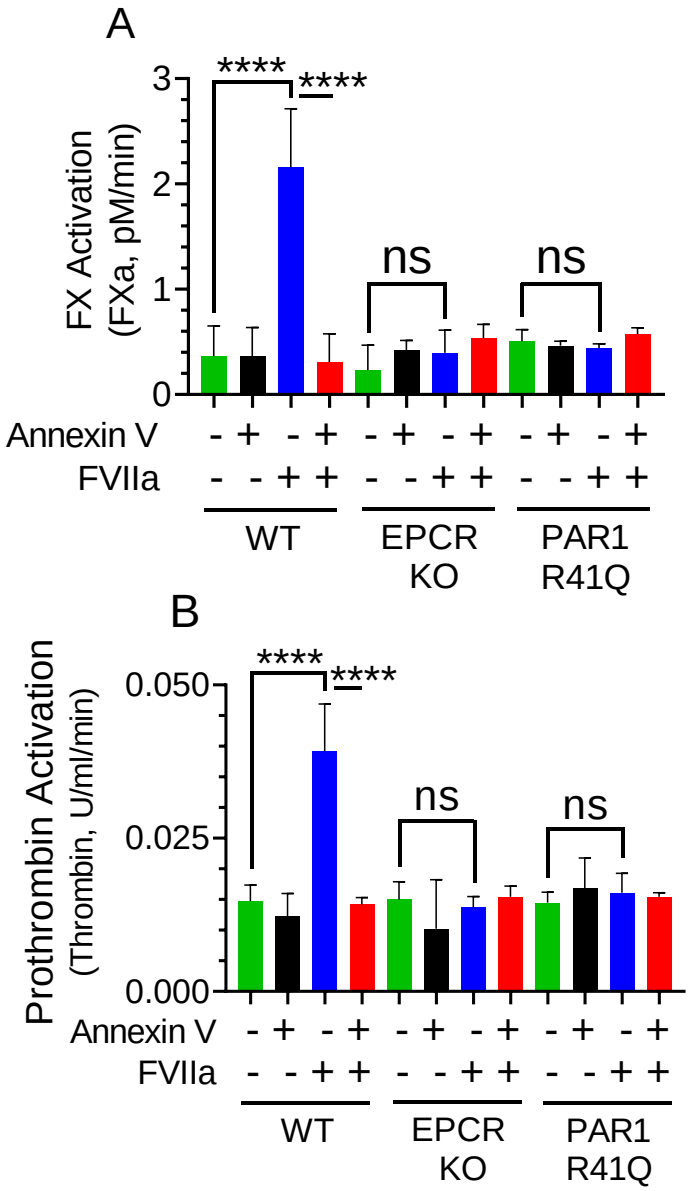
Supplemental Figure 8. Time-dependent externalization of PS in endothelial cells exposed to FVIIa. HUVEC were treated with control vehicle or FVIIa (100 nM) for varying times (0, 0.5, 2, 6, and 24 h). (A) Control and FVIIa-treated cells were incubated with control vehicle or annexin V (400 nM) for 1 h, and then cell surface prothrombinase activity was measured by adding FVa (10 nM), FXa (1.0 nM), and prothrombin (1.4 μM) to the cells, and measuring thrombin generated in the reaction mixture in a chromogenic assay. (B). EVs, isolated from HUVEC after treatment with control or FVIIa for varying times, were analyzed for their ability to support prothrombin activation. Prothrombin assay conditions were the same as described in panel A. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not statistically significantly different.



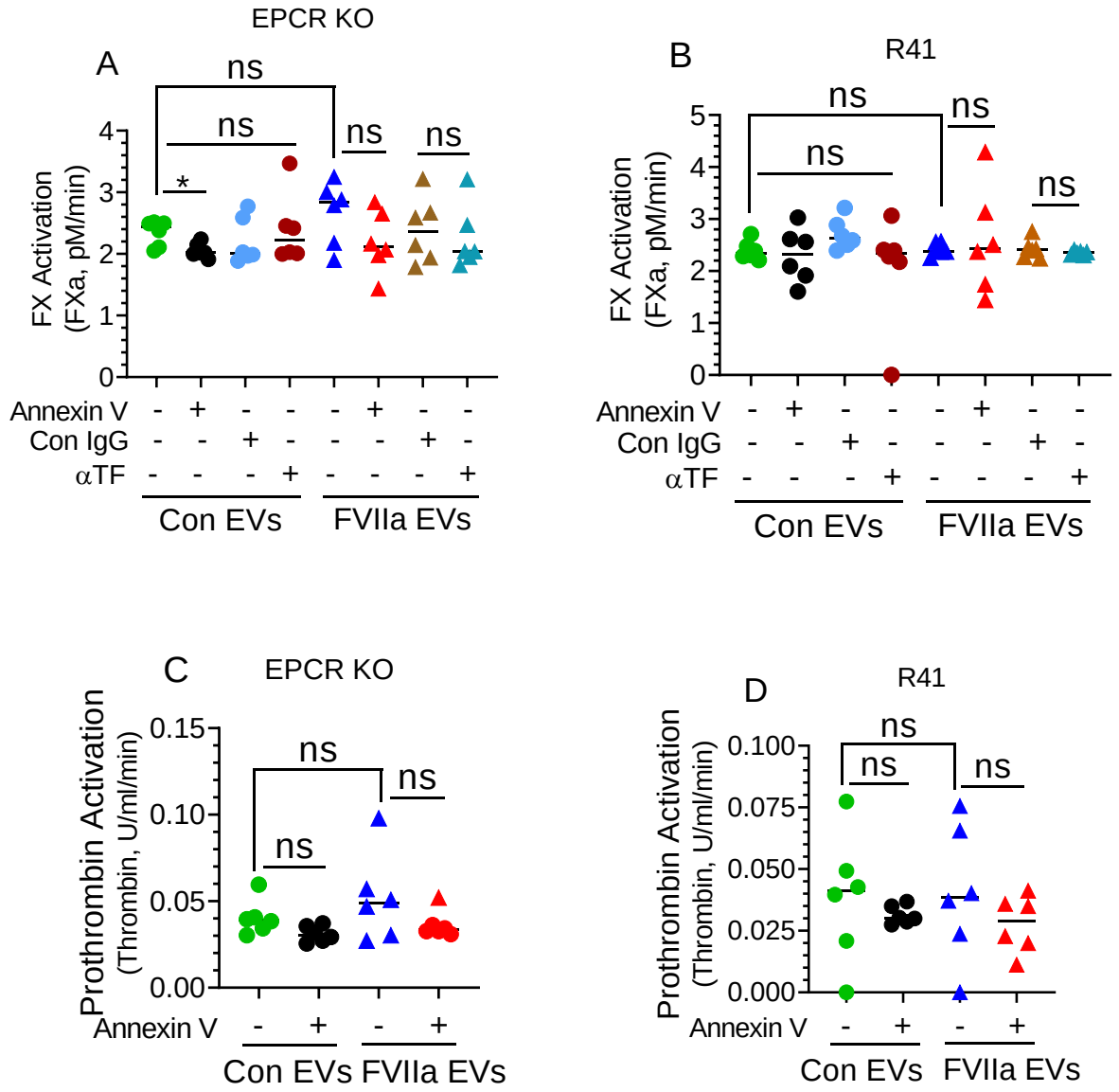
Supplemental Figure 9. FVIIa treatment neither releases vWF nor induces the externalization of P-selectin to the cell surface. (A) Confluent monolayers of HUVEC were treated with a control vehicle or FVIIa (100 nM) for 24 h or Ca^{2+} Ionophore (10 µM; positive control) for 30 min. After isolating the EVs from the conditioned medium, the supernatant fraction was precipitated with 10% TCA to concentrate the proteins. Both EVs and supernatant fractions were subjected to immunoblot analysis to probe for vWF. (B-D) HUVEC monolayers were treated with a control vehicle, FVIIa (100 nM), or thrombin (1 U/ml; positive control) for 5 min. Cell surface P-selectin expression was determined by cell surface ELISA (B) or immunofluorescence microscopy (C). Total cellular P-selectin levels were analyzed by immunoblot analysis (D). ****, p<0.0001.



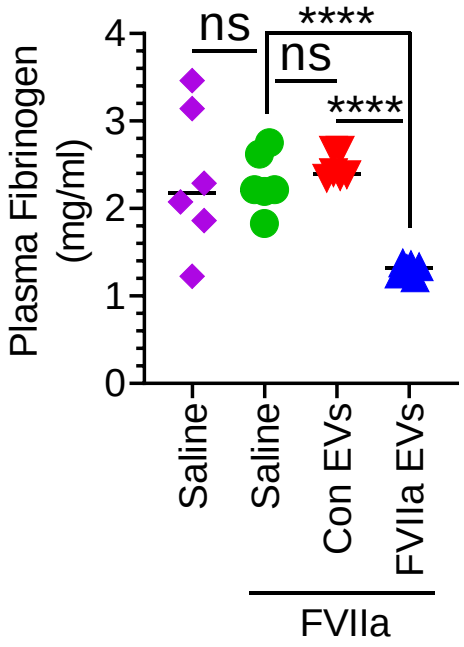
Supplemental Figure 10. FVIIa-generated EVs from WT, but not EPCR-KO or PAR1-R41Q murine brain endothelial cells, exhibit enhanced procoagulant activity. Cultured murine brain endothelial cells, isolated from WT, EPCR-KO, or PAR1-R41Q mice, were treated with control vehicle or FVIIa (100 nM) for 24 h. EVs isolated from the culture supernatant were quantified, and equal numbers of EVs (1 x10^8/ml) were used to measure their ability to support the activation of factor X and prothrombin as described in Supplemental Figure legend 6. (A) FX activation; (B) Prothrombin activation. ****, p<0.0001; ns, not statistically significantly different.



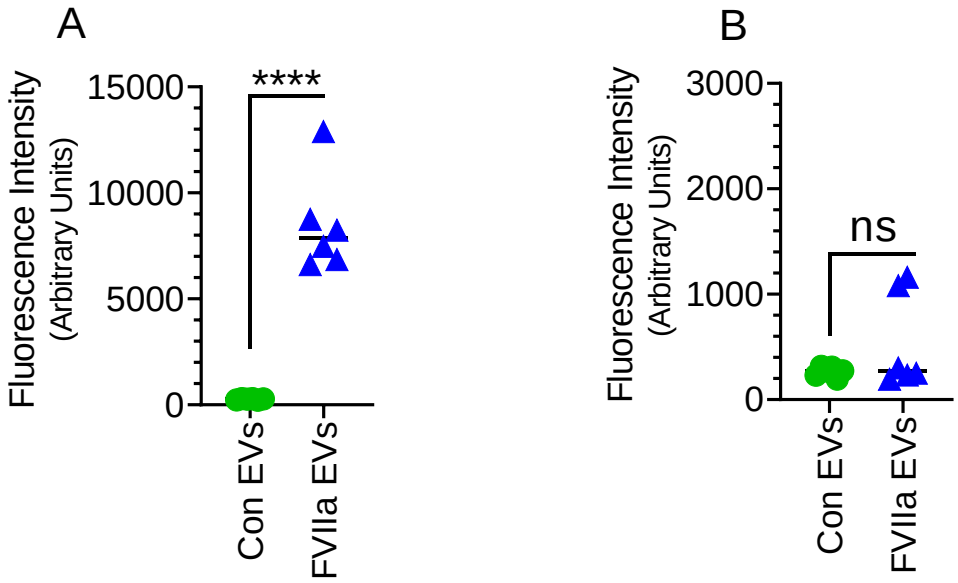
Supplemental Figure 11. EVs isolated from FVIIa-treated EPCR-KO or PAR1-R41Q mice do not exhibit higher procoagulant activity compared to control EVs. EPCR-KO and PAR1-R41Q mice were administered with FVIIa (0.25 mg/kg) via the tail-vein injection. After 2 h, blood was collected into citrate anticoagulant, and EVs were isolated from the plasma as mentioned in Materials and Methods. EVs were assayed for their ability to support factor X (A, B) or prothrombin (C, D) activation. Assay conditions for factor X and prothrombin were the same as described in the legend of Supplemental Figure 6. *, $p<0.05$; ns, not statistically significantly different.



Supplemental Figure 12. Administration of FVIIa-derived EEVs to FVIII$^{-/-}$ mice lowers plasma fibrinogen levels, indicating they support coagulation. 1.5 X 10^9 EVs from control vehicle- or FVIIa-treated endothelial cells were administered to FVIII$^{-/-}$ mice via the tail-vein injection. Mice were injected with FVIIa (0.25 mg/kg) and subjected to the saphenous vein injury. After 15 min of the injury, blood samples were collected via the submandibular vein, and plasma fibrinogen levels were determined. ****, p<0.0001; ns, not statistically significantly different.



Supplemental Figure 13. FVIIa-generated EEVs accumulate at the wound site. FVIII$^{-/-}$ mice were administered via the tail vein with equal numbers (1.5 X 10^9) of PKH67-labeled, control- and FVIIa-generated EVs from bEnd.3 endothelial cells. Mice were injected with FVIIa (0.25 mg/ml) and immediately subjected to the saphenous vein incision to induce bleeding. Blood from the wound site was collected, and equal amounts of blood (50 µl) were used to measure fluorescence intensity by spectrofluorimetry to evaluate the concentration of EVs (A). Blood samples were also collected from the systemic circulation via the submandibular vein puncture and analyzed for fluorescence (B). ****, p<0.0001; ns, not statistically significantly different.



Supplemental Figure 14. FVIIa-generated EVs do not contain tissue factor. HUVEC monolayers were serum-starved for 1 h and then treated with control vehicle, FVIIa (100 nM), or TNF-α (20 ng/ml; positive control). After 24 h, EVs from the supernatant media were isolated and subjected to immunoblot analysis to probe for tissue factor (TF). GAPDH was used as a loading control of cells from which EVs were originated.