Cdc42 and RhoA have opposing roles in regulating Membrane Type 1-Matrix Metalloproteinase localization and Matrix Metalloproteinase-2 activation.

Eric Ispanovic, Damiano Serio and Tara L. Haas
School of Kinesiology and Health Sciences, York University, 4700 Keele St. Toronto, Ontario, Canada M3J 1P3

Running Title: Cdc42 and RhoA regulation of MT1-MMP and MMP-2

Address correspondence to:
Tara L. Haas
Rm 341 Farquharson
School of Kinesiology and Health Sciences
York University
4700 Keele St.
Toronto, ON
M3J 1P3
e-mail: thaas@yorku.ca
Abstract

Proteolysis of the basement membrane and interstitial matrix occur early in the angiogenic process, and require matrix metalloproteinase (MMP) activity. Skeletal muscle microvascular endothelial cells exhibit robust actin stress fibres, low levels of MT1-MMP expression and minimal MMP-2 activation. Depolymerization of the actin cytoskeleton increases MT1-MMP expression and MMP-2 activation. Rho family GTPases are regulators of actin cytoskeleton dynamics, and their activity can be modulated in response to angiogenic stimuli such as vascular endothelial growth factor, so we investigated their roles in MMP-2 and MT1-MMP production. Endothelial cells treated with H1152 (an inhibitor of ROCK) induced stress fibre depolymerization and an increase in cortical actin. Both MMP-2 and MT1-MMP mRNA increased, which translated into greater MMP-2 protein production and activation. ROCK inhibition rapidly increased cell surface localization of MT1-MMP and increased PI3K activity which was required for MMP-2 activation. Constitutively active Cdc42 increased cortical actin polymerization, PI3K activity, MT1-MMP cell surface localization and MMP-2 activation similarly to inhibition of ROCK. Activation of Cdc42 was sufficient to decrease RhoA activity. Capillary sprout formation in a 3D collagen matrix was increased in cultures treated with RhoAN19 or Cdc42QL and, conversely, decreased in cultures treated with dominant negative Cdc42N17. VEGF stimulation also induced activation of Cdc42 while inhibiting RhoA activity. Further, VEGF-dependent activation of MMP-2 was reduced by inhibition of Cdc42. These results suggest that Cdc42 and RhoA have opposing roles in regulating cell surface localization of MT1-MMP and MMP-2 activation.

Keywords: VEGF, PI3K, RhoGTPase, cytoskeleton, endothelium, angiogenesis.
**Introduction**

Angiogenesis is the growth of new capillaries from pre-existing mature ones. It occurs through a cascade of events in which disruption of the endothelial adherens junctions, by way of actin cytoskeleton reorganization, and proteolysis of the basement membrane and interstitial matrix are critical steps. Matrix metalloproteinases (MMPs), a family of zinc and calcium dependent enzymes (6; 56), are key participants in several steps of the angiogenic response including regulation of endothelial cell permeability, migration, invasion and tubule formation (13; 22). MMP-2 and membrane type 1 (MT1)-MMP are produced by endothelial cells and can degrade types I and IV collagen (2; 37). MMP-2 deficient mice have reduced tumor, corneal and retinal angiogenesis (25; 27; 41) while MT1-MMP deficient mice fail to gain weight, have deficient connective tissue metabolism and die 3-4 weeks after birth (23; 62). Combined MMP-2 and MT1-MMP deficiency causes embryonic lethality (due to respiratory failure, abnormal blood vessels and immature muscle fibres), highlighting the synergistic potential of these MMPs as key targets in controlling angiogenesis (40).

We, and others, have shown that the MMPs, specifically MMP-2 and MT1-MMP, are upregulated in response to reorganization of the actin cytoskeleton (24; 50; 59). The RhoGTPases are known regulators of the actin cytoskeleton (39) and contribute to the transformation of extracellular stimuli into angiogenic responses (9). Angiogenic factors such as VEGF and thrombin recruit downstream pathways including the mitogen activated protein kinases (MAPKs), PI3K and various transcription factors through RhoGTPase activation (28; 32-34; 54).

RhoA induces stress fiber formation and is required for VEGF induced increases in endothelial cell permeability, migration and stabilization of capillary tubes (1; 12; 38). Shear...
stress increases RhoA activity (46; 57) and stress fiber formation (7) and results in increased focal adhesion and junctional complex formation (45).

Rac1 controls lamellipodia and is linked with angiogenic signaling cascades. It is responsible for endothelial branching morphogenesis and capillary assembly in Matrigel overlay assays (11) through its contributions to endothelial migration (3) and MMP-2 activation (63). Downstream targets of Rac1 include the MAPKs and the AP-1 family of transcription factors, which in turn regulate MMP production (34; 54).

Cdc42 activation occurs with VEGF activation (31), and triggers the formation of filopodia and regulates cell polarization through microtubule organization. Activation of Cdc42 induces lumen and vacuole formation in endothelial cells and confers an invasive phenotype to T-lymphocytes (36; 48). Nobes and Hall proposed that a hierarchy of crosstalk occurs in which Cdc42 lies upstream of Rac1 and RhoA and that Cdc42 activates Rac1 while suppressing RhoA activity (39).

We hypothesized that manipulation of RhoGTPase activity would result in changes in MMP-2 and MT1-MMP expression and activity in microvascular endothelial cells. We show that RhoA suppresses MMP-2 expression while limiting the amount of cell surface MT1-MMP and, thereby, MMP-2 activation. Conversely, Cdc42 activation promotes angiogenesis and increases the amount of cell surface MT1-MMP, thereby increasing MMP-2 activation. Further, we demonstrate that these pathways contribute to VEGF dependent activation of MMP-2.
**Materials and Methods**

**Cell Culture** – Rat microvascular endothelial cells (SMEC) were isolated from extensor digitorum longus muscles and cultured as previously described (21). Cells were used for experiments between passages 4–11 and plated on type I collagen (12.5 μg collagen/ml coating buffer) coated culture dishes. For inhibitor studies, SMEC were pre-treated for 3 hours with 10 μM LY294002 or 50 μM SP600125 and then treated with 10 μM H1152, 10 μM GGTI-298 or 1 μM Cytochalasin D for 24 hrs. All inhibitors were purchased from Calbiochem. VEGF (recombinant human, Invitrogen) treatments were 25 ng/mL for varying times.

**3-Dimensional Collagen Culture** – SMEC were embedded in a 3-Dimensional type I collagen matrix (2.5 mg/mL Vitrogen) as previously described (8). SMEC were cultured in the presence or absence of 10 μM LY294002 for 24 hrs at 37 ºC.

**Gelatin Zymography** – Cells were lysed using 120 mM Tris-HCl (pH 8.7), 0.1% Triton X-100, and 5% glycerol supplemented with protease inhibitors (Sigma, Catalog # P8340) and 100 mM sodium orthovandate (lysis buffer). 10 μg of whole cell protein extracts were analyzed by gelatin zymography as previously described (20). Gels were visualized and imaged using the Fluorchem gel doc system and analyzed using Alphaease (Alpha Innotech) software. Images were inverted for publication for ease of visualization. Total MMP-2 protein was calculated as the sum of the latent (72 kDa) and active (62 kDa) bands was expressed as fold increase vs. Control. Active MMP-2 (62 kDa) was expressed as fold increase vs. Control.

**Northern Blot** – Total RNA was isolated and analyzed by Northern Blot using conventional techniques as described previously (20). Films were scanned and densitometry was performed using Alphaease (Alpha Innotech) software. Loading was normalized to the 28S rRNA band.
**Transient Transfection and Promoter Assays** - Cells were plated on type I collagen coated 12 well dishes at a density of 50 000 cells/well and 18 hours later transient transfections were performed, using LipofectAMINE 2000 (Invitrogen) reagent as per manufacturer’s instructions. Cells were transfected with 0.5µg of full length MMP-2 promoter (21) and 0.05µg of pRenilla (Promega). 24 hrs post transfection, cell were treated with either H1152 or GGTI-298 for 24 hrs. Forty-eight hours after transfection, cells were lysed with 1x passive lysis reagent (Promega) and reporter assays were performed using the Dual-Glo Luciferase Assay system (Promega). Firefly luciferase values were normalized to Renilla luciferase values. Normalized MMP-2 luciferase values from conditions that were treated with H1152 or GGTI-298 were then compared relative to those control untreated conditions.

**Western Blot** – Whole cell extracts were analyzed by Western Blotting as described previously described (24). Primary antibodies were: MT1-MMP (1:1000, Novus), AKT (1:1000) and Phospho-AKT (1:1000, Cell Signaling). Films were scanned and densitometry was performed using Alphaease (AlphaInnotech) software. Phospho-AKT values were normalized to total-AKT values to account for variability in loading.

**Immunofluorescence Staining** – Cells were plated on type I collagen coated glass coverslips, incubated overnight at 37°C and then treated with 10 µM H1152 for varying time points. Cells were fixed with 3.75% paraformaldehyde, then blocked and permeabilized in PBS + 5% normal goat serum + 0.05% Triton-X 100. Cells were incubated with primary phospho-JNK antibody (1:300, Upstate), followed by secondary goat anti-rabbit Alexa-568 (1:400, Molecular Probes). Actin was visualized with FITC-phalloidin (4 µM, Sigma) and nuclei were counterstained with 4’, 6’-diaminodino-2-phenylindole (DAPI, 1:1500, Molecular Probes). Cells were visualized by
fluorescence microscopy (Zeiss Axiovert 200M). Images were captured using a cooled digital CCD Camera (Quantix 57) and imaging software (Metamorph, Universal Imaging).

**TAT-RhoGTPase fusion protein creation and transduction** — RhoGTPase DNA plasmids (RhoAN19, RhoAQL, Rac1N17, Rac1QL, Cdc42N17, Cdc42QL, gifts from Dr. Ken Yamada) were subcloned into the psecTAG-TAT vector (amino acids 47-57 of the HIV-Tat, a gift from Dr. Tibor Barka) (5). Cos7 cells (provided Dr. Imogen Coe, York University) were transfected with 6 μL of TransPassD1 (New England Biolabs) and 5 μg TAG-TAT-RhoGTPase or TAT-TAT plasmid DNA in OptiMEM for 3 hrs, as per manufacturer’s instructions. OptiMEM was replaced with cDMEM and cells were maintained at 37ºC overnight after which media was replaced with fresh OptiMEM for 3hrs. The Cos7 conditioned OptiMEM, containing the secreted TAT or TAT-RhoGTPase proteins, was collected, spun down and the supernatant stored at –20ºC. SMECs were incubated with equal volumes of the OptiMEM containing mutant RhoGTPase protein after which the cells were lysed as described above. As the TAT peptide is known to elicit changes in endothelial cell cytoskeleton organization (58) and signaling (4), experimental conditions utilizing the TAT-RhoGTPase fusion proteins were compared to TAT peptide treated controls. In some experiments, cyclohexamide (20 μg/mL, Sigma) was added during the OptiMEM incubation to eliminate the effects of mRNA translation on MMP-2 protein levels.

**Cell Surface Immunofluorescence Staining** — Cells were plated on type I collagen coated glass coverslips and incubated overnight at 37ºC and then treated with 10 μM H1152 or TAT-Cdc42QL containing media for 2hrs. Cells were kept on ice and washed with physiological saline solution (PSS; 130 mM NaCl, 1.18 mM KH2PO4, 1.17 mM MgSO4 7H2O, 14.9 mM NaHCO3 pH = 7.3, 5.5 mM dextrose, 0.026 CaNa2EDTA, 1.6 mM CaCl2), blocked for 1 hr
with 5% BSA in PSS, and incubated with MT1-MMP 1° Ab (1:300, Chemicon, Ab815) in PSS for 1.5 hrs. Following 3 washes with PSS, cells were fixed with 3.75% paraformaldehyde and then permeabilized in PSS + 0.05% Triton-X 100. Cells were then incubated with secondary goat anti-rabbit Alexa-568 (1:400 dilution, Molecular Probes). Actin was stained with FITC-phalloidin (4 µM, Sigma). Cells were visualized by confocal microscopy (Olympus Fluoview 300 with Argon laser, 488 nm, and HeNe laser, 543 nm, pinhole aperture = 2) using Fluoview software (Olympus). Z-sections were captured (0.1 µm, 10 slices) and complete Z-stacks were used for actin staining images. For cell surface MT1-MMP images, only the first three slices of the Z-section were included in the final Z-stack.

Surface Biotinylation – SMEC were cultured on type I collagen for 24 hrs, pre-treated with 50 µM LY294002 and then stimulated with 25 ng/mL VEGF or 10 µM H1152 for 60 min or treated with TAT-Cdc42QL containing media for 2 hrs. Cells were washed with ice cold PBS and incubated with 1 mg/mL Sulfo-NHS-biotin in PBS for 30 min on ice. The reaction was terminated by washing the cells with 100 mM glycine for 20 min. Cells were lysed as described above and 75 µg of protein from total cell lysates was incubated with Streptavidin-Agarose beads (Pierce) overnight at 4°C with gentle rocking. Beads were collected by centrifugation and the pellet was washed several times with PBS containing 0.1% NP-40. 50 µL of 1X loading buffer was added and the samples were boiled and the proteins were separated by 10% SDS-PAGE followed by MT1-MMP Western blotting as previously described above. Blots were then stripped and re-probed for β1 integrin (Cell Signaling) as a loading control.

Capillary Sprout Formation Assay – Capillary segments were isolated from the rat epididymal fat pad and cultured within a 3-D type I collagen matrix (Vitrogen) as previously described (20). Fragments were cultured overnight at 37°C after which time culture media was replace with low
serum media (Opti-MEM, Gibco, 0.5% serum) containing constitutively active or dominant negative TAT-RhoGTPase proteins or a TAT peptide control for three hours. Low serum media was then replaced with cDMEM and the fragments were cultured for an additional 48 hrs. Phase contrast images were taken and sprout length measured using Metamorph imaging software (Universal Imaging).

*RhoGTPase activity assays* – SMEC were lysed with IP lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.3 M NaCl, 2% NP-40) and 100 µg of protein was incubated with Rhotekin-GST or PAK-GST protein beads (Cytoskeleton) for 2 hours at 4°C with gentle rocking for RhoA or Rac1 and Cdc42 activity measurements respectively. Beads were collected by centrifugation and the pellet was washed 3x with Wash Buffer (25 mM Tris pH 7.5, 30 mM MgCl$_2$, 40 mM NaCl). 50 µL of 1X reducing loading buffer was added and the samples were boiled and the proteins were separated by SDS-PAGE through a 15% acrylamide gel followed by Western blotting for RhoA, Rac1 or Cdc42 (1:1000, Cell Signaling).

*Statistics* – Data were normalized to control and are presented as fold change ± SEM vs. control. Student *t*-test or one-way ANOVA followed by Tukey post-hoc tests, were applied to determine statistical significance (p<0.05).
**Results**

*Inhibition of RhoA/ROCK induces MMP-2 activation.*

We previously demonstrated that depolymerization of the actin cytoskeleton with Cytochalasin D induces MMP-2 and MT1-MMP mRNA expression (24). To further investigate the signaling pathways involved in this response, we treated SMEC with the non-specific Rho-GTPases inhibitor GGTI-298 (an inhibitor of geranylgeranyl-transferase-1 that decreases activity of all Rho-GTPases). Pretreatment of SMEC with GGTI-298 abolished the Cytochalasin D induced increase in MMP-2 and MT1-MMP mRNA (Fig 1 A). In contrast, inhibition of ROCK, a RhoA effector responsible for actin cytoskeleton organization, increased basal levels of both MMP-2 and MT1-MMP mRNA. Co-treatment with the ROCK inhibitor, H1152, and Cytochalasin D did not result in a greater increase in MMP-2 or MT1-MMP mRNA than seen with either treatment on its own (Fig 1 B). To test if the increase in MMP-2 mRNA in response to H1152 treatment was due to increased mRNA synthesis, we assayed MMP-2 promoter activity. Promoter activity increased significantly following ROCK inhibition while treatment with GGTI-298 had no effect (Fig 1 C).

These changes in mRNA were translated into increases in MMP-2 protein production and activation. Treatment of endothelial cells with H1152 increased MMP-2 protein production and activation as evidenced by gelatin zymography (Fig 1 D). Treatment of endothelial cells with a dominant negative RhoA (RhoAN19) coupled to the 11 amino acid cell entry domain of the HIV-Tat peptide (TAT) demonstrated that inhibition of RhoA was sufficient to induce MMP-2 activation (Fig 1 E). Together, these results suggest that Rac1/Cdc42 are required for MMP-2 and MT1-MMP mRNA expression while RhoA activity suppresses their expression.

*JNK regulates MMP-2 and MT1-MMP mRNA expression following ROCK inhibition.*
Based on our previous findings that JNK regulates MMP-2 and MT1-MMP mRNA expression in endothelial cells (24), we tested if JNK contributes to the increase in mRNA expression following ROCK inhibition. Inhibition of ROCK with H1152 caused depolymerization of actin stress fibres and an increase in cortical actin (Fig 2 B, arrows). H1152 treatment caused nuclear translocation of phospho-JNK (Fig 2 B, arrowheads). Correlating with this response, we observed that the H1152 induced increase in MMP-2 mRNA expression was attenuated partially by JNK inhibition while the increase in MT1-MMP was blocked completely by pre-treatment with SP600125 (Fig 2 C).

*Reorganization of the actin cytoskeleton induces MT1-MMP cell surface localization.*

Because activation of MMP-2 is dependent critically on the amount of MT1-MMP on the cell surface (42), we examined the amount of cell surface MT1-MMP following ROCK inhibition. Endothelial cells treated for 2 hrs with H1152 were incubated with anti-MT1-MMP antibody prior to permeabilization to specifically visualize cell surface MT1-MMP. Increased cell surface MT1-MMP was detectable following H1152 treatment, and MT1-MMP localized to areas of actin staining (Fig 3 A). Cell surface biotinylation followed by Western blotting confirmed that ROCK inhibition increased cell surface MT1-MMP in the absence of a change in total cellular MT1-MMP (Fig 3 B). Cell surface levels of β1 integrin levels were assessed to confirm equal loading of the surface biotinylated samples (data not shown). These results further suggest that high levels of RhoA activity are inhibitory to MT1-MMP cell surface localization.

*PI3K activity is required for MMP-2 activation.*

We have shown that MMP-2 protein production and activation can be modified by PI3K signaling (24). We found that P-AKT levels increased as early as 30 min post ROCK inhibition
and remained elevated for 2 hrs (Fig 4 A). This time course of P-AKT levels corresponds to the timing of increased cortical actin polymerization as demonstrated in Fig 2 B.

Endothelial cells were pretreated with LY294009 (10 μM) for 2 hrs followed by ROCK inhibition with H1152 to determine if the changes in MMP-2 activity were dependent on PI3K. H1152 treatment was brief (2 hrs) to minimize the contribution of de novo synthesized MMP-2. Gelatin zymography demonstrated that PI3K is required for the activation of MMP-2 observed following ROCK inhibition (Fig 4 B). A small, but non-significant, increase in total MMP-2 protein was observed between Control and H1152 treated cells. Interestingly, inhibition of PI3K failed to attenuate the increase in MT1-MMP cell surface localization following ROCK inhibition (H1152 = 1.8 ± 0.24 fold increase over Control vs. H1152 + LY = 1.6 ± 0.21 fold increase over Control, p = 0.48, n = 3). At the dose used, LY294009 effectively inhibited the H1152 induced increase in P-AKT levels as verified by Western blot (data not shown). It has been suggested that cell surface localization of MT1-MMP is not the critical step for its activation, but, rather, that MT1-MMP clustering on the cell surface is more important (15). This may explain why inhibition of PI3K was sufficient to inhibit MMP-2 activation following H1152 treatment without inhibiting cell surface localization of MT1-MMP.

To test if PI3K modulates endothelial cell sprouting, we isolated capillary segments and cultured them within a 3-D type I collagen matrix in the absence or presence of LY294009 (Fig. 4 C). The typical formation of endothelial cell sprouts from the pre-existing capillaries was significantly in those treated with the PI3K inhibitor (Fig 4 D). Correspondingly, the increase in total MMP-2 protein observed in endothelial cells following 24 hrs in 3-D culture was attenuated partially by PI3K inhibition (3.3 ± 0.4 fold v. 2-D control, p < 0.05, and 2.3 ± 0.4 v. 2-D control,
p > 0.05, respectively). Notably, MMP-2 protein activation was significantly reduced by pre-treatment with LY294009, when compared to control 3-D cultures (Fig 4 E).

*Overexpression of Cdc42 increases activation of MMP-2.*

To further define the roles of individual RhoGTPase members in MMP-2 activation, we utilized RhoGTPase fusion proteins of both the dominant negative and constitutively active forms of RhoA, Rac1 and Cdc42, coupled to the 11 amino acid cell entry domain of the HIV-Tat peptide (TAT). Western Blot analysis verified the function of the TAT-RhoGTPase fusion proteins. Activation of Cdc42 and Rac1 by treatment with TAT-Cdc42QL and TAT-Rac1QL respectively are shown as representative experiments (Fig 5 A).

Capillary segment cultures were used to observe the effects of the RhoGTPases on capillary sprouting. TAT-RhoAN19, TAT-Rac1QL and TAT-Cdc42QL treated cultures had a greater average sprout length while treatment with TAT-Cdc42N17 significantly decreased average sprout length when compared to TAT control (Fig 5 B). TAT-RhoAQL and TAT-Rac1N17 treatments showed a trend towards decreased *de novo* sprout formation.

Transduction of endothelial cells with TAT-Cdc42QL for 4 hrs resulted in a significant increase in MMP-2 protein activation (Fig 5 C) without a concurrent increase in total MMP-2 protein. This increase in active MMP-2 was not attenuated in Cdc42QL transduced cells treated with the translational inhibitor, cycloheximide (1.66 ± 0.1 fold above TAT-only in untreated vs. 1.50 ± 0.2 fold above TAT-only in cycloheximide treated cells; n=6-7). There was a trend towards increased MMP-2 activation in SMEC treated with TAT-Rac1QL, but no change in MMP-2 activation was seen in cells treated with TAT-RhoAQL. Increased levels of P-AKT also were observed following transduction of cells with Cdc42QL (Fig 5 D), similar to that observed in response to inhibition of the RhoA effector, ROCK (Fig.4A).
Given the effect of Cdc42QL on MMP-2 activation, we examined MT1-MMP localization. Treatment of endothelial cells with TAT-Cdc42QL caused cell retraction, formation of filopodia-like protrusions and an increase in cortical actin (Fig 6 A). Cell surface MT1-MMP localization also increased as evidenced by immunostaining (Fig 6 A) and cell surface biotinylation (Fig 6 B).

_Cdc42 activation inhibits RhoA activity._

The results of the above experiments suggest that RhoA and Cdc42 oppose each other in the regulation of MMP-2 production and activation. As crosstalk between the RhoGTPases has been reported, we investigated if a signaling hierarchy exists in our system. Inhibition of RhoA signaling using H1152 failed to increase Cdc42 activity (data not shown). Conversely, activation of Cdc42 with TAT-Cdc42QL significantly decreased RhoA activity (Fig 7A).

_VEGF induces activation of MMP-2 via Cdc42_

VEGF is reported to induce reorganization of the actin cytoskeleton, dependent on the activity of RhoGTPases. Here, we confirm that 1 hour VEGF stimulation causes reorganization of the actin cytoskeleton to form peripheral regions of cortical actin and induces filopodia-like cellular protrusions (Fig 8A, upper panels). Similar changes in actin cytoskeleton reorganization followed Cdc42 activation (Fig 8A, lower panels). VEGF stimulation of endothelial cells caused an increase in Cdc42 activity (Fig 8B, upper panel). Concurrently, RhoA activity was decreased following VEGF stimulation (Fig 8B, lower panel). VEGF-dependent changes in actin organization and RhoGTPase activity were accompanied by MMP-2 activation (Fig 8C). Lastly, inhibition of Cdc42 activity using TAT-Cdc42N17 was sufficient to decrease MMP-2 activation following VEGF stimulation (Fig 8D).
**Discussion**

This study provides evidence that Cdc42 is a positive modulator of the angiogenic response in endothelial cells through its effects on MMP-2 and MT1-MMP. Activation of Cdc42 increased MMP-2 protein production and induced its activation by increasing cell surface MT1-MMP. Conversely, RhoA plays an inhibitory role by suppressing MMP-2 and MT1-MMP mRNA expression and limiting MT1-MMP surface expression. These opposing changes in GTPase activity also can be observed following stimulation with VEGF. Furthermore, VEGF induced activation of MMP-2 was reduced significantly by inhibition of Cdc42.

Reorganization of the actin cytoskeleton participates in the initiation of angiogenesis. We previously reported that reorganization of the actin cytoskeleton modulates both MMP-2 and MT1-MMP production through signal pathways involving JNK and PI3K (24). We extend these findings in the current study by elucidating the involvement of specific RhoGTPases in these events. In our study, strong stress fiber formation, and low levels of MMP-2 expression and activation were characteristic of endothelial cells plated on type I collagen. Our results point to two temporally regulated mechanisms by which inhibition of RhoA signaling increases MMP-2 activation. First, depolymerization of stress fibres rapidly increases the amount of MT1-MMP on the cell surface and facilitates activation of MMP-2. Inhibition of ROCK, a target of RhoA signaling also mobilizes P-JNK to the nucleus and increases MMP-2 and MT1-MMP mRNA expression, which provides sustained MMP-2 and MT1-MMP protein production and further activation of MMP-2.

The level of RhoA activity has been linked previously to MMP-2 activity as Matsumoto *et al* (35) showed that in LPA stimulated cells, low levels of RhoA activity increased MMP-2 activity but increased RhoA activity inhibited MMP-2 activity. Similarly, confluent non-migratory
HUVECs exhibit high levels of RhoA activity, and RhoA inhibition increases MT1-MMP activity (16). However, mechanisms linking these effects were not established by these studies. Zucker et al. (64) showed that actin depolymerization increases the number of cell surface receptors for TIMP-2 (i.e. MT1-MMP) without altering the binding affinity for TIMP-2. MT1-MMP is internalized by both clathrin-dependent (26; 43) and clathrin-independent (through caveolae) (16; 43) endocytosis, both of which rely on actin scaffolding. Disruption of the actin cytoskeleton may affect normal internalization of MT1-MMP (17), quickly increasing MT1-MMP molecules on the cell surface. These observations suggest that high levels of RhoA and stress fiber formation contribute to a stabilized, non-proteolytic endothelial phenotype.

Increasing Cdc42 activity caused the same effect on MMP-2 activation as inhibition of RhoA/ROCK activity. Overexpression of Cdc42 induced an increase in MT1-MMP on the cell surface and increased angiogenesis in 3-D type I collagen. Previous studies indicated a significant permissive role for Cdc42 in tumor cell migration and invasion (19; 30; 55). Our results extend these findings by providing a mechanism by which these events may be mediated, namely Cdc42 dependent MMP-2 activation via increased cell surface MT1-MMP. It is possible that in addition to inhibition of MT1-MMP internalization, activation of Cdc42 may increase MT1-MMP cell surface localization by increasing exocytosis of MT1-MMP from intracellular stores to the plasma membrane. For example, Cdc42 activation plays a positive role in exocytosis of von Willebrand factor in endothelial cells (29; 51) and the second phase of insulin secretion in pancreatic β-cells (53).

The Rho-family of GTPases signal to one another in the process of coordinating the appropriate intracellular responses to extracellular signals. We demonstrated that Cdc42 activation suppressed basal RhoA activity. This is in agreement with the hierarchy of
RhoGTPase signaling proposed by Nobes et al. (39). We observed that Cdc42 activation led to decreased RhoA activity, stress fibre depolymerization, increased MT1-MMP cell surface localization and greater MMP-2 activation. Concurrently, Cdc42 activation can increase Rac1 activation, which may contribute to increase endothelial sprouting.

Coordination of Cdc42 and RhoA signaling was recently demonstrated in podosome formation in primary endothelial cells (49). Cdc42 activation resulted in podosome formation, which was accompanied by localized stress fiber depolymerization and correlated with decreased RhoA activity (49). Conversely, Cdc42 dependent Rac1 activation has been reported wherein Cdc42 activates IRSp53, an intermediary protein that allows for Rac1 to bind to the WAVE-Arp 2/3 complex, and is required for Rac1 dependent lamellipodia formation (18).

Several studies indicate Cdc42 dependent activation of PI3K and AKT (10; 60), which is consistent with our observation that PI3K is required for MMP-2 activation following RhoA inhibition. When considered with the report that increased RhoA activity inhibited AKT-dependent phosphorylation of eNOS in endothelial cells (52) and that Cdc42 activation decreases RhoA activity, we postulate that activation of Cdc42 alleviates the RhoA induced suppression of PI3K/P-AKT signaling. The activation of these pathways then increases cell surface MT1-MMP and induces MMP-2 activation.

The mechanism by which P-AKT regulates MT1-MMP on the cell surface remains to be elucidated. Galvez et al. suggested that clustering of MT1-MMP molecules is more important than internalization in regulating MT1-MMP activation of MMP-2 (14). They proposed that MT1-MMP clustering is dependent on cortical actin polymerization, which is regulated by PI3K. Our data are consistent with this hypothesis as ROCK inhibition increased P-AKT levels at time
points corresponding to increased MMP-2 activation, and inhibition of PI3K abolishes the increases in MMP-2 activation without altering the amount of MT1-MMP on the cell surface.

Our results provide evidence that VEGF-induction of MMP-2 activity utilizes a combination of increased Cdc42 activity and decreased RhoA activity. The signal cascade leading to MMP-2 activation proposed here is in line with events following VEGF stimulation of endothelial cells. Cdc42 activation has been reported in response to VEGF stimulation (61) and is activated during endothelial cell haptotaxis on type I collagen (47). Interestingly, activation of Cdc42 can induce VEGF promoter activity via a c-Jun dependent mechanism (44) and we have shown previously that c-Jun is necessary for MMP-2 mRNA expression following VEGF stimulation (24). Together, these results suggest that VEGF can induce both rapid and sustained production activation of MMP-2 through upregulation of Cdc42 activity.

In summary, inhibition of RhoA or activation of Cdc42, in microvascular endothelial cells induced a shift from stress fibres to cortical actin and increased MT1-MMP localization to the cell surface. Longer term consequences of RhoA inhibition or Cdc42 activation included increased MMP-2 and MT1-MMP mRNA expression and greater capillary sprouting. These findings further elucidate the process of endothelial cell activation and initiation of angiogenesis.
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Reference List


Figure legend

**Fig 1.** Long-term inhibition of RhoA/ROCK signaling induces MMP-2 production and activation. (A) Endothelial cells pretreated with 10 μM GGTI-298 or (B) 10 μM H1152 (B) were treated with 1 μM CytoD for 24 hrs. MMP-2 (white bars) and MT1-MMP (black bars) mRNA were quantified using Northern Blot and expressed relative to 18S rRNA. (C) Normalized MMP-2 promoter activity in cells treated with H1152 or GGTI-298 for 24 hrs. (D) Cells were treated with 1 μM CytoD or 10 μM H1152 for 24 hrs and MMP-2 production (72 kDa + 62 kDa bands, white bars) and activation (62 kDa band only, black bars) were assessed by gelatin zymography. Values are mean ± SEM, * = p < 0.05 vs. Control, # = p <0.05 vs. Cytochalasin D, n = 3. (E) Cells were treated with TAT-RhoAN19 or TAT peptide alone for 4 hours and MMP-2 production (white bars) and activation (black bars) was measured by gelatin zymography. Values are mean ± SEM, * = p < 0.05 vs. TAT, n = 3.

**Fig 2.** Inhibition of ROCK induces MMP-2 and MT1-MMP mRNA expression in a JNK dependent pathway. (A) Endothelial cells in the absence (left) or presence (right) of 10 μM H1152 were stained for phospho-JNK (red), F-actin (green), and DAPI (blue, nuclei). Arrows = cortical actin, Arrowheads = nuclear P-JNK, Scale bar = 20 μm. (B) Cells pretreated with 100 μM SP600125 were treated with 10 μM H1152 for 24 hrs. MMP-2 (white bars) and MT1-MMP (black bars) mRNA was measured by Northern Blot and expressed relative to 18S rRNA. Values are mean ± SEM, * = p < 0.05 vs. Control, # = p < 0.05 vs. H1152, n = 3.

**Fig 3.** Short-term inhibition of ROCK induces increased cell surface MT1-MMP. (A) Cells were treated for 2 hr with 10 μM H1152 and then immunostained for MT1-MMP prior to permeablization (right), followed by F-actin (left), Scale bar = 20 μm. (B) Cell surface and total cellular MT1-MMP, following 2 hr treatment of cells with 10 μM H1152 was measured by cell
surface biotinylation and Western Blot for MT1-MMP. Values are mean ± SEM, * = p < 0.05 vs. Control, n = 3.

**Fig 4.** PI3K is required for MMP-2 activation and capillary sprout formation.

(A) Endothelial cells were treated with 10 μM H1152 for varying time points and P-AKT, measured by Western Blot, was normalized to total-AKT. Values are mean ± SEM, * = p < 0.05 vs. Control, n = 3. (B) Cells were pretreated with 10 μM LY294002 (2 hrs) and then treated for 2 hrs with 10 μM H1152 to inhibit ROCK. MMP-2 production and activation were measured by gelatin zymography. Values are mean ± SEM, * = p < 0.05 vs. Control, # = p < 0.05 vs. H1152, n = 3. (C) Capillary segments were cultured within a 3-D type I collagen matrix in the presence or absence of 10 μM LY294002. Asterisks denote original capillary segment while arrows denote sprouting cells. (D) Average sprout length was quantified using Metamorph software. Values are mean ± SEM, * = p < 0.05 vs. Control, n = 3. (E) Endothelial cells were pretreated with 10 μM LY294002 for 3 hrs and then cultured within a 3-D type I collagen matrix for 24 hours. MMP-2 production and activation were measured by gelatin zymography. Values are mean ± SEM, * = p < 0.05 vs. Control, n = 3.

**Fig 5.** Overexpression of Cdc42 increases MMP-2 activity and capillary sprouting.

(A) Endothelial cells were treated with TAT-fusion protein of the RhoGTPases or TAT peptide control. Functional activity of the TAT-fusion proteins was verified by measuring Cdc42 and Rac1 activity in cells treated with TAT-CdcQL and TAT-Rac1QL, respectively, for 4 hours by immunoprecipitation using PAK-GST coated beads and Western Blotting. (B) Capillary segments were cultured within a 3-D type I collagen matrix in the presence of TAT-RhoAN19/QL, TAT-Rac1N17/QL, TAT-Cdc42N17/QL or TAT peptide control and the average sprout length was quantified using Metamorph software. (C) Endothelial cells were treated with
TAT-RhoAQL, TAT-Rac1QL or TAT-Cdc42QL for 4 hours and MMP-2 production (white bars) and activation (black bars) was measured by gelatin zymography. Endothelial cells were treated with TAT-Cdc42QL and P-AKT was detected by Western Blot, and normalized to total-AKT (D). Values are mean ± SEM, * = p < 0.05 vs. TAT, n = 3.

**Fig 6.** Overexpression of Cdc42 increases cell surface localization of MT1-MMP. (A) Endothelial cells were treated for 4 hr with TAT-Cdc42QL and then immunostained for MT1-MMP prior to permeabilization (right) and F-actin (left), Scale bar = 20 µm. (B) Cell surface MT1-MMP following 4 hr TAT-CdcQL treatment was measured by cell surface biotinylation and Western Blot. Values are mean ± SEM, * = p < 0.05 vs. TAT, n = 3.

**Fig 7.** Overexpression of Cdc42 decreases RhoA activity. RhoA activity in endothelial cells treated with TAT-RhoAN19 or TAT-CdcQL for 4 hours was measured by immunoprecipitation using Rhotekin-GST protein beads and Western Blotting, n = 3.

**Fig. 8** VEGF induces cytoskeletal reorganization and MMP-2 activation via Cdc42GTPase. (A) Cells were treated with 25 ng/mL VEGF or with TAT-Cdc42QL for 4 hr and stained for F-actin, arrows indicate peripheral actin, scale Bar = 20 µm. (B) Cells were treated with 25 ng/mL VEGF for varying time points and Cdc42 and RhoA activity was measured by immunoprecipitation using PAK-GST or Rhotekin-GST coated beads, respectively, and Western Blotting. (C) Cells were treated with 25 ng/mL VEGF for 1 hr and MMP-2 activation was measured by gelatin zymography. (D) Cells were pre-treated with TAT or TAT-Cdc42N17 and then treated with 25 ng/mL VEGF for 1 hr. MMP-2 activation was measured by gelatin zymography. Values are mean ± SEM, * = p < 0.05 vs. control, # = p < 0.05 vs. TAT, n = 3.
Figure 1

A

MMP-2
MT1-MMP
18S

B

MMP-2
MT1-MMP
18S

C

Control
CD
GGT1
GGT1 + CD

D

Control
CD
H1152
H1152 + CD

E

TAT
RhoAN19

72 kDa
62 kDa

MMP-2 Protein (Relative to Control)

MMP-2 Protein Activity (Relative to Control)
Figure 2

A

B

mRNA (Relative to Control)

Control  H1152  H1152 + SP

MMP-2

MT1-MMP

18S

* * #

0.0  0.5  1.0  1.5  2.0
Figure 3

A

![Image of Actin and MT1-MMP staining for Control and H1152 conditions.](image)

B

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<th>Total</th>
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63 kDa

![Graph showing cell surface MT1-MMP expression relative to control.](image)
Figure 4

A

P-AKT

AKT

Time (min)

Control 30 60 120

P-AKT (Relative to Control)

Control LY +H1152

Active MMP-2 (Relative to Control)

Control LY H1152 H1152 + LY

B

C

Control

LY

D

Average Sprout Length (µm)

Control LY

E

Active MMP-2 Protein (Relative to Control)

3D 3D + LY
Figure 5

A

TAT Cdc42
Cdc42-GTP 21 kDa
TAT Rac1
Rac1-GTP 21 kDa

C

TAT RhoA Rac Cdc42

MMP-2 Protein (Relative to Control)

TAT RhoAQL Rac1QL Cdc42QL

B

Avg sprout length (um)

TAT RhoQL RhoN19 RacQL RacN17 CdcQL CdcN17

D

P-AKT (Relative to Control)

TAT Cdc42QL
Figure 6

A

![Cell Surface MT1-MMP](image)

B

![Surface and Total MT1-MMP](image)

![Cell Surface MT1-MMP](image)
Figure 7

A

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<th>TAT</th>
<th>RhoA N19</th>
<th>Cdc42 QL</th>
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GTPase Activity (Relative to control)

- TAT
- RhoAN19
- Cdc42QL

* *
Figure 8

A

Control  VEGF

TAT  Cdc42QL

B

VEGF

Con  0.5 hr  1 hr

Cdc42-GTP

21 kDa

RhoA-GTP

21 kDa

C

Con  VEGF

72 kDa  62 kDa

Active MMP-2

(Density to Control)

D

VEGF

TAT  Cdc42N17

72 kDa  62 kDa

Active MMP-2

(Density to Control)