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Fluid shear stress promotes proprotein convertase-dependent activation of MT1-MMP

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Abstract

During angiogenesis, endothelial cells (ECs[1]) initiate new blood vessel growth and invade into the extracellular matrix (ECM). Membrane type-1 matrix metalloproteinase (MT1-MMP) facilitates this process and translocates to the plasma membrane following activation to promote ECM cleavage. The N-terminal pro-domain within MT1-MMP must be processed for complete activity of the proteinase. This study investigated whether MT1-MMP activation was altered by sphingosine 1-phosphate (S1P) and wall shear stress (WSS), which combine to stimulate EC invasion in three dimensional (3D) collagen matrices. MT1-MMP was activated rapidly and completely by WSS but not S1P. Proprotein convertases (PCs) promoted MT1-MMP processing, prompting us to test whether WSS or S1P treatments increased PC activity. Like MT1-MMP, PC activity increased with WSS, while S1P had no effect. A pharmacological PC inhibitor completely blocked S1P- and WSS-induced EC invasion and MT1-MMP translocation to the plasma membrane. Further, a recombinant PC inhibitor reduced MT1-MMP activation and decreased lumen formation in invading ECs, a process known to be controlled by MT1-MMP. Thus, we conclude that PC and MT1-MMP activation are mechanosensitive events that are required for EC invasion into 3D collagen matrices.

Keywords

angiogenesis; mechanotransduction; sprouting; furin

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[1]Abbreviations used: ECs, endothelial cells; MT1-MMP, Membrane type-1 matrix metalloproteinase; ECM, extracellular matrix; WSS, wall shear stress; S1P, sphingosine 1-phosphate; PC, pro protein convertase; 3D, threedimensional; HUVEC, human umbilical vein endothelial cell; GFP, green fluorescent protein; GAPDH, glyceraldehyde phosphate dehydrogenase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

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Introduction

Angiogenesis is the formation of new capillaries from pre-existing vessels and is important during physiological and pathological events such as wound healing, embryonic development, the female reproductive cycle, and tumor vascularization [1]. During angiogenesis, endothelial cells (ECs) respond to biochemical factors such as vascular endothelial growth factor (VEGF) [2], basic fibroblast growth factor (bFGF) [3], placental growth factor [4], and sphingosine 1-phosphate (S1P) [5], as well as mechanical shear forces created by blood flow [6]. Wall shear stress (WSS) rates have been estimated at 1–8 dyn/cm^2 for the microcirculation, the site of angiogenic initiation [7]. Although multiple signaling pathways are activated by growth factors, lipids, and mechanical forces [4,6,8,9], the precise underlying signals and intracellular events that control EC sprouting in response to shear forces remain incompletely understood.

Membrane type-1 matrix metalloproteinase (MT1-MMP) is a member of the matrix metalloproteinase family of enzymes and is vital during angiogenesis. Mice deficient in MT1-MMP demonstrate defective vascular infiltration of cartilage [10] and corneal angiogenesis [10]. Also, MT1-MMP exclusively promoted endothelial-dependent vessel formation *in vitro* and *in vivo* [11], indicating a clear requirement for MT1-MMP in initiating new blood vessel growth. In addition, MT1-MMP is required for EC tubulogenesis and lumen formation [12,13,14,15]. Together, these studies establish a critical role for MT1-MMP in angiogenic responses.

To be functional, the propeptide sequence of MT1-MMP must be removed by one of several intracellular proprotein convertases (PCs), which include furin, PC1/3, PC2, PACE4, PC4, PC5/6, and PC7 [16,17]. Of these, furin, PC5/6, PC7, and PACE4 cleave the first 111 amino acids of MT1-MMP at a defined site (Arg-Arg-Lys-Arg^{111}) to unmask the catalytic domain and promote surface localization of MT1-MMP [18,19]. Furin, PC1, PC6, and PC7 mRNA have been detected in ECs [20], and furin colocalized with MT1-MMP in the trans-Golgi network [21]. Although it is well-recognized that MT1-MMP promotes vessel outgrowth and lumen formation [11,13] and proprotein convertases activate MT1-MMP [18,19], it is not known whether WSS affects MT1-MMP activation. We observed here that WSS activated proprotein convertases, and proprotein convertases activated MT1-MMP to facilitate EC invasion of 3D collagen matrices.

Materials and Methods

Endothelial cell invasion stimulated by shear stress

Human umbilical vein endothelial cells (HUVECs) (Lonza BioProducts) were used at passage 4–6. Collagen type I was isolated as previously described [22]. Experiments applying 1μM S1P and 5.3 dyn/cm^2 WSS were conducted as previously described [23,24].

Quantification of endothelial sprouting responses stimulated by shear stress

Invading cell cultures were fixed in 3% glutaraldehyde in PBS overnight and stained (15min) with 0.1% toluidine blue in 30% methanol. Invasion density was quantified as the average number of structures invading beneath the monolayer per standardized 1mm^2 field

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(n>3 fields). The percentage of cells forming lumens and the lumen diameter were quantified from images taken of a side view of invasion. For each treatment group, n>100 cells were measured.

Proprotein convertase inhibition

ECs were pre-incubated with 25μM of proprotein convertase inhibitor, decanoyl-RVKR-chloromethylketone (ALX-260-022, Enzo) or vehicle control (DMSO) during attachment to collagen matrices (1hr) and for the duration of WSS application (24hr).

Proprotein convertase activity

Cells were seeded on polymerized collagen matrices containing S1P, exposed to 5.3 dyn/cm^2 WSS, and allowed to invade for 3hr before homogenization in lysis buffer [100mM HEPES (pH 7.5), 0.5% TX-100, 1mM $CaCl_2$, 1mM 2-mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche), and Halt Phosphatase Inhibitor Cocktail (Pierce)] at 4°C for 10min. Samples were vortexed every 5min for 20min and centrifuged at 13,000×g at 4°C for 10min. Supernatants were collected and stored at −80°C until use. Assay buffer, fluorogenic substrate peptides (Boc-RVRR-AMC, ALX-260-040, Enzo), and reactants were prepared according to manufacturer's instructions and measured for fluorescence intensity at excitation/emission wavelengths of 380(±20nm)/520(±20nm) using a Victor X3 plate reader (PerkinElmer Life Sciences) in triplicate wells.

Immunoblotting

Immunoblotting was conducted as previously described [23,24]. Band intensities were measured using ImageJ. Antisera used in this study were raised against GAPDH (ab8245, Abcam), MT1-MMP (SC30074, Santa Cruz; MAB3328, Millipore), Zyxin (cs3553, Cell Signaling), and antitrypsin (ab9400, Abcam). In all figures, densitometric analyses of band intensities were compiled from 3 independent experiments. Pro and active MT1-MMP levels were normalized to GAPDH. Data presented are mean values ±S.D.

Cell transfection and immunofluorescence analyses

Transient transfections of MT1-MMP-GFP expression plasmids were performed and quantified as previously described [24,25]. Transfection efficiency was approximately 20%. ECs were serum-starved for 1hr and then untreated or treated with the proprotein convertase inhibitor in the presence of 1μM S1P and WSS for 2hr. Cells were fixed in 4% paraformaldehyde before quantifying MT1-MMP-GFP localization to the cell periphery as previously described [24,25]. Data shown were averaged from 3 experiments (n=39 cells total/group).

Proprotein convertase inhibitor cloning and lentiviral transduction

Full-length α1-antitrypsin (α_1-AT), α1-antitrypsin variant Pittsburgh (α_1-PIT), and α_1-antitrypsin variant Portland (α_1-PDX) constructs were kind gifts from Gary Thomas (Oregon Health Sciences University) [26]. The inserts were subcloned into the pIEx-5 vector (Novagen) using the *Acc65I* and *HindIII* sites, generating a C-terminal S-tag. Positive clones confirmed by sequence analysis were subcloned into the pENTR4 vector (Invitrogen) using

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the *Acc65I* and *XhoI* sites and recombined into the pLenti6/V5 DEST vector (Invitrogen) using the GATEWAY system. Lentiviruses were generated as previously described [24,25]. ECs were transduced for 3d and selected with blasticidin (1μg/ml) for 8d. Blasticidin was removed for 24hr and invasion assays were conducted.

Statistical analyses

All data are presented as the mean ± standard deviation (S.D.) or standard error of the mean (SEM) for each group. Individual statistical analyses were performed using SAS software. Lowercase letters denote groupings from one-way ANOVA followed by post hoc pairwise comparison testing using Tukey's method ($p<0.05$).

Results

MT1-MMP activation increases with time during EC invasion in 3D collagen matrices

We have shown that surface translocation of MT1-MMP facilitates EC invasion in multiple assay systems [24,25]. However, whether WSS directly affects MT1-MMP activation has not been investigated. We used an established model [23,24], where 5.3 dyn/cm^2 WSS combined with 1μM S1P promoted EC sprouting. Robust sprouting required S1P and WSS to promote EC invasion of 3D collagen matrices (Fig. 1A). Extracts were collected from invading cultures at 0, 1, 6, 12, and 18hr following stimulation with S1P and WSS. MT1-MMP was converted from the pro (63kDa) to an active, lower molecular weight form (60kDa) almost completely by 12hr (Fig. 1B). Quantification of band intensities from multiple experiments revealed a significant decrease in pro MT1-MMP with time in S1P- and WSS-treated ECs (Fig. 1C), which corresponded with an increase in active MT1-MMP over time (Fig. 1D). To test the effects of S1P and WSS separately, ECs were treated with or without WSS in the presence or absence of S1P. We observed increased active MT1-MMP in all groups exposed to WSS, while S1P appeared to have no effect on MT1-MMP activation (Fig. 2A). Quantification of both pro and active forms of MT1-MMP revealed that pro MT1-MMP levels were decreased significantly at 2hr compared to control treatment (Fig. 2B). At 6hr WSS treatment, pro MT1-MMP was nearly undetectable (Fig. 2B). In accordance with decreased levels of pro MT1-MMP, active MT1-MMP levels increased significantly with 2hr and 6hr WSS treatment compared to controls (Fig. 2C). No differences in levels of pro (Fig. 2B) or active (Fig. 2C) MT1-MMP were observed between Control and S1P treatment groups, indicating that S1P did not enhance MT1-MMP activation. Altogether, these data indicate that treatment of EC monolayers on 3D collagen matrices with 5.3 dyn/cm^2 WSS enhanced conversion of pro MT1-MMP to the active form, while treatment with S1P had no effect on MT1-MMP activation.

WSS increases proprotein convertase (PC) activity

Because proprotein convertases (PCs) convert pro MT1-MMP to the active form by cleaving the propeptide sequence of MT1-MMP [18,19,27], we first tested whether proprotein convertases were expressed in ECs. Using validated primer sets [28], we observed ECs express furin, PC1/3, PC5/6, and PC7 (Fig. 3A), which agrees with a previous report [20]. We next determined whether PC activity increased with time during S1P- and WSS-induced EC invasion using a fluorogenic substrate. ECs were treated with WSS and S1P and allowed

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to invade 3D collagen matrices for 0, 1, 3, and 6hrs. PC activity initially increased from 0 to 3hr, reaching a maximum value at 3hr, and then decreased slightly at 6hrs (Fig. 3B). To test whether S1P or WSS activated PCs, ECs cultured on 3D collagen matrices containing or lacking S1P were treated with or without WSS for 3hr. PC activity increased significantly with WSS stimulation but was not affected by S1P treatment (Fig. 3C), which was consistent with the trend observed for conversion of pro MT1-MMP to the active form (*cf.* Fig. 2A).

Proprotein convertase activity is required for S1P- and WSS-induced EC invasion and MT1-MMP translocation to the plasma membrane

The results above indicate that WSS enhanced PC activity and the conversion of pro MT1-MMP to the active form. We next determined whether PC activity was necessary for EC invasion in 3D collagen matrices. ECs were stimulated with S1P and WSS in the presence of vehicle control or PC inhibitor. Photographs capturing a side view of invading structures are shown in Figure 3D. No decrease in EC viability was observed in the presence of the PC inhibitor (not shown), which significantly blocked EC invasion responses (Fig. 3E). Compared to controls, PC inhibition reduced the conversion of pro MT1-MMP to the active form at 6 and 12hr (Fig. 3F), supporting that proprotein convertases regulated MT1-MMP activation in invading ECs.

Béliveau and colleagues observed that S1P stimulated MT1-MMP translocation to the plasma membrane [29], and we have previously shown that increased MT1-MMP-GFP plasma membrane translocation correlated with increased EC sprouting responses [24,25]. We next determined whether proprotein convertase activity was required for MT1-MMP-GFP translocation to the membrane in response to stimulation by S1P and WSS. As expected, the PC inhibitor almost completely abolished PC activity (Fig. 3G) and blocked localization of MT1-MMP-GFP to the plasma membrane (Fig. 3H) in response to stimulation by S1P and WSS. Quantitative analysis of these images indicated that PC inhibition significantly reduced the ability of S1P and WSS to stimulate MT1-MMP-GFP membrane localization (Fig. 3I). Altogether, these data reinforce that PC activity is required for successful plasma membrane translocation of MT1-MMP [18], which is vital for ECM proteolysis during endothelial sprouting responses.

Exogenous expression of a PC inhibitor attenuated lumen formation in invading ECs

To reinforce the findings from pharmacological PC inhibition, we investigated whether expression of a protein-based PC inhibitor altered EC invasion in response to WSS and S1P. ECs were transduced to express GFP or various antitrypsin constructs. Human α_1-antitrypsin (α_1-AT) is a natural inhibitor of elastase and trypsin [30,31], while the α_1-AT Pittsburgh (α_1-PIT) mutant (Arg^{358}) acts as specific inhibitor of thrombin [26]. The α_1-AT Portland (α_1-PDX) mutant contains a bait sequence (Arg^{355}-X-X-Arg^{358}) that binds to furin and PC5/6 to function as a competitive inhibitor [26,32,33]. Transduced ECs were stimulated with S1P and WSS for 2 or 24hrs on 3D collagen matrices. At 2hrs, expression of all antitrypsin constructs was detected (Fig. 4A), and GFP expression was confirmed visually (not shown). As expected, α_1-PDX expression resulted in increased levels of pro-MT1-MMP, indicative of reduced PC-dependent processing of pro MT1-MMP to the active

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form (Fig. 4A). No difference in invasion density was observed with expression of α_1-AT, α_1-PIT, or α_1-PDX (Fig. 4B). MT1-MMP is required for EC lumen formation [12,13] and associates with several signaling complexes known to be crucial for lumen formation and endothelial sprouting [25,34,35]. Fitting with these findings, expression of α_1-PDX significantly reduced average lumen diameter (Fig. 4C) and the percentage of cells forming lumens (Fig. 4D). Representative photographs are shown in Figure 4E. Together, these findings support that WSS promotes PC-dependent activation of MT1-MMP.

Discussion

We report that WSS induced PC-dependent activation of MT1-MMP. Our data show a corresponding increase in PC activity and MT1-MMP processing that was stimulated by 5.3 dyn/cm^2 WSS, while S1P had no effect on PC activity or MT1-MMP processing. These results support that PC-dependent activation of MT1-MMP is a mechanosensitive event that facilitates proper plasma membrane localization of MT1-MMP to allow successful endothelial sprouting in 3D collagen matrices.

The data presented here suggest subsets of PCs may be responsible for WSS-dependent processing of MT1-MMP in ECs. Furin, PC1/3, PC5/6, and PC7 mRNA were expressed (Fig. 3A), confirming a previous report [20]. Although the PC responsible for MT1-MMP processing and activation has not been definitively determined, furin, PC5/6, PC7, and PACE4 cleave MT1-MMP [19], and α_1-PDX specifically inhibits furin and PC5/6 [32,33], suggesting a role for furin and/or PC5/6 in WSS-induced MT1-MMP activation. Notably, our data agree with previous reports that α_1-PDX partially blocked MT1-MMP activation [18,19]. Although we cannot rule out a role for PC7 or furin-independent MT1-MMP activation [18,36], the data presented here support that furin and/or PC5/6 are, at least partially, responsible for WSS-dependent MT1-MMP processing needed for EC invasion of collagen matrices.

While the present study investigated alterations in PC activity in response to WSS, others have reported that alterations in flow affect furin expression. Expression of furin and its associated substrate, transforming growth factor-β, increased in the endothelium of the carotid vein proximal to implantation of a carotid arteriovenous shunt *in vivo* [37]. In addition, 15 dyn/cm^2 WSS increased furin mRNA expression in bovine aortic endothelial cells *in vitro* [37]. In separate studies, both increasing and decreasing flow levels within carotid and femoral arteries upregulated furin and MT1-MMP mRNA expression in arterial extracts containing endothelial and smooth muscle cells [38]. These data indicate that furin and MT1-MMP mRNA expression levels were regulated by changes in flow and correlated with arterial remodeling, but isolated effects on the endothelium were not determined. Our study has not examined whether WSS affected the expression of PC mRNA in invading ECs, and thus we cannot rule out a contribution of elevated PC expression contributing to the increased PC activity observed here. However, because the assay system used here consists exclusively of ECs, we can confirm a definitive EC-specific effect of WSS in upregulating PC activity.

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WSS has been estimated in post-capillary venules to range from 1–8 dyn/cm^2 [7]. We found that 5.3 dyn/cm^2 WSS was effective at converting pro MT1-MMP to the active form. To promote matrix degradation, MT1-MMP must be activated and present at the plasma membrane [11,13]. Although we find here that S1P did not change PC activity (Fig. 3B), S1P has been reported to promote MT1-MMP plasma membrane localization in ECs [24,25,39], and treatment with both S1P and 5.3 dyn/cm^2 WSS resulted in sustained localization of MT1-MMP to the plasma membrane and robust sprouting [24] that was blocked by PC inhibition. When considering key events required for successful EC invasion, WSS enhanced MT1-MMP activation (shown here), while S1P combined with WSS was needed for membrane retention [24,25]. Altogether, these data illuminate intracellular signaling events downstream of physiological stimulation with WSS and S1P, which combine to promote robust EC sprouting.

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Highlights

- Physiological levels of WSS seen at post-capillary venules robustly convert pro MT1-MMP to the active form
- Proprotein convertase activity is required for WSS-mediated conversion of pro to active MT1-MMP and EC invasion responses
- Proprotein convertase activity and subsequent MT1-MMP activation are mechanosensitive and enable EC invasion

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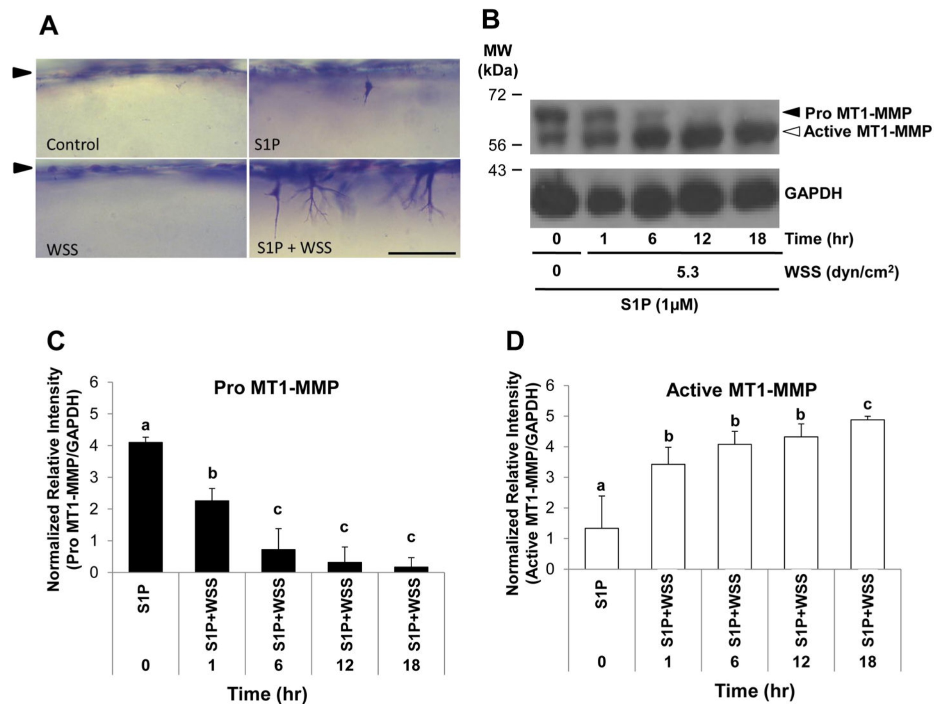

Figure 1. MT1-MMP activation occurred during S1P- and WSS-stimulated EC invasion in 3D collagen matrices

(A) Representative photographs of invasion responses. Scale bar,100μm. **(B)** Western blots are shown from ECs treated with or without 5.3 dyn/cm^2 WSS and allowed to invade for 0, 1, 6, 12, and 18hr in the presence of 1μM S1P. Cell extracts were probed with antibodies directed to MT1-MMP (*top blot*) or GAPDH (*bottom blot*). Black and white arrowheads indicate pro and active MT1-MMP, respectively in all figures. Densitometric analyses of band intensities for **(C)** Pro MT1-MMP and **(D)** Active MT1-MMP levels normalized to GAPDH using ImageJ software from 3 independent experiments (means ±S.D.). In all figures, lowercase letters denote groupings from one-way ANOVA followed by post hoc pairwise comparison testing using Tukey's method ($p<0.05$).

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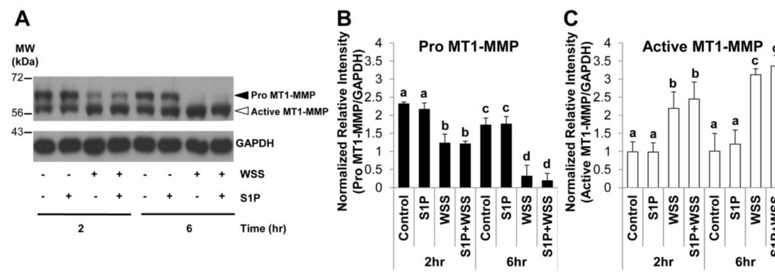

Figure 2. WSS, but not S1P, stimulated conversion of pro MT1-MMP to the active form in ECs seeded on 3D collagen matrices

(A) Representative western blots are shown from ECs treated with or without 5.3 dyn/cm^2 WSS for 2 or 6hr in the presence or absence of 1μM S1P. Cell extracts were probed with antibodies directed to MT1-MMP (*top blot*) or GAPDH (*bottom blot*). Densitometric analyses of band intensities for **(B)** Pro MT1-MMP and **(C)** Active MT1-MMP levels normalized to GAPDH.

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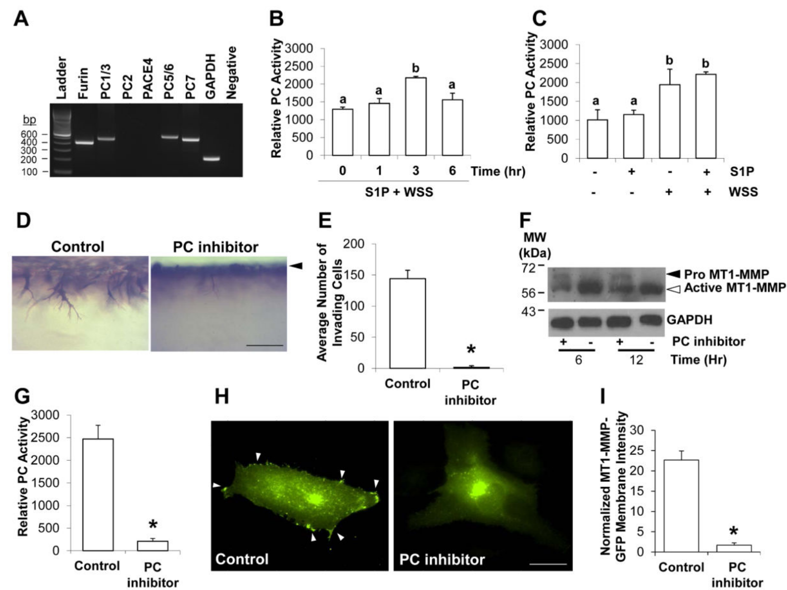

Figure 3. WSS increased proprotein convertase activity, which was required for EC invasion

(A) Representative RT-PCR using primers validated to amplify proprotein convertases [28]. ECs derived from 3 independent donors were tested with identical results. A representative example is shown. **(B)** ECs were treated with 5.3 dyn/cm^2 WSS for 0, 1, 3, and 6hrs in the presence of 1μM S1P and lysed to measure proprotein convertase activity. **(C)** ECs were treated with or without 5.3 dyn/cm^2 for 3hr in the presence or absence of 1μM S1P and lysed to measure proprotein convertase activity. **(D)** Photographs showing a side view of invading ECs stimulated with S1P and 5.3 dyn/cm^2 WSS in the presence of vehicle control or 25μM PC inhibitor for 24hr. Scale bar, 100μm. Arrowhead indicates original monolayer. **(E)** Quantification of invasion density from 4 individual experiments (n=20 total wells). Data shown are average number of invading cells +/–S.D. **(F)** Cell extracts were collected at 6 and 12hr and probed with antibodies directed to MT1-MMP (*top blot*) or GAPDH (*bottom blot*). **(G)** Quantification of PC activity from cell lysates collected at 3hr. **(H)** ECs were transiently transfected with a vector expressing MT1-MMP-GFP and treated with 5.3 dyn/cm^2 WSS and 1μM S1P for 2hr. Photographs of ECs expressing MT1-MMP-GFP are shown treated with or without 25μM PC inhibitor. White arrowheads indicate MT1-MMP-GFP at the cell periphery. Scale bar, 10μm. **(I)** Quantification of MT1-MMP-GFP localization to the cell periphery as described in "Materials and Methods" from 3 independent experiments. * indicates significant difference from control (Student's t-test; n=39 cells, $p<0.01$).

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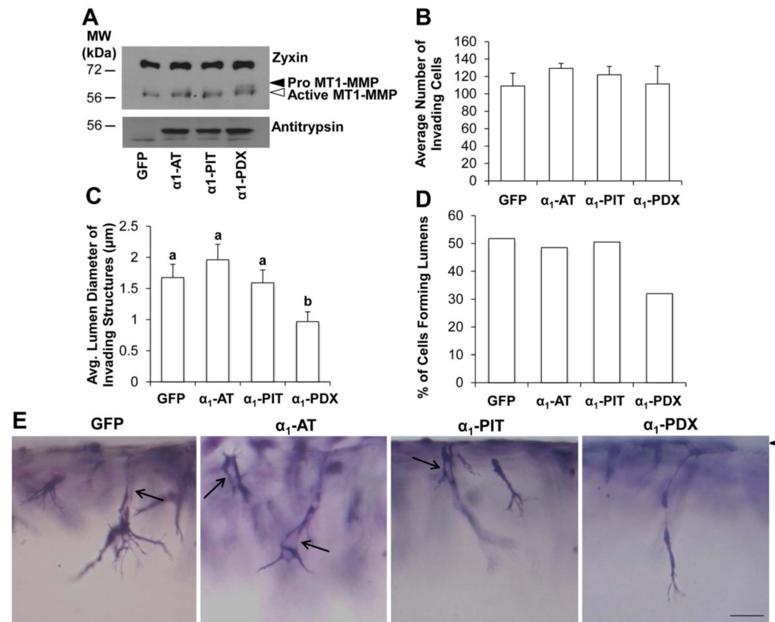

Figure 4. Exogenous expression of proprotein convertase inhibitor decreased lumen formation

ECs were transduced to express GFP, α_1-AT, α_1-PIT, or α_1-PDX and allowed to invade 2 or 24hr in the presence of 5.3 dyn/cm^2 WSS and 1μM S1P. **(A)** Cell extracts (2hr) were immunoblotted with antibodies against Zyxin to normalize for total protein (*top blot*), MT1-MMP (*middle blot*), and Antitrypsin (*bottom blot*) to demonstrate overexpression of antitrypsin-derived constructs. **(B)** Quantification of invasion density (24hr invasion). **(C)** Quantification of the average lumen diameter of invading cells (24hr invasion; n>100 cells, means ±SEM). **(D)** Percentage of invading structures which contained a lumen (n>100 cells). **(E)** Photographs illustrating a side view of EC invasion after 24hr. Arrowhead indicates monolayer; arrows indicate lumens. Scale bar, 50μm.