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RESEARCH PAPER

# Activated thrombin-activatable fibrinolysis inhibitor attenuates the angiogenic potential of endothelial cells: potential relevance to the breast tumour microenvironment

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**Abstract** Thrombin-activatable fibrinolysis inhibitor (TAFI) is a basic carboxypeptidase zymogen present in blood plasma. Proteolytic activation of TAFI by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin, or plasmin results in an enzyme (TAFIa) that removes carboxyl-terminal lysine residues from protein and peptide substrates, including cell-surface plasminogen receptors. TAFIa is therefore capable of inhibiting plasminogen activation in the pericellular milieu. Since plasminogen activation has been linked to angiogenesis, TAFIa could therefore have anti-angiogenic properties, and indeed TAFIa has been shown to inhibit endothelial tube formation in a fibrin matrix. In this study, the TAFI pathway was manipulated by providing exogenous TAFI or TAFIa or by adding a potent and specific inhibitor of TAFIa. We found that TAFIa elicited a series of anti-angiogenic responses by endothelial cells, including decreased endothelial cell proliferation, cell invasion, cell migration, tube formation, and collagen degradation. Moreover, TAFIa decreased tube formation and proteolysis in endothelial cell culture grown alone and in co-culture with breast cancer cell lines. In accordance with these findings, inhibition of TAFIa increased secretion of matrix metalloprotease proenzymes by endothelial and breast cancer cells. Finally, treatment of endothelial cells with TAFIa significantly inhibited plasminogen activation. Taken together our results suggest a novel role for TAFI in inhibiting tumour angiogenic behaviors in breast cancer.

**Keywords** TAFI · Angiogenesis · Breast cancer · Plasminogen · Endothelial cells

**Abbreviations**

| | |
|---|---|
| BME | Basement membrane extract |
| CM | Conditioned media |
| ε-ACA | ε-aminocaproic acid |
| ECM | Extracellular matrix |
| HUVECs | Human umbilical vein endothelial cells |
| MMPs | Matrix metalloproteinases |
| PAS | Plasminogen activation system |
| PTCI | Potato tuber carboxypeptidase inhibitor |
| TAFI | Thrombin-activatable fibrinolysis inhibitor |
| TAFIa | Activated thrombin-activatable fibrinolysis inhibitor |
| TM | Thrombomodulin |
| tPA | Tissue-type plasminogen activator |
| uPA | Urokinase plasminogen activator |
| uPAR | Urokinase plasminogen activator receptor |
| VEGF | Vascular endothelial growth factor |

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## Introduction

Sustained angiogenesis is of one the hallmarks of cancer [1, 2] and is necessary for the growth of a tumour past a dormant avascular stage [3–6]. Tumour angiogenesis provides nutrients and oxygen to a tumour and also can act as an escape route allowing metastasis to occur [6, 7]. It is firmly established that highly angiogenic tumours have a higher metastatic potential than less vascular tumours [8].

The plasminogen activation system (PAS) is an important contributor to the metastatic process, including angiogenesis [9, 10]. The PAS is an enzymatic cascade responsible for cleavage and activation of plasminogen to its active form plasmin by urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [11]. Plasminogen activation is enhanced on the cell surface by plasminogen receptors, some of which possess carboxyl-terminal lysine residues, allowing for plasminogen binding [12]. Plasmin is a serine protease that cleaves a wide range of substrates, including several matrix metalloproteinases (MMPs) and a variety of extracellular matrix (ECM) proteins [13], events that stimulate cell invasion, migration, and ultimately sprouting during angiogenesis [14].

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B2 and procarboxypeptidase U, is a plasma zymogen that plays a role in attenuating fibrinolysis [15] and in regulating pericellular plasminogen activation [16]. TAFI is activated by thrombin, plasmin and, most efficiently, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM). TAFIa is a basic carboxypeptidase that cleaves carboxyl-terminal lysine residues from various protein and peptide substrates, including fibrin degradation products and plasminogen receptors.

Recently, we have demonstrated that TAFIa possesses anti-metastatic potential, inhibiting cell invasion, cell migration and collagen degradation of breast cancer cells, through attenuation of plasminogen activation [17]. Likewise, Higuchi et al. found that TM-stimulated activation of TAFIa decreased invasion of HT1080 fibrosarcoma cells by reducing pericellular plasminogen activation [18]. A study by Reijerkerk et al. reported no difference in metastasis or tumour growth in TAFI knockout mice [19]. However, the cell lines used in the latter study (Lewis lung carcinoma and B16-BL melanoma) do not support TM-mediated TAFI activation on their surface; therefore, this study does not clearly rule out a role for TAFI in cancer. The role of TAFI in tumour angiogenesis has not been previously explored, although TAFIa has been shown to reduce degradation of ECM [20] and to inhibit wound healing angiogenesis in plasma clots [21]. Collectively, these findings suggest that TAFIa has the potential to play an anti-angiogenic and anti-metastatic role in cancer progression. We therefore set out to further assess the potential of TAFI to regulate angiogenesis in a setting that, unlike a plasma clot, more closely mimics the breast cancer tumour microenvironment.

## Materials and methods

### Cell lines and cell culture

SUM149 cell line was a gift from Dr. Stephen Ethier (Barbara Ann Karmanos Cancer Institute), MDA-MB-231 cell line was purchased from Sigma, and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. SUM149 cells were grown in Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotic/antimycotic (Gibco), 10 µg/mL insulin (Sigma–Aldrich) and 0.5 µg/mL hydrocortisone (Sigma–Aldrich). MDA-MB-231 cells were supplemented with DMEM/F-12 supplemented with 10% FBS, and 1% antibiotic/antimycotic. HUVECs were supplemented with Endothelial Cell Basal Medium-2 (EBM-2) (Lonza) completed with 2% FBS (Lonza) and Endothelial Cell Growth Media-2 (EGM-2) SingleQuots Kit (Lonza). HUVECs were used at passage five for all experiments. Human embryonic kidney (HEK 293) cells were cultured in minimum essential media (MEM) (Gibco), containing 5% FBS and 1% antibiotic/antimycotic. All cells were maintained at 37 °C in a humidified 95% air/5% $CO_2$ atmosphere.

### TAFI purification and activation

TAFI was cloned into a mammalian expression plasmid in-frame with a carboxyl-terminal 6×His tag encoding sequence, as previously described [17]. This plasmid was stably transfected into HEK 293 cells and conditioned media (CM) was collected from cell lines grown in triple flasks (Nunc). Recombinant TAFI (rTAFI) was purified utilizing a $Ni^{2+}$-Sepharose column (GE Healthcare Life Sciences), as previously described [17]. TAFI (0.8 µM) was activated to TAFIa using 25 nM thrombin (Haematologic Technologies), 100 nM thrombomodulin (Haematologic Technologies) and 5 mM $CaCl_2$ for 10 min at room temperature. Following incubation, thrombin was inhibited using 200 nM H-D-Phe–Pro–Arg chloromethylketone (PPAck; Calbiochem). TAFIa was then used in experimental procedures as described below, at a final concentration of 50 nM. For experiments using non-activated TAFI, the activation mixtures were assembled as described above, in the absence of thrombin. For control experiments, the activation mixtures were assembled in the absence of TAFI. Therefore, all experiments were conducted with the

equivalent concentrations of added $CaCl_2$, thrombomodulin, and chloromethylketone.

### Endothelial cell invasion and migration assays

Transwell invasion assays were used to assess the effect of inhibiting TAFIa on endothelial cell invasion. BD Falcon cell culture inserts (8 μm pore size; PET track-etched membranes) (BD Biosciences) were coated with 2 mg/mL Cultrex® Basement Membrane Extract (BME) without Phenol Red (Trevigen) in a 24-well plate for 1 h. BME was diluted to 2 mg/mL from a stock solution with serum and supplement-free EBM-2 medium (Lonza). Following incubation, the inserts were washed three times with HEPES Buffered Saline Solution (HBSS) (Lonza). Supplement free EBM-2 containing 0.5% FBS was added to the bottom of each well in the 24-well plate. Test substances added to this medium were 50 ng/mL vascular endothelial growth factor (VEGF), 25 μg/mL potato tuber carboxypeptidase inhibitor (PTCI) [16] or 1 μM aprotinin [22]. For experiments using TAFI, medium in the bottom chamber contained 50 ng/mL VEGF and 50 nM activated or non-activated TAFI, or no added TAFI. Coated inserts were placed in the wells and 50,000 HUVECs/well in 100 μL of serum- and supplement-free EBM-2 were added into the coated inserts. Cells were allowed to invade into the BME for 20 h, after which non-invaded cells were removed with a cotton swab, and the invaded cells were fixed in methanol. The invaded cells were stained with 0.25% (w/v) Crystal Violet (Sigma–Aldrich). The cells in five different fields of view were counted under a 20×objective using an Olympus CKX415F2 Inverted Microscope or an Olympus IX51 Inverted Microscope.

Cell migration assays were carried out in the same manner as the cell invasion assay, except cell culture inserts were not coated with BME.

### Cell metabolism assay

Cell proliferation of HUVECs was determined using the WST-1 assay (Roche Diagnostics), which measures metabolic activity of viable cells. HUVECs were seeded in a 96-well plate at 10,000 cells/well complete EBM-2 media. Cells were treated in quadruplicate with either 50 ng/mL VEGF or 25 μg/mL PTCI. For experiments with TAFI, cells were treated with 50 nM of either activated or non-activated TAFI, in the presence or absence of 50 ng/mL VEGF. Cells were treated for 24 h, following which WST-1 was added to each well and incubated for 2 h at 37 °C. The cleavage of WST-1 to formazan by metabolically active cells was measured at 450 nm using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices).

### Tube formation assays

In preparation for tube formation assays, 35 mm glass bottom culture dishes (MatTek) were coated with BME and incubated for 10 min at 37 °C to allow the BME to solidify. HUVECs grown in T25 flasks to ~80% confluency were washed four times with HBSS (Lonza), and labelled with 5 μM Cell Tracker™ Orange CMTMR (Life Technologies Inc.) in EBM-2 with 0.75% FBS for 1 h at 37 °C. Labelled cells were then washed four times with HBSS (Lonza) and incubated in complete EBM-2 for 1 h at 37 °C. Following incubation, 20,000 HUVECs in EBM-2 with 0.75% FBS were seeded on top of the BME-coated glass bottom dishes and allowed to attach for 40 min at 37 °C. Subsequently, complete EBM-2 media containing 2% BME with and without treatment (50 ng/mL VEGF or 25 μg/mL PTCI) was added. For TAFI treatments, 50 nM of either activated or non-activated TAFI or the control activation mixture was added to complete EBM-2 containing 2% BME. Cells were incubated at 37 °C for 18 h to allow formation of tube-like structures [23, 24]. Cells were then imaged using an Olympus IX81 confocal microscope. Tube formation in eight–ten different fields of view was imaged under a 10× objective. Relative tube length was measured as the total length of all tubes within the field of view divided by total number of tubes within the field of view.

For co-culture experiments, SUM149 or MDA-MB-231 cells washed with phosphate buffered saline (PBS), and labelled with 5 μM CellTrace™ Far Red DDAO-SE (Life Technologies Inc.) in serum free DMEM/F-12 for 1 h at 37 °C. Labelled cells were then washed with PBS, and incubated in complete DMEM/F-12 for 1 h at 37 °C. Following incubation, breast cancer cells (20,000 SUM149 cells or 7000 MDA-MB-231 cells in serum free DMEM/F-12) were seeded on top of BME coated glass bottom dishes, and allowed to attach for 40 min at 37 °C. Complete DMEM/F-12 media containing 2% BME was added and cells were incubated at 37 °C for 24 h. Thereafter, media was removed and 20,000 HUVECs pre-labeled with Cell Tracker™ Orange CMTMR were seeded on top of the embedded breast cancer cells. Cells were treated with either VEGF (50 ng/mL), PTCI (25 μg/mL) or 50 nM TAFI, as described above. After 18 h of incubation at 37 °C, tube formation was assessed as described above.

### Dye-Quenched (DQ)-collagen IV proteolysis assays

Proteolysis of DQ-collagen IV by HUVECs was assessed as previously described [25, 26]. Briefly, 35 mm dishes were coated with BME containing 25 μg/mL DQ-collagen IV (Life Technologies Inc.) and incubated for 10 min at 37 °C. HUVECs were then seeded on top of the BME/DQ-collagen IV mixture as described above

for the tube formation assay with and without 50 ng/mL VEGF or 25 µg/mL PTCI or 50 nM TAFI/TAFIa treatments. Cells were incubated at 37 °C for 18 h, and then imaged using an Olympus IX81 confocal microscope. Three to four *z*-stacks each containing fourteen 2.5 µM slices were imaged using a 20× objective with a zoom of three, and three adjacent slices were selected for analysis (one of which generally including the slice at the equatorial plane). The amount of proteolysis was determined using ImageJ software by calculating total green fluorescence of the DQ-collagen type IV degradation products in each the three slices divided by HUVEC area and averaging over the selected slices.

Proteolysis of DQ-collagen IV by co-cultured HUVECs and breast cancer cells was assessed as previously described [27]. BME mixed with 25 µg/mL DQ-collagen IV was used to coat a 35 mm glass bottom culture dish as described above. Breast cancer cells, pre-labeled with CellTrace™ Far Red DDAO-SE, were seeded on top of the BME/DQ-collagen IV mixture using the same cell number, procedure and treatments as described for the co-culture tube formation assay above. Cells were incubated at 37 °C for 18 h. Areas where HUVEC tube-like structures were in contact with a breast cancer spheroid were imaged and the extent of proteolysis measured as described above for the HUVEC monocultures.

### Plasminogen activation assay

Plasminogen activation assays were conducted as previously described [17, 28]. Briefly, HUVECs were seeded to confluency in black, clear-bottom 96 well plates. TAFI was activated to TAFIa as described above. Cells were washed twice with HBS supplemented with 0.4% (w/v) BSA (HBS-BSA). Plasminogen was purified from human plasma using lysine-Sepharose affinity chromatography, as previously described [29]. Plasminogen activation on the cell surface was conducted using both uPA and tPA. For the uPA experiments, a solution containing various concentrations of TAFIa (0–50 nM), 500 nM plasminogen, 20 nM uPA (EDM Millipore) and 40 mM of the fluorogenic plasmin substrate H-D-Val–Leu–Lys-AMC (Bachem) was added to the cells. For the tPA experiments, various concentrations of TAFIa (0–50 nM), 500 nM plasminogen, 5 nM tPA (Alteplase; Kingston General Hospital) and 40 mM H-D-Val–Leu–Lys-AMC was used. For experiments with the lysine analogue ε-aminocaproic acid (ε-ACA), 10 nM TAFIa and 50 µM ε-ACA were used. Hydrolysis of the substrate was monitored for 60 min at 37 °C, at excitation and emission wavelengths of 370 and 470 nm, respectively.

### Gelatin zymography

Subconfluent monocultures of SUM149 cells or HUVECs were treated with or without 50 ng/mL VEGF or 25 µg/mL PTCI for 24 h. Following treatment, SUM149 cells and HUVECs were washed with PBS and then serum starved for 24 h in serum free DMEM or EBM-2 with 0.2% FBS media, respectively. CM was collected from both cell types. Lysates from both cell types were collected by adding lysis buffer (50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). Cell lysate protein concentration was determined using a BCA assay (Life Technologies Inc.) according to the manufacturer's instructions.

In some experiments, HUVECs grown to 60% confluence were washed with HBSS (Lonza) and CM from untreated, VEGF, or PTCI treated SUM149 cells (prepared under conditions as described above) was mixed 1:1 with complete EBM-2 medium and added to the HUVECs. Control HUVECs were treated with complete EBM-2 medium alone. Following 24 h of incubation with SUM149 CM, the HUVECs were washed with HBSS and placed in EBM-2 media with 0.2% FBS for 24 h. The supernatant and lysate were then collected, and lysate protein concentration was determined as described above.

MMP2 and MMP9 enzymatic activities in medium from SUM149, HUVEC, or HUVEC treated with SUM149-CM were determined by SDS–PAGE gelatin zymography as described by Mohamed et al. [30]. Samples were mixed with 2× SDS sample buffer and equal protein amounts (20 µg/lane of SUM149 samples, 6 µg/lane of HUVEC samples) were loaded, without heating or reducing, and subjected to SDS–PAGE on 10% polyacrylamide gels containing 1% (w/v) gelatin at 125 V for 2.5 h at 4 °C. Human recombinant MMP2 (5 ng) and MMP9 (2.5 ng) (R&D Systems) were also loaded as positive controls. Following electrophoresis, gels were incubated twice in renaturing buffer (2.5% Triton X-100) for 15 min. Thereafter, gels were washed twice with deionized water for 15 min. Gels were then placed in developing buffer (50 mM Tris–HCl pH 7.8, 5 mM $CaCl_2$, 0.05% Brij 35) and incubated overnight at 37 °C. Subsequently, gels were stained for 1 h with Coomassie blue (50% methanol, 10% acetic acid, 40% water, 0.5% Coomassie brilliant blue R-250), and destained (50% methanol, 10% acetic acid, 40% water) until clear bands of proteolytic activity appeared. Relative proteolytic activity of each sample was determined by densitometry using FluoroChem® Q imaging system software (Alpha Innotech Corporation).

### Statistical methods

Results are expressed as a mean ± SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA test with post-hoc Tukey HSD with IBM SPSS Statistics V22.0 software. Statistical significance was presumed at $p < 0.05$.

## Results

### TAFIa inhibits endothelial cell invasion and migration

During angiogenesis, endothelial cells locally degrade the vascular basement membrane and invade into the surrounding stroma [3, 31]. Here, we performed an in vitro invasion assay by seeding HUVECs in the top chamber of a BME coated Transwell insert, to assess the effect of TAFIa on endothelial cell invasion. TAFIa was inhibited using a specific competitive inhibitor, potato tuber carboxypeptidase inhibitor (PTCI). For the purposes of comparison, we also used vascular endothelial growth factor (VEGF), a known stimulator of angiogenesis. Our results show that PTCI significantly increased invasion of HUVECs almost twofold relative to the control, while VEGF increased invasion of HUVECs approximately threefold relative to the control (Fig. 1a, b). To examine whether the effects of PTCI were a result of increased plasmin generation, we assessed the effect of PTCI in the presence of the plasmin inhibitor, aprotinin. The increase in invasion observed with PTCI was abolished by addition of aprotinin (Fig. 1b). We next assessed the direct effect of TAFI on endothelial cell invasion. Treatment with both activated and non-activated TAFI resulted in a more than twofold decrease in HUVEC invasion compared to the control treated with VEGF (Fig. 1e).

Cell migration is another important process that occurs during angiogenesis. Migration assays were conducted in the absence of BME and hence reflect only the motility of the cells, since there is no BME barrier that must be proteolyzed. To assess the effect of TAFI on cell migration, TAFIa was inhibited using PTCI or the cells were treated with TAFI/TAFIa. As shown in Fig. 1c, treatment with PTCI results in a significant increase in cell migration, an effect that was abolished by the addition of aprotinin (Fig. 1c). Treatment with either activated or non-activated TAFI significantly decreased endothelial cell migration by almost twofold compared to VEGF alone (Fig. 1f). Together, these results demonstrate a role for TAFIa in mediating endothelial cell invasion and migration.

### Effect of TAFIa on cell proliferation

We investigated whether the effects of PTCI on HUVEC invasion and migration may have been due to an increase in proliferation. After incubation for 18 h, both VEGF and PTCI increased HUVEC metabolic activity, as measured by the WST-1, as an index of cell proliferation (Fig. 2a). We next examined the effect of TAFI and TAFIa on HUVEC metabolic activity, in the presence and absence of VEGF. In the absence of VEGF, neither TAFI nor TAFIa had a significant effect on metabolic activity (Fig. 2b). In the presence of VEGF, both the TAFI and TAFIa significantly decreased metabolic activity (Fig. 2b). However, the effects of TAFIa or TAFIa inhibition were of a much lower magnitude than their effects on cell invasion and migration (Fig. 1).

### TAFIa inhibits tube formation of endothelial cells grown alone and in co-culture with breast cancer cells

Another index of the angiogenic potential of endothelial cells is the formation of tube-like structures in vitro, in which the cells undergo a process of attachment, migration, and differentiation of endothelial cells into tube-like structures on a matrix in a manner that mimics the in vivo process of tube formation [24, 32]. Previous studies have shown that inhibition of TAFIa increases endothelial tube formation in a 3D plasma clot consisting of a fibrin matrix [21]. While this result demonstrated a role for TAFI in wound healing angiogenesis, the role of TAFI in endothelial tube formation during tumour angiogenesis has yet to be studied. HUVECs were seeded on top of BME, and were treated with either VEGF or the TAFIa inhibitor PTCI (Fig. 3a). PTCI promoted in vitro tube formation of HUVECs as observed by the significant increased relative tube length (1.3-fold) compared to the control, and resembling the effect of VEGF (Fig. 3b). We next examined the direct effect of TAFI on endothelial tube formation. Likewise, treatment of endothelial cells with both TAFI and TAFIa significantly decreased tube formation (Fig. 3c) to 0.75-fold of untreated control (Fig. 3d) .

Next, we assessed the effect of TAFIa inhibition on tube formation of HUVECs grown in co-culture with either SUM149 cells (Fig. 4) or MDA-MB-231 cells (Supplementary Fig. 1), both of which are highly aggressive triple negative breast cancer cell lines. The breast cancer cells form spheroid structures on top of the BME, while the endothelial cells form tube-like structures. This in vitro co-culture model functions to mimic the in vivo tumour microenvironment, which consists of cancer cells as well as stromal cells such as endothelial cells [33]. Interaction between HUVECs and breast cancer cells can be seen in merged images combining differential interference contrast

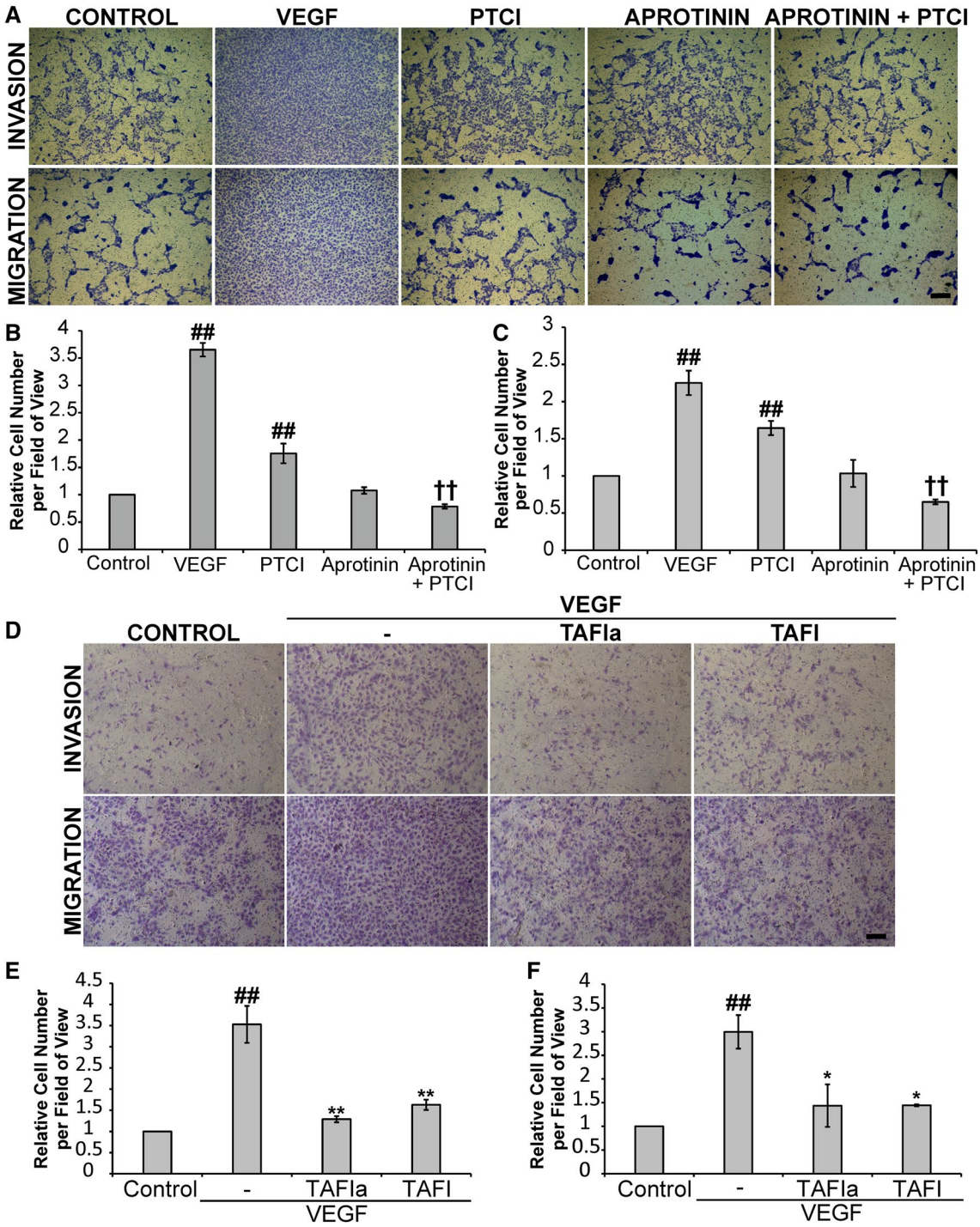


**Fig. 1** TAFI decreases endothelial cell invasion and migration. **a** HUVECs were added to a Transwell chamber coated with BME (for invasion), or without BME (for migration), in the presence and absence of 50 ng/mL VEGF or 25 μg/mL PTCI or 1 μM aprotinin. Cells were allowed to invade for 20 h after which cells were fixed, stained and imaged. Images above were obtained at 4× magnification. *Scale bar*, 200 μM **b** Quantification of HUVEC invasion. **c** Quantification of HUVEC migration. For both cell invasion and migration, cells were counted from five different fields of view at 20× magnification, and cell numbers expressed relative to control. **d** HUVECs were added to a Transwell chamber coated with BME (for invasion), or without BME (for migration), in the presence 50 ng/mL VEGF with 50 nM of either activated (TAFIa) or non-activated TAFI. Cells were allowed to invade for 20 h and then were fixed, stained and imaged. **e** Quantification of HUVEC invasion *Panels B*. **f** Quantification of HUVEC migration *Panels C*. Values are expressed as mean ± SEM from four independent experiments. $^{\#\#}p < 0.01$ versus control, $^{\dagger\dagger}p < 0.01$ versus PTCI alone $^{*}p < 0.05$, $^{**}p < 0.01$ versus VEGF alone

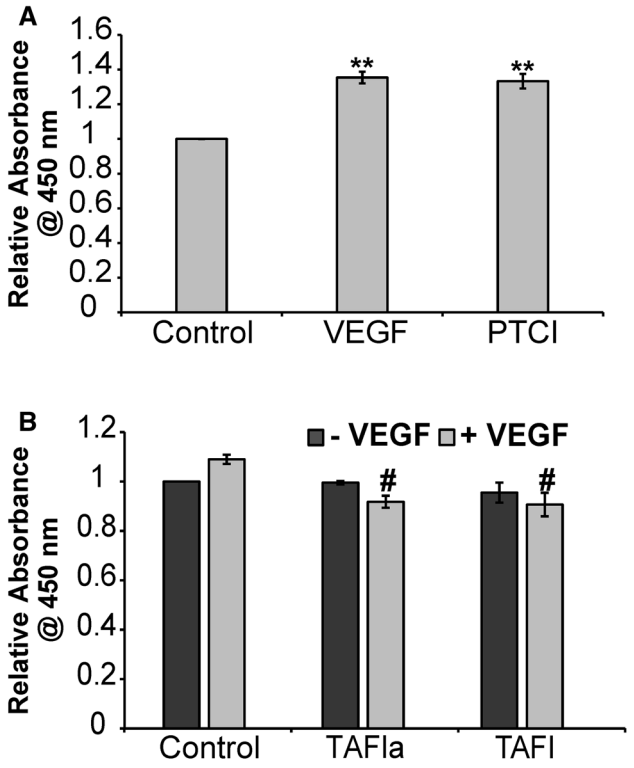


**Fig. 2** Effect of TAFI on cell proliferation of endothelial cells. **a** HUVECs were treated for 24 h with 50 ng/mL VEGF or 25 µg/mL PTCI. The WST-1 assay was performed as an index of proliferation. Absorbance values for the formazan product were measured at 450 nm using a plate-reading spectrophotometer. **b** HUVECs with treated with 50 nM of either activated or non-activated TAFI in the presence or absence of 50 ng/mL VEGF for 24 h. Values are expressed as mean ± SEM from ≥3 independent experiments performed in quadruplicate. *$p < 0.05$, **$p < 0.01$ *versus* control, #$p < 0.05$, versus control with VEGF

(DIC), HUVEC fluorescence, and breast cancer cell fluorescence images (Fig. 4a–c; Supplementary Fig. 1A). Co-cultures were treated with VEGF, PTCI, TAFI, or TAFIa. PTCI significantly increased relative tube length compared to the control with a 1.4-fold increase in both the SUM149:HUVEC co-cultures (Fig. 4b) or the MDA-MB-231:HUVEC co-cultures (Supplementary Fig. 1A), and at levels similar to treatment with VEGF. Treatment with both TAFI and TAFIa decreased endothelial tube length to approximately 0.75-fold of untreated control (Fig. 4d).

### TAFIa decreases proteolysis of DQ-collagen IV by endothelial cells grown alone and in co-culture with breast cancer cells

One of the initial steps of angiogenesis involves the degradation of vascular basement membrane and ECM by endothelial cells [3, 31]. Pericellular plasminogen activation has been implicated in angiogenesis and metastasis and associated with the activation of MMPs, release of growth factors from the ECM, and degradation of the ECM [34]. VEGF upregulates components of the plasminogen activation system in the leading edge of migrating endothelial cell [13, 35, 36]. Moreover, tube formation spatially correlates with ECM proteolysis in vitro [37]. TAFIa inhibits pericellular plasminogen activation in vitro and in vivo [38–40]. Therefore, we tested the effect of TAFIa on proteolysis of DQ-collagen IV, since collagen-IV is the most abundant type of collagen found in the basement membrane [41]. HUVECs were seeded on top of BME mixed with DQ-collagen IV, and treated with either VEGF or PTCI. Once DQ-collagen IV—a quenched fluorescent substrate—is cleaved by proteases, fluorescent DQ-collagen IV degradation products are produced (green) (Fig. 5a). Degradation products of DQ-collagen IV were localized predominantly intracellularly, although treatment with VEGF did slightly increase pericellular [outside the cell or on the cell membrane (37)] localization of DQ-collagen IV degradation products (Fig. 5a). Treatment of HUVECs with PTCI significantly increased proteolysis of DQ-collagen IV, two-fold relative to the control (Fig. 5b). We next examined the effect of TAFI and TAFIa on DQ-collagen IV proteolysis of HUVECs (Fig. 5c). TAFIa decreased DQ-collagen IV proteolysis to 0.7-fold of control (Fig. 5d). Non-activated TAFI decreased proteolysis to 0.67-fold of control (Fig. 5d).

To examine the effect of TAFIa on proteolysis of DQ-collagen IV by both endothelial and breast cancer cells in the same environment, we co-cultured HUVECs with SUM149 cells (Fig. 6) or MDA-MB-231 cells (Supplementary Fig. 2). Co-cultures were treated with PTCI, VEGF, TAFI or TAFIa. Although both intracellular and pericellular proteolysis was observed in the co-cultures (Fig. 6a; Supplementary Fig. 2A), HUVEC:MDA-MB-231 co-cultures exhibited predominately pericellular proteolysis (Supplementary Fig. 2C). In both co-cultures, treatment with VEGF or PTCI significantly increased both pericellular and intracellular proteolysis as compared to the control (Fig. 6b; Supplementary Fig. 2D). We next examined the effect of TAFI or TAFIa treatment on proteolysis in the HUVECs: SUM149 co-cultures. Treatment with either TAFI or TAFIa resulted in a decrease in DQ-collagen proteolysis to 0.7-fold of control.

### TAFIa inhibits plasminogen activation on endothelial cells

Plasminogen activation on the cell surface is accelerated by the presence of carboxyl-terminal lysine residues found on several plasminogen receptors. These lysine residues represent plasminogen binding sites on the cell surface. As a carboxypeptidase, TAFIa has been shown to cleave carboxyl-terminal lysine residues from various substrates, including

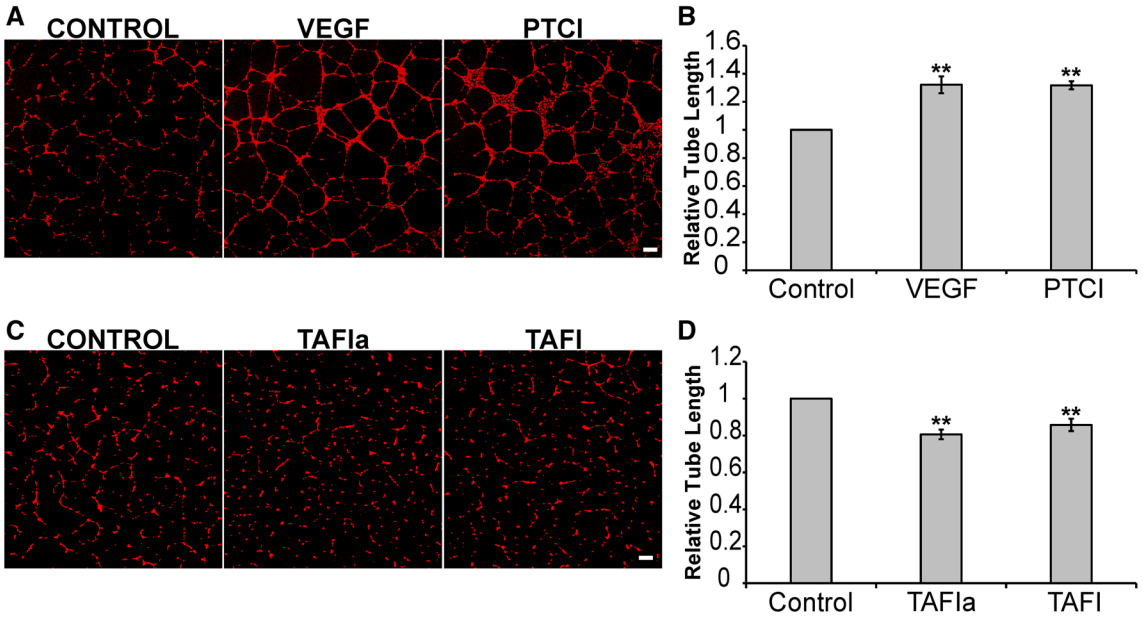


**Fig. 3** TAFIa decreases endothelial tube formation. **a** HUVECs labelled with Cell Tracker Orange™ (*red*) were seeded on *top* of BME in a 35 mM glass bottom culture dish and treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 h after seeding HUVECs using confocal microscopy. Images above were obtained at 40× magnification. *Scale bar*, 200 µM. **b** Quantification of endothelial tube formation. To quantify tube formation 8–10 images were obtained at 100× magnification. Tube length was determined by measuring total tube length divided by total tube number, and is expressed relative to that in the control untreated cells. **c** HUVECs labelled with Cell Tracker Orange™ (*red*) were seeded on *top* of BME and treated with 50 nM of either activated or non-activated TAFI for 18 h. Cells were imaged as in *Panel (A)*. **d** Quantification of tube length following treatment with TAFIa, performed as for *Panel (B)*. Values are expressed as mean ± SEM from ≥3 independent experiments. *p < 0 0.05, **p < 0.01 versus control

plasminogen receptors. As shown in Fig. 7, various concentrations of TAFIa can inhibit both uPA and tPA mediated plasminogen activation on the surface of HUVECs. uPA-mediated and tPA-mediated plasminogen activation decreased 26 and 45% in the presence of 10 nM TAFIa, respectively. Additionally, we observed that the inhibitory effects of TAFIa on tPA-mediated plasminogen activation are dependent on the cell surface, as TAFIa does not inhibit plasminogen activation the absence of cells (Fig. 7b). Furthermore, we examined whether the effect of TAFIa on plasminogen activation was lysine dependent. Treatment with ε-ACA resulted in inhibition of plasminogen activation, similar to treatment with TAFIa (Fig. 7c). Importantly, treatment with TAFIa in combination with ε-ACA did not result in a further decrease in plasminogen activation. Taken together, our findings indicate that TAFIa inhibits plasminogen activation on the HUVEC cell surface, in a manner dependent on removal of carboxyl-terminal lysines from cell-surface receptors (Fig. 7c).

### Effect of inhibition of TAFIa on MMP abundance

Since inhibition of TAFIa was shown to increase proteolysis of DQ-collagen IV by endothelial cells and by endothelial cells co-cultured with breast cancer cells, we next wanted to assess whether inhibition of TAFIa affected the abundance of MMPs. Pericellular plasminogen activation is involved in ECM remodeling, as well as the release and activation of MMPs from the ECM [22]. MMP2 and MMP9 have been shown to be implicated in tumour angiogenesis [42]. MMP2 and MMP9 are gelatinases, but also degrade other ECM proteins such as collagen type IV [43]. VEGF has been shown to be in a positive feedback loop with MMP9 in RPE cells [44], therefore it was used as a positive control in these experiments.

Gelatin zymography, which allows the gelatinolytic activity of latent and active MMP2 and MMP9 to be measured [45], was used to determine the effect of inhibition of TAFIa on the abundance of MMP2 and MMP9 after treatment of SUM149 breast cancer cells with PTCI or VEGF. Bands from the SUM149 media samples migrated similarly to recombinant pro-MMP2 and pro-MMP9 controls, which suggests that they are likely pro-MMP9 and pro-MMP2 (Fig. 8a). It was determined that PTCI significantly increased abundance of both pro-MMP2 (1.7-fold) and pro-MMP9 (2.6-fold) compared to the control (Fig. 8a). We could not detect the presence of active MMP2 or MMP9 (Fig. 8a).

Similar experiments were performed to assess the effect of inhibition of TAFIa on MMP2 and MMP9 abundance

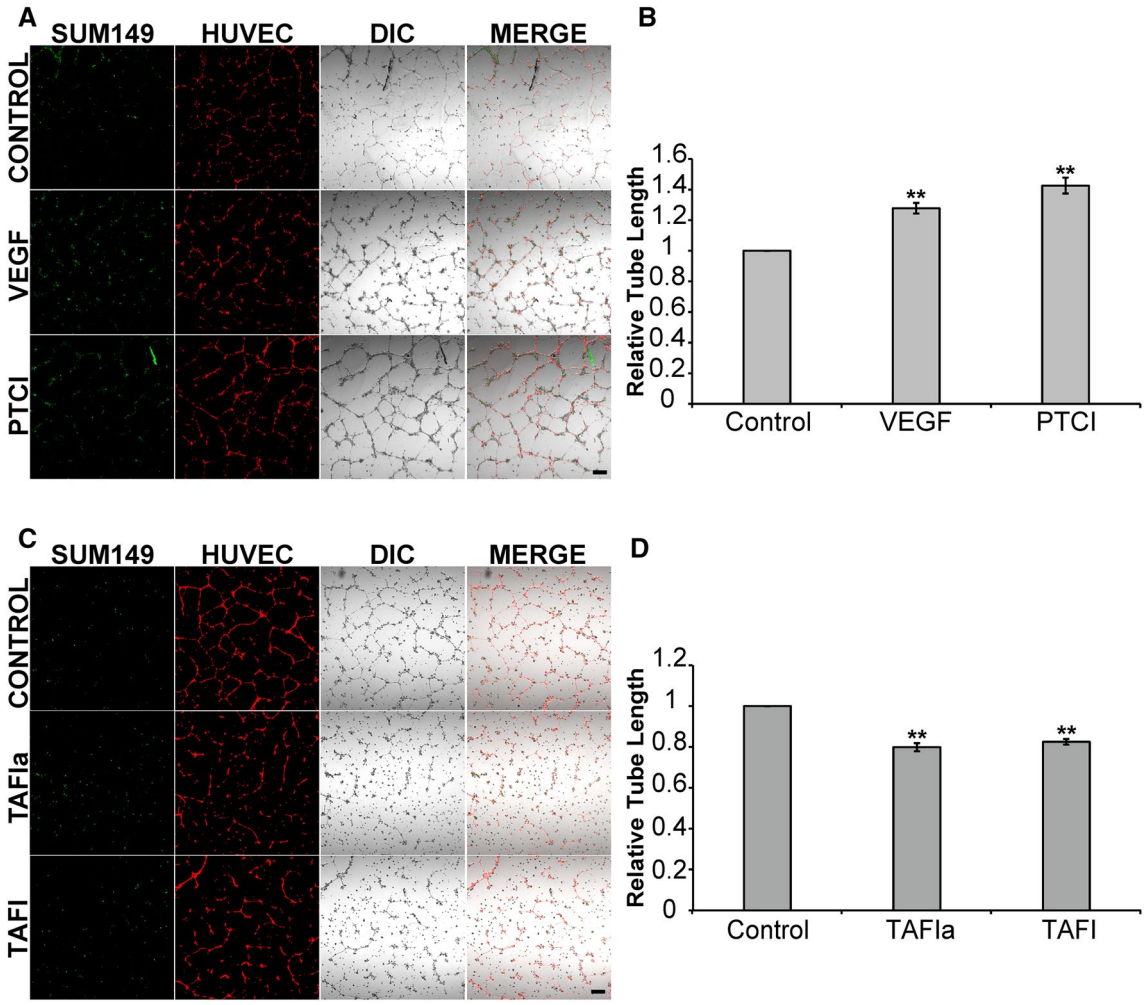


**Fig. 4** TAFIa decreases endothelial tube formation in a co-cultures of HUVECs and SUM149 cells. **a** SUM149 cells labelled with Cell Trace™ Far Red (*green*) were seeded on *top* of BME in a 35 mM glass bottom culture dish. After 24 h, HUVECs labelled with Cell Tracker™ Orange (*red*) were seeded on *top* of the SUM149 cells and BME. Co-cultures were treated with either 50 ng/mL VEGF or 25 μg/mL PTCI and imaged after 18 h using confocal microscopy at 40× magnification. *Scale bar,* 200 μM. **b** To quantify tube formation, 8–10 images were obtained at 100× magnification. Average tube length was determined by measuring total tube length divided by total tube number, and is expressed relative to that in the control untreated cells. **c** HUVECs labelled with Cell Tracker™ Orange (*red*) and SUM149 cells were labelled with Cell Trace™ Far Red (*blue*) were seeded on *top* of BME and treated with 50 nM of either activated or non-activated TAFI for 18 h. Cells were imaged as in *Panel (A)*. **d** Quantification of tube length following treatment with TAFI or TAFIa, performed as for *Panel (B)*. Values are expressed as mean ± SEM from ≥3 independent experiments. *$p < 0.05$, **$p < 0.01$ versus control

in the medium of HUVECs. It was determined that PTCI does not significantly affect the abundance of pro-MMP2 (Fig. 8b). We could not detect the presence of pro-MMP9 or MMP9 (Fig. 8b).

Next, we used CM from PTCI- or VEGF-treated SUM149 cells to treat HUVECs, to examine if breast cancer cells could mediate the proteolytic activity of HUVECs in a paracrine manner. Treatment of HUVECs with CM from untreated SUM149 cells resulted in a 1.2-fold increase in pro-MMP2 abundance compared to the untreated HUVEC control, although this was not significant (Fig. 8c). Treatment of HUVECs with CM from PTCI treated SUM149 cells significantly increased the abundance of pro-MMP2, 1.6-fold relative to the untreated HUVEC control (Fig. 8c).

## Discussion

Our current studies examining the role of TAFIa in angiogenic responses of endothelial cells were prompted by reports that TAFIa is able to inhibit pericellular plasminogen activation [38] and to reduce endothelial cell tube formation in a fibrin matrix [21]. We examined the role of TAFIa in angiogenesis in the context of breast cancer

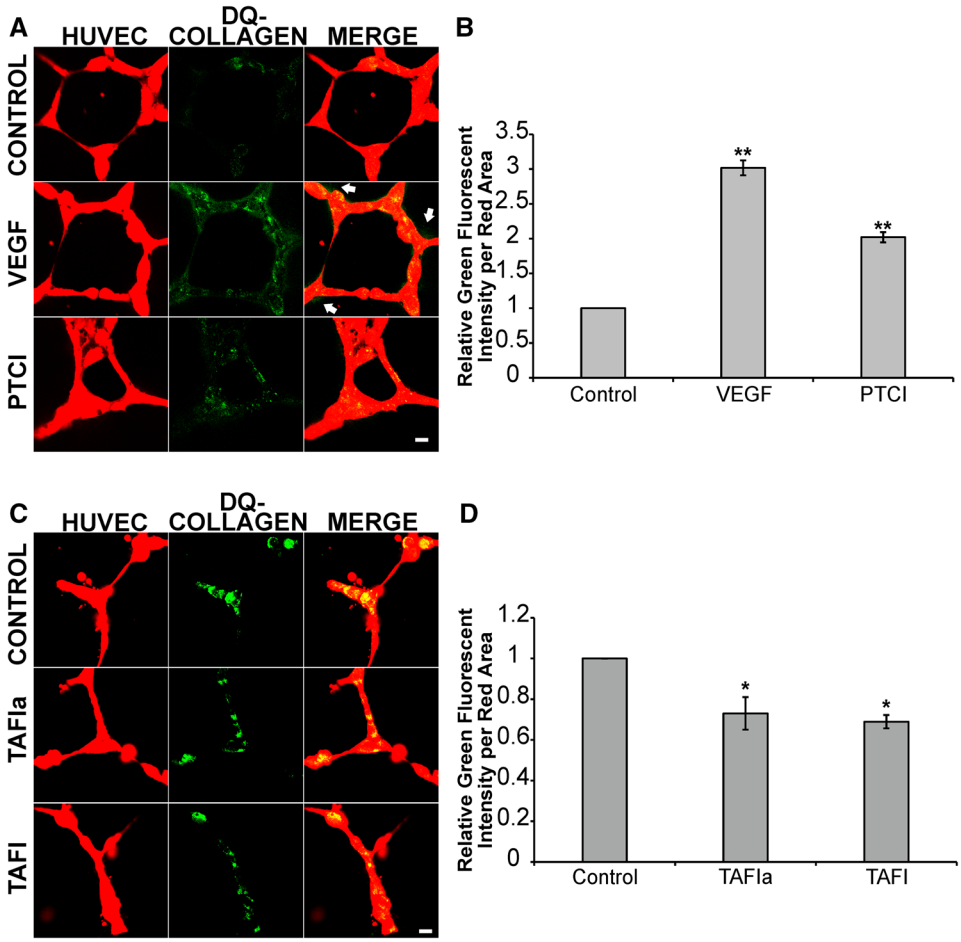


**Fig. 5** TAFIa decreases proteolysis of DQ-collagen IV by endothelial cells. **a** HUVECs labelled with Cell Tracker™ Orange (*red*) were seeded on *top* of BME mixed with DQ-collagen IV in a 35 mM glass bottom culture dish and treated with either 50 ng/mL VEGF or 25 μg/mL PTCI. Cells were imaged 18 h after seeding HUVECs using confocal microscopy. DQ-collagen IV cleavage products (*green*) were present predominantly intracellularly. Merge images show intracellular (*yellow*) and pericellular (*green*) proteolysis. Images were obtained at 600× magnification. Representative images from the equatorial plane of a Z-stack are shown. *Scale bar*, 20 μM. **b** To quantify proteolysis, three equatorial planes from 3 to 4 different images in each independent experiment were examined. Total integrated intensity of DQ-collagen IV fluorescence divided by total combined cell area of HUVECs was averaged across the three planes and expressed relative to the control. **c** HUVECs labelled with Cell Tracker Orange (*red*) were seeded on *top* of BME mixed with DQ-collagen IV and treated with 50 nM of either activated or non-activated TAFI for 18 h. Cells were imaged as in *Panel (A)* **d** Quantification of DQ-collagen proteolysis following treatment with TAFI or TAFIa, performed as for *Panel (B)*. Values are expressed as mean ± SEM from ≥3 independent experiments. *$p < 0.05$, **$p < 0.01$ versus control

because we recently demonstrated that TAFIa inhibits pro-metastatic behaviours and pericellular plasminogen activation in both SUM149 and MDA-MB-231 breast cancer cells [17]. We therefore used SUM149 and MDA-MB-231 cells in co-cultures with HUVECs because they provide an appropriate cellular model to evaluate tumour angiogenesis.

Results from our in vitro studies suggest that TAFIa plays a role in inhibition of tumour angiogenesis. Addition of TAFI or TAFIa resulted in a decrease in crucial components of angiogenesis: namely, endothelial cell proliferation, invasion, tube formation, and ECM proteolysis, while the opposite effects were observed when endogenous TAFIa was inhibited with PTCI. Although TAFIa appeared to have a small effect on cell proliferation (Fig. 2), this is unlikely to account for the much larger effect of TAFI on cell migration and invasion, which we ascribe to effects of TAFIa on cell motility and basement membrane degradation, respectively. Endothelial tube formation and ECM proteolysis were also decreased by TAFI/TAFIa in co-cultures of endothelial cells with breast cancer cells. We hypothesize that the ability of TAFIa to inhibit pericellular plasminogen activation likely underlies these effects, as we have shown in this study – for the first time – that TAFIa can inhibit plasminogen activation on HUVECs (Fig. 7).

For angiogenesis to occur the vascular basement membrane must be locally degraded by proteolytic enzymes

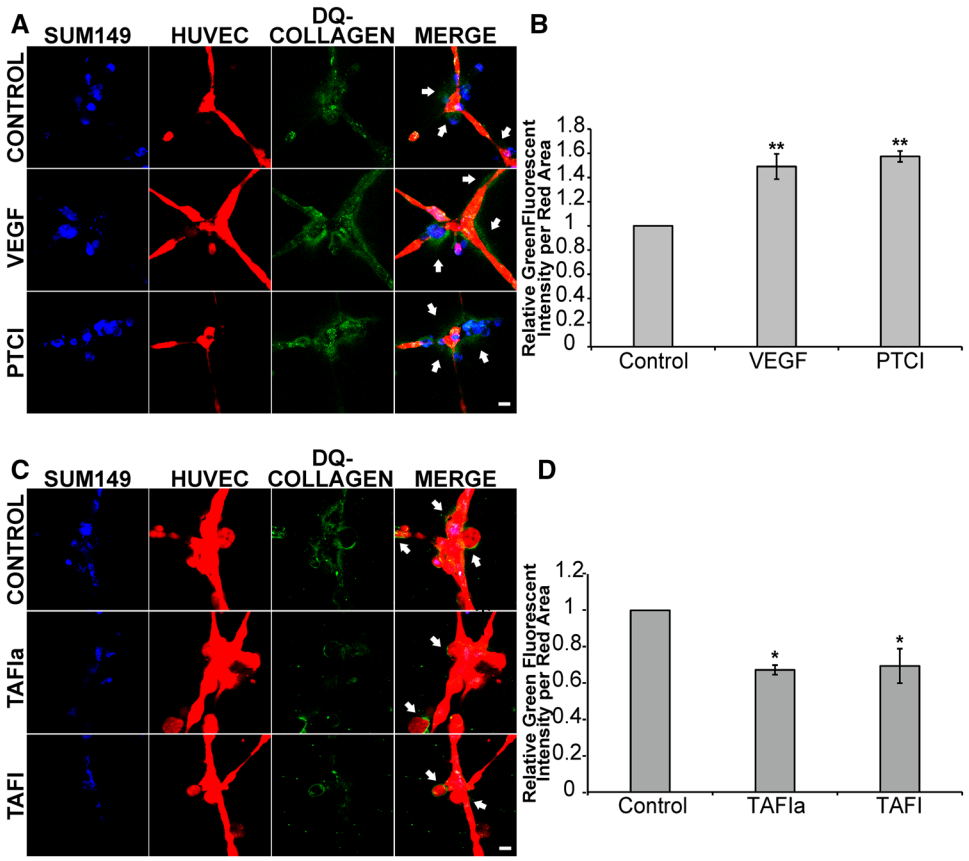


**Fig. 6** TAFIa decreases DQ-collagen IV proteolysis by co-cultured HUVECs and SUM149 cells. **a** SUM149 cells labelled with Cell Trace Far Red (*pseudocoloured blue*) were seeded on *top* of BME mixed with DQ-collagen IV in a 35 mM glass bottom culture dish. After 24 h HUVECs labelled with Cell Tracker Orange (*red*) were seeded on *top* of the SUM149 cells and BME mixed with DQ-collagen IV. The co-cultures were treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 h after HUVECs were seeded using confocal microscopy. DQ-collagen IV cleavage products (*green*) were present both intracellularly and pericellularly. Merged images show pericellular proteolysis (*green*) surrounding the co-cultured cells. Images were obtained at 600× magnification. Representative images from the equatorial plane of a Z-stack are shown. *Scale bar*, 20 µM. **b** To quantify proteolysis, three equatorial planes from three to four different images in each independent experiment were examined. Total integrated intensity of DQ-collagen IV fluorescence divided by total combined cell area of HUVECs and SUM149 cells was averaged across the three planes and expressed relative to the control. **c** HUVECs labelled with Cell Tracker Orange (*red*) and SUM149 cells were labelled with Cell Trace Far Red (*blue*) were seeded on *top* of BME containing DQ-collagen IV and treated with 50 nM of either activated or non-activated TAFI for 18 h. Cells were imaged as in *Panel (A)* **d** Quantification of DQ-collagen proteolysis following treatment with TAFIa, performed as for *Panel (B)*. Values are expressed as mean ± SEM from ≥3 independent experiments. *$p < 0.05$, **$p < 0.01$ versus control

[46]. ECM proteolysis promotes the angiogenic process in part by stimulating cell invasion and migration [47]. Importantly, growth factors, such as VEGF, stimulate this process [48]. In this current study we have shown that TAFIa inhibits VEGF-mediated invasion and migration of endothelial cells. Additionally, we examined the effect of TAFIa on degradation of DQ-collagen IV, a quenched fluorescent derivative of type IV collagen [49], by HUVECs grown alone and in co-culture breast cancer cells. The co-culture systems act to better recapitulate a tumour microenvironment [27]. TAFIa decreased proteolysis of DQ-collagen IV, thus supporting our hypothesis that TAFIa is able to inhibit extracellular proteolysis through inhibition of plasmin formation. Indeed, pericellular proteolysis can be caused by components of the plasminogen activation system [49, 50], and plasmin can liberate and activate MMPs from the ECM [50]. Importantly, we have shown that the effects of PTCI on HUVEC invasion and migration can be completely blocked by the inclusion of the specific plasmin inhibitor aprotinin (Fig. 1).

Although the proteases and pathways involved in pericellular proteolysis compared to intracellular proteolysis differ in some respects [49, 50], changes in intracellular proteolysis, as we apparently observed in some cases (Figs. 5, 6) can, in fact, reflect changes in extracellular proteolysis. Pericellular proteolysis has been shown to involve MMPs, serine proteases (including components of the plasminogen activation system), and cysteine proteases

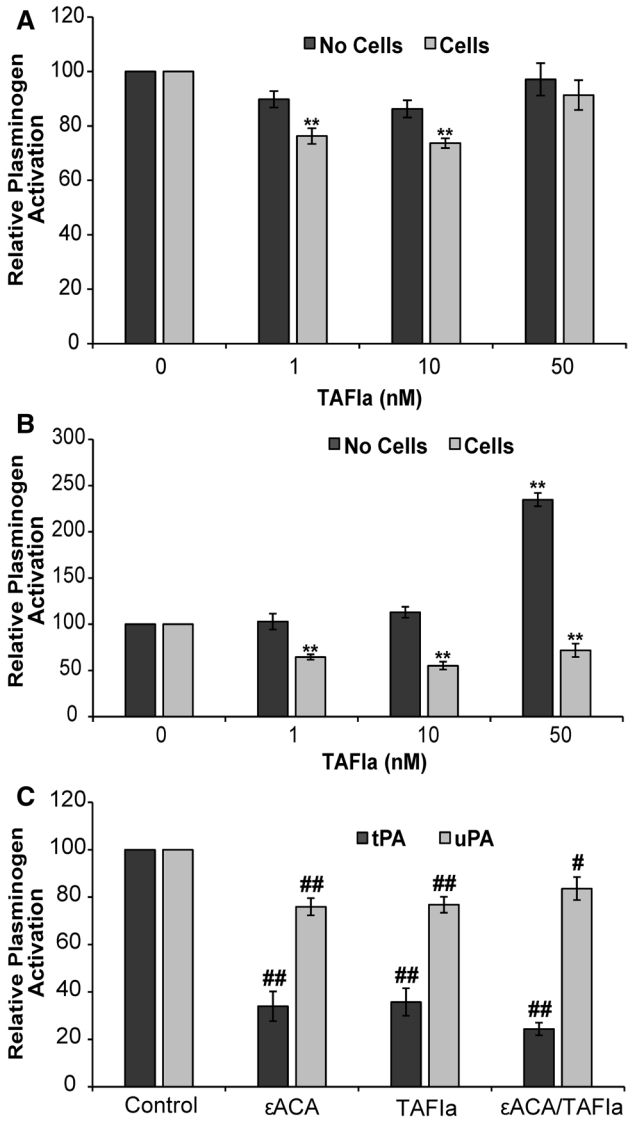


**Fig. 7** Inhibition of plasminogen activation by TAFIa on endothelial cells. Plasminogen activation mixture containing various concentrations of TAFIa (1–50 nM), 500 nM plasminogen, **a** 20 nM uPA or **b** 5 nM tPA and a fluorogenic plasmin substrate was added to HUVECs. Experiments were also completed in the absence of cells. **c** Plasminogen activation on HUVECs was completed in the presence of 50 μM ε-ACA and/or 10 nM TAFIa with uPA (20 nM) or tPA (5 nM). The rate of plasmin generation was monitored over 1 h at 37 °C. The data are represented as the relative changes in fluorescence versus time squared. The data represent results of 3–4 independent experiments and are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus no TAFIa; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus control

[49, 50]. Intracellular proteolysis could reflect endocytosis of DQ-collagen IV by the live cells, and degradation of the substrate in the lysosome [50–52]. More likely, in our study, is that the observed intracellular proteolysis occurs after partial degradation of DQ-collagen IV with pericellular proteases, after which endocytosis and further degradation in the lysosome can occur [53]. Indeed, it must be emphasized that PTCI acts extracellularly to inhibit TAFIa, and hence promote plasmin formation, in this milieu.

Treatment of the HUVEC monoculture with PTCI increased intracellular proteolysis as compared to the control (Fig. 5), whereas treatment with PTCI increased pericellular proteolysis as compared to the control in both the co-culture systems (Fig. 6; Supplementary Fig. 2). Likewise, treatment with TAFI or TAFIa resulted in a decrease in intracellular proteolysis of the HUVEC monoculture, and decreased pericellular proteolysis in the co-culture system. It is not clear, however, if TAFIa has a greater effect on the breast cancer cells in terms of inhibition of pericellular proteolysis than it does on the endothelial cells, since it is unclear which cells are giving rise to the proteolytic activity in the co-cultures. Interestingly, there was a greater effect of PTCI and TAFIa in HUVEC invasion, migration and DQ-collagen proteolysis than there was in HUVEC tube formation, either in the presence or absence of breast cancer cells (compare Figs. 1, 5, 4). This could suggest that TAFIa plays a larger role in the proteolysis and invasion aspects of angiogenesis, while other processes involved in tube formation are resistant to the effects of TAFI, such as intracellular signaling events regulating cytoskeletal remodeling or differentiation [54].

Our working model is that TAFI inhibits pericellular plasmin generation, with the consequence that there is reduced matrix and latent growth factor proteolysis by both plasmin and by plasmin-activated MMPs. Indeed, uPA bound to uPAR can enhance endothelial cell organization into tubes [55]. We looked for evidence that manipulation of TAFI did, in fact, alter levels of MMPs, using a zymography approach. We were not able to detect the cleaved, active MMP2 and MMP9 (Fig. 8), perhaps because these enzymes are rapidly bound by inhibitors in the extracellular milieu. Intriguingly, however, we found that inhibition of TAFIa did increase the levels of pro-MMP2 and pro-MMP9 in breast cancer cell conditioned medium. Furthermore, our co-culture systems may be reporting paracrine signaling between breast cancer cells and endothelial cells [56], where CM harvested from SUM149 cells slightly increased abundance of pro-MMP2 from HUVECs (Fig. 8). Interestingly, treatment of those SUM149 cells with PTCI to inhibit TAFIa in the period prior to collection of CM significantly increased the ability of the CM to increase pro-MMP2 abundance in HUVEC CM. The mechanisms underlying these effects remain to be explored, but may relate to the effect of TAFIa on the liberation and activation of growth factors from the extracellular matrix. Whether TAFIa regulates the expression of MMP genes is also unknown at this time.

Throughout our studies, we found that addition of non-activated TAFI or TAFIa had virtually identical effects. This is in contrast to our previous study of metastatic

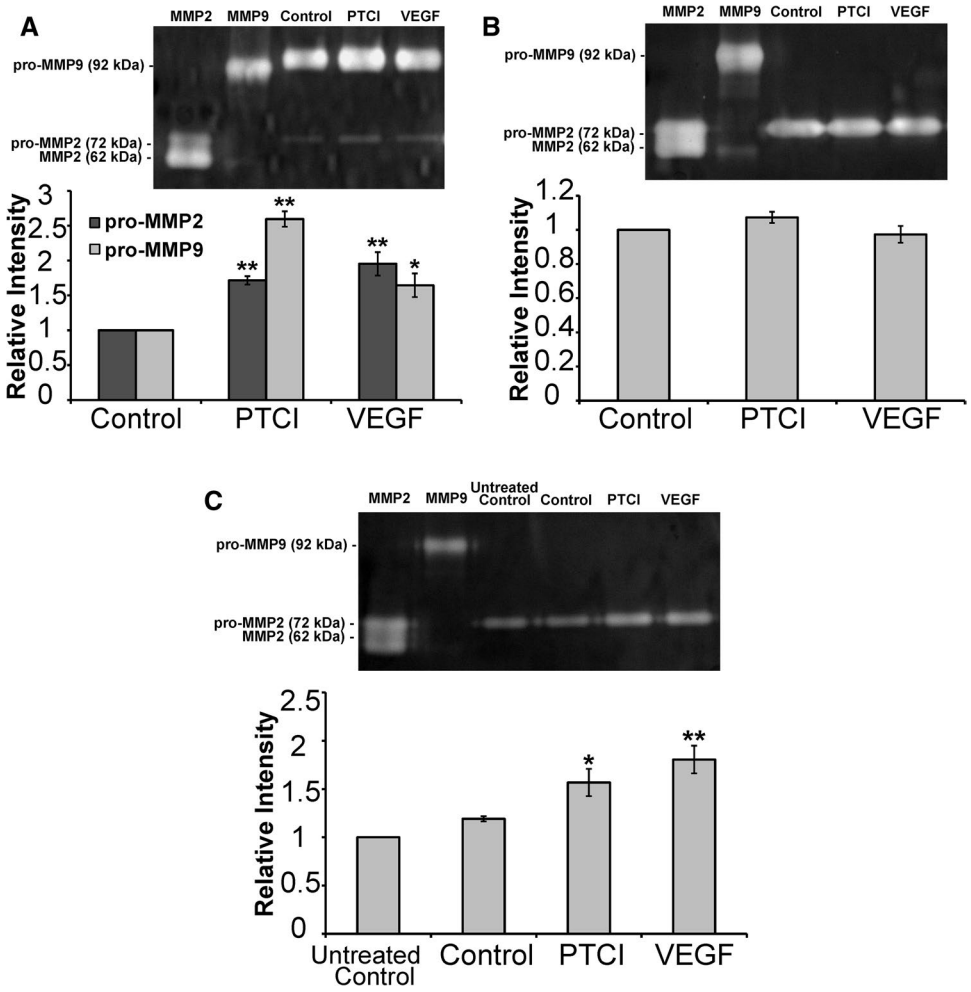


**Fig. 8** Inhibition of TAFIa in SUM149 cells increases abundance of pro-MMP2 and pro-MMP9. **a** Representative zymogram depicting gelatinolytic activity of pro-MMP9 (92 kDa), pro-MMP2 (72 kDa) present in CM harvested from SUM149 breast cancer cells treated with either 25 µg/mL PTCI or 50 ng/mL VEGF for 24 h. Densitometry was performed and reported as relative intensity as compared to the control. **b** Representative zymogram depicting gelatinolytic activity of pro-MMP2 (72 kDa) secreted by HUVECs treated with either 25 µg/mL PTCI or 50 ng/mL VEGF for 24 h. Densitometry was performed and reported as relative intensity as compared to the control. **c** Representative zymogram depicting gelatinolytic activity of pro-MMP2 (72 kDa) present in CM harvested from HUVECs treated with CM from SUM149 cells treated with either 25 µg/mL PTCI or 50 ng/mL VEGF. Densitometry was performed and reported as relative intensity as compared to the untreated control. Data represent results of ≥3 independent experiments and are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus control

responses by breast cancer cells [17] in which TAFIa was generally more effective than TAFI. A major difference in these studies is that HUVECs strongly express thrombomodulin which is a highly effective cofactor for TAFI activation [57]. Notably, we found that PTCI was able to partially blunt the effect of TAFI and TAFIa on VEGF-stimulated HUVEC invasion to equivalent extents (data not shown). Therefore, in the current study, it is clear that the concentration of TAFI itself is a limiting factor, whereas thrombomodulin is limiting in the breast cancer cells. Indeed, reduced tumour thrombomodulin is a negative predictor of prognosis in metastatic breast cancer [58].

In conclusion, our results show that TAFIa plays a significant role in modulating several angiogenic process, including cell invasion, migration, proteolysis and endothelial tube formation. Our findings suggest that stimulation of TAFI activation in the tumour microenvironment might be a viable strategy to inhibit tumour angiogenesis.

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