Expanded Materials and Methods section

Antibodies and reagents

Monoclonal antibody against VE-cadherin and peroxidase-conjugated immunglobulins to mouse or rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. ADAM10 was detected using a polyclonal antiserum from Calbiochem. β-Tubulin antibody (E7) was from DSHB (University of Iowa). Goat anti-mouse IgG conjugated to Alexa Fluor 594 was from Molecular Probes. The γ-secretase inhibitors DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl esterand) and inhibitor X (L-685,458) were purchased from Calbiochem. TAPI, GM6001, CF109203X, GÖ6976, APMA (aminophenylmercuric acetate), Ionomycin, staurosporine and thrombin (from human plasma) were obtained from Sigma. Hydroxamate-based inhibitors GI254023 and GW280264 were described elsewhere.[1] Recombinant human VE-cadherin and ADAM10 were obtained from R&D Systems.

Cell culture and transfection

HUVECs were obtained from Provitro and cultured in Endothelial Cell Growth Medium from PromoCell. All experiments were performed with HUVECs from passages 2-4. COS-7 cells were grown in DMEM (high glucose) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (PAA Laboratories). Murine VE-cadherin in pcDNA3.1 vector, kindly provided by D. Vestweber, was fused with C-terminally hemagglutinin (HA)-tag. COS-7 cells were transfected in six-well tissue culture plates using FuGENE 6 (Roche) according to the manufacturer's instructions. HUVECs were transfected with jet PEI^{TM}-HUVEC transfection reagent from Polyplus transfection (Hamburg, Germany) according to manufacturer's instructions.

siRNA transfection

Inhibition of ADAM10 expression was performed using the following RNAi oligonucleotides (Stealth^{TM} RNAi, Invitrogen): HSS100165, HSS100166, HSS100167. Unspecific RNAi (Stealth^{TM} RNAi Negative Control LO GC, Invitrogen) with low GC content was used as a negative control. HUVECs were transfected with a total of 40 pmol of an equimolar mixture of the three constructs. Cells were seeded on transwell filters 4 hours after transfection. The cells were harvested for immunoblot analysis after 48 hours. For downregulation of ADAM10 in human T cells (PHA blasts) $5X10^6$ cells were transfected with a total of 150 pMol of an equimolar mixture of the three constructs, using the AMAXA electroporation system according to manufacturers` instructions. After 6 hours of incubation the medium was replaced. T cells were seeded on top of the HUVECs 24h after transfection.

Immunocytochemistry

HUVECs were grown to confluence on collagen-coated glass coverslips. Cells were incubated in serum-free RPMI with stimuli for different time points. Cells were fixed and permeabilised with methanol at –20°C for 10 min. After washing with PBS, cells were blocked with 3% BSA in PBS for 45 min and incubated with primary antibody for 1 hour at room temperature. Following washing with PBS, cells were incubated with secondary antibodies (goat anti-mouse IgG Alexa Fluor 594) for 1h.

Statistical analysis

All values are expressed as mean of triplicates ± S.D. or as mean ± standard error of the mean (SEM). The SEM values indicate the variation between mean values obtained from at least three independent experiments. The assumptions for normality (Kolmogorov-Smirnov test) and equal variance (Levene median test) were verified with the SigmaStat 3.1 software

(Erkrath, SYSSTAT, Germany). The analysis of variance were performed with one-way ANOVA. Multiple parametric statistical comparisons between experimental groups versus a control group were accomplished with Dunnett´s method. All pairwise multiple comparison procedures were performed with Tukey´s test. p-values < 0.05 were classified as statistically significant.

Supplementary Figure Legends

Supplementary Figure 1. Dose-dependent decrease of VE-cadherin proteolysis. HUVECs were treated with DMSO or different concentrations of ADAM10 inhibitor (GI254023X). Cell pellets were analyzed for VE-cadherin CTF generation by immunoblot. Tubulin was used as loading control.

Supplementary Figure 2. Densitometric analysis of ADAM10 siRNA transfection and VE-cadherin proteolysis in HUVECs. Cell pellets of untransfected, mock-transfected or ADAM10 siRNA-transfected HUVECs were harvested 48 hours after transfection and analyzed for ADAM10 expression and for VE-cadherin CTF generation by immunoblot. Total ADAM10 expression and VE-cadherin CTF generation of 4 different experiments was quantified by densitometric analysis. ADAM10 siRNA transfection significantly decreased ADAM10 expression and VE-cadherin shedding. $^{*}P<0.05$ ADAM10 siRNA vs control siRNA treated cells).

Supplementary Figure 3. VE-cadherin proteolysis is ADAM10-dependent. (A) HUVECs were pretreated with DMSO or ADAM10 inhibitor GI254023X (10 µM) for 30 min. Then, cells were stimulated with APMA (100 µM), a potent activator of MMPs and ADAMs, for 30 min. Cell pellets were analyzed for VE-cadherin CTF generation by immunoblotting (upper panel). CTF generation was calculated as percentage of total VE-cadherin by densitometric analysis (right panel). Data are expressed as mean±SEM; n=4 independent experiments. $^{*}P<0.05$ DMSO+APMA vs DMSO, $^{\circ}P<0.05$ GI+APMA vs DMSO+APMA treated cells. HUVECs were transiently transfected with control siRNA or hADAM10 siRNA (lower panel). 48 hours after transfection cells were stimulated with APMA (100 µM), for 30 min. Cell pellets were analyzed for VE-cadherin CTF generation and ADAM10 expression by immunoblotting. p: precursor of ADAM10; m: mature form of ADAM10. Tubulin was used as loading control.

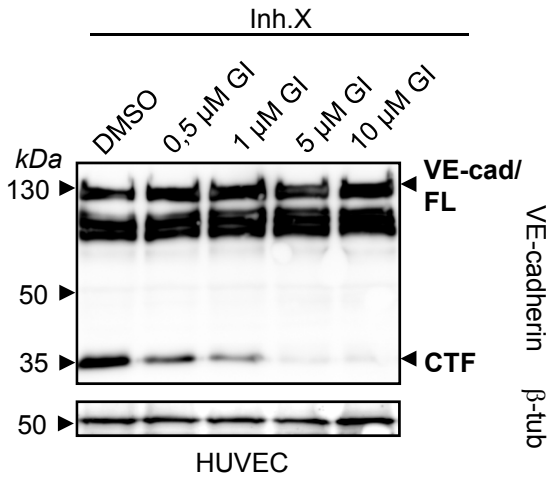
B) Recombinant VE-cadherin is cleaved by ADAM10 in vitro. Recombinant VE-cadherin (1 mg, R & D Systems) was incubated with recombinant ADAM10 (100 ng, R & D Systems) in reaction buffer (25 mM Tris/0.005% Brij-35/2.5 mM ZnCl2, pH 8.8) at 37°C (10 ml per sample). Before starting the reaction, a 10-µl sample was taken, representing the intact VE-cadherin. At the indicated time points, samples were taken and immediately mixed with Laemmli buffer and analyzed by SDS/PAGE under reducing conditions. ADAM10 and VE-cadherin were analyzed by immunoblot with anti-HIS-antibodies.

Supplementary Figure 4. PKC inhibition does not influence VE-cadherin proteolysis. HUVECs were incubated with PKC inhibitor CF109203X (5 µM) and GÖ6976 (1 µM) over night in the presence of γ-secretase inhibitor X (1 µM). Cells were lysed and analyzed by immunoblot using anti-VE-cadherin antibody. Tubulin was used as loading control.

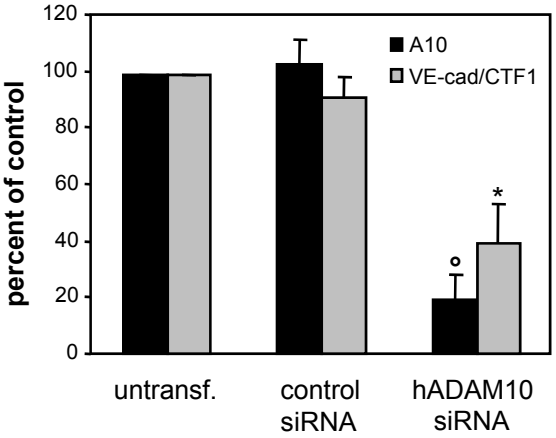
Supplementary Figure 5. Ionomycin and Thrombin induce ADAM10-dependent VE-cadherin shedding. HUVECs were transiently transfected with control siRNA or hADAM10 siRNA. 48 h after transfection cells were treated with A) ionomycin (IO, 10 µM) or with B) thrombin (1 U/ml) for 30 min in the presence of the γ-secretase inhibitor X (1 µM). Cells were analysed for VE-cadherin CTF generation and ADAM10 expression by immunoblotting. Tubulin was used as loading control.

REFERENCE

1. Ludwig A, Hundhausen C, Lambert MH, Broadway N, Andrews RC, Bickett DM, Leesnitzer MA, Becherer JD. Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell surface molecules. *Comb Chem High Throughput Screen*. 2005;8:161-171.

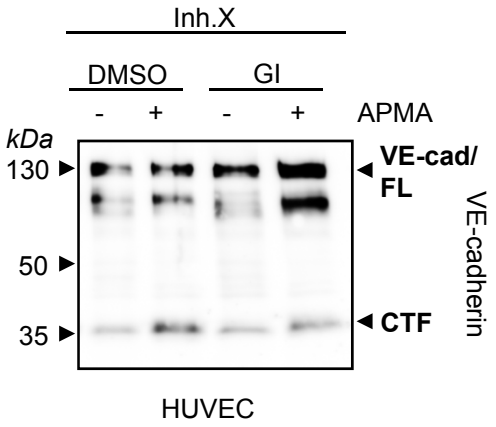


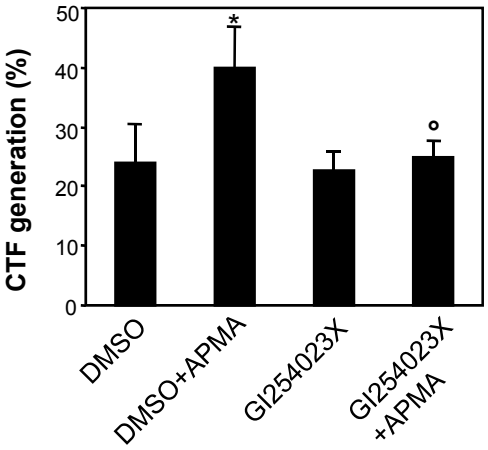
Supplementary Figure 1

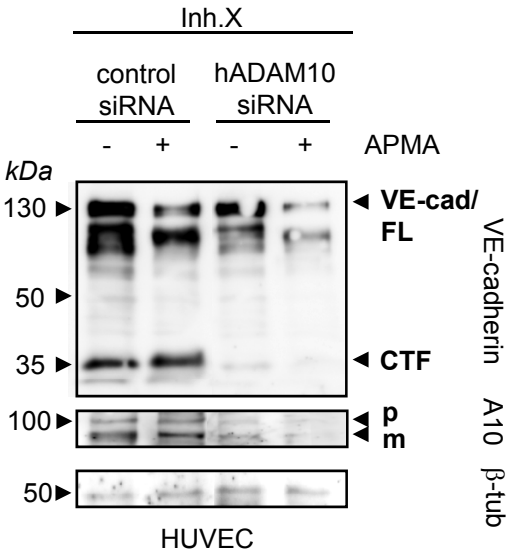


Supplementary Figure 2

A







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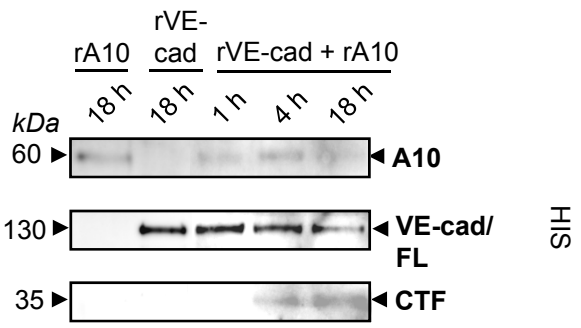
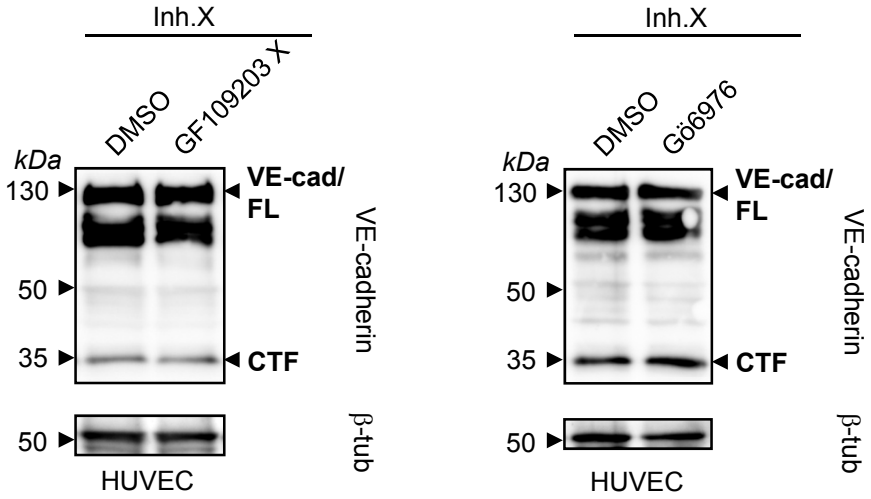
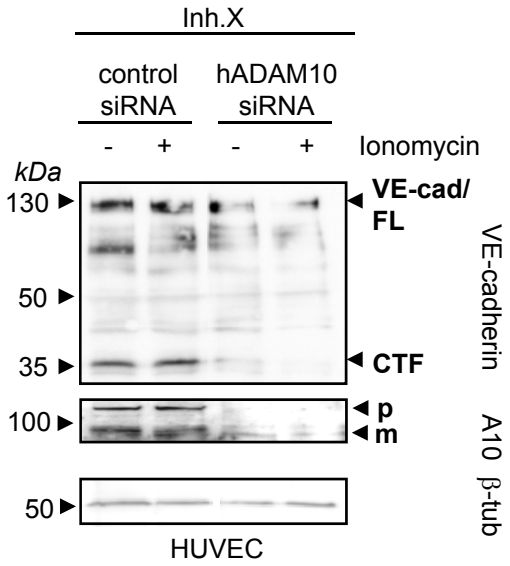


Supplementary Figure 3



Supplementary Figure 4

A



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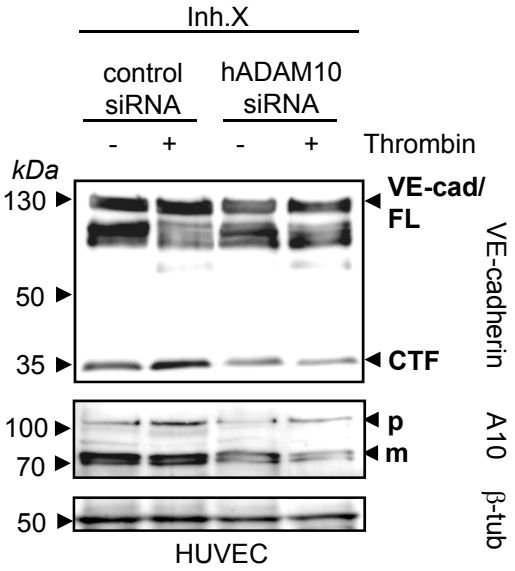

Supplementary Figure 5