TERMINAL SEQUENCE TO EXPOSE A NEW NH2-TERMINAL-ACTIVATING PEPTIDE (26). Unlike other GPCRs, proteases cleave PARs’ NH2-terminal sequence to expose a secondary extracellular domain to trigger PAR activation (26).

PAR2 activation by FVIIa/TF leading to cellular migration has not been extensively characterized. Cellular migration is a key component of tumor metastases and invasion. In fact, several studies have correlated levels of TF expression in primary colorectal, breast, and pancreatic cancer and the levels of TF correlate with the aggressiveness of the cancer phenotype (31). Stimulation of angiogenesis is one mechanism of this pathway that leads to increased tumor invasion. FVIIa/TF-PAR2 signaling of breast cancer cells induces numerous proangiogenic factors, such as VEGF, Cyr61, VEGF-C, and CTGF (24). FVIIa/TF-PAR2 is also critical for influencing tumor cell behavior through playing a key role in metastases. In this process, PAR2 recruits β-arrestin and ERK to pseudopodia of migrating cells (16). This β-arrestin recruitment promotes breast cancer migration and results in dephosphorylation of coflin (52). This activation of the coflin pathway results in severing actin filaments that are required for reorganization of the cytoskeleton and breast cancer invasion (47). In this context, a role for FVIIa/TF-PAR2 has been described in cellular migration, resulting in metastasis and tumor aggressivity (3). However, the mechanism by which cellular migration and metastasis occurs in response to FVIIa/TF-PAR2 activation is not well understood.

In this study, we have utilized porcine cerebral microvascular endothelial cells (pCMVECs) as a model system to delineate mediators involved in FVIIa/TF-PAR2-induced cellular migration. Our results reveal that PAR2 stimulation induces Ras homolog gene family member A (RhoA) and cortactin, which were found to be key players in FVIIa/TF-PAR2-evoked cell migration.

MATERIALS AND METHODS

Materials. Mitomycin C was purchased from Fluka BioChemika (Buchs, Switzerland). Monoclonal cortactin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 555 conjugated cortactin antibody was from Upstate (Lake Placid, NY). Monoclonal β-actin antibody and fibronectin were from Sigma-Aldrich (Oakville, ON). Matrigel was from BD Biosciences (Mississauga, ON). FVIIa was from NiaState (Mississauga, ON). Falcon Cell Culture Insert (8-μm pore size) was from Falcon (Fremont Lakes, NJ). shRNA for PAR2, RhoA, and cortactin were from Open Biosystems (Huntsville, AL). SLIGRL peptide was synthesized by Elim Biopharmaceuticals (Hayward, CA). Trypsin was from Worthington (Lakewood, NJ). Full-length human TF and cytoplasm domain-truncated TF-containing constructs have been previously described (8). Flag-PAR2-HA/pcDNA3 was kindly provided by Dr. N. W. Bunnett (7), University of California, San Francisco, CA.
Cell line and culture. pCMVECs (17) were cultured in complete medium of DMEM (GIBCO, Grand Island, NY) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and was incubated in a 5% CO₂, 37°C humidified tissue culture incubator. For synchronization, the cells were grown in starving medium (DMEM without FBS and supplemented with penicillin and streptomycin) for 4 h or overnight. Human kidney epithelial cell line BOSC-23 was used as a TF-expressing positive control (13). Full-length human TF was expressed in pCMVECs using a pcDNA3.1 vector construct (see Supplemental Material, Supplemental Fig. S1A, published with the online version of this article).

Coverslip migration assay. Microscope coverslips were sterilized by flaming and put on the bottom of each well in a 24-well culture dish. The TF-transfected pCMVECs were seeded on each coverslip-loaded well at the density of 10⁵ cells/well and incubated overnight in 0.5 ml DMEM complete medium (Supplemental Fig. S1B, i). The cells were then synchronized by incubation with DMEM starving medium for 4 h. To discriminate between cell proliferation and migration, mitomycin C was supplied to the cells at 10 μg/ml for 30 min in a 37°C incubator (Supplemental Fig. S1C and Ref. 21). The cell-coated coverslips were then picked up carefully by using a sterile jeweler’s microforceps rinsed with culture medium and placed in a 12-well culture dish, which provided a sufficiently large space. Cells in the 12-well culture dish were maintained in 2 ml 2% FBS-containing DMEM medium containing testing mediators (such as: FBS, 100 nM FVIIa, 20 μM SLIGRL, or 20 nM trypsin) (Supplemental Fig. S1B, ii). pCMVECs were then induced to migrate outward from the coverslip to the culture dish (Supplemental Fig. S1B, iii). After 72-h incubation the original cell-covered coverslips were removed. Cells remaining in the culture dish are migrated cells (Supplemental Fig. S1B, iv). FBS was used as a mediator to stimulate cellular migration (Supplemental Fig. S1D). In FVIIa-induced pCMVEC migration measurements, 50 μl of Matrigel containing 2.5 μM fibronectin was pasted on the bottom edge of each well of a 12-well culture dish to form a gradient chemotactractant force to direct cellular migration outward from the coverslip.

The cellular migration rate was estimated by using the 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay (32). This rapid, reproducible and automated method measures the end product of the enzymatic cleavage of the tetrazolium rings in the MTT salt by mitochondrial dehydrogenase. The amount of the resulting dark blue formazan crystal product is directly proportional to the number of live cells and can be easily quantified in a multwell scanning spectrophotometer (ELISA) reader at an optical density of 545 nm with a reference wavelength of 690 nm as previously reported (51). In this novel system, FVIIa-induced migration was optimized by comparing varying doses of FVIIa, and an optimal dose of 100 nM FVIIa was elucidated by using the coverslip migration assay. Since FVIIa-mediated cell migration has been demonstrated to be PAR dependent, we used the PAR2-activating protease trypsin and an activating peptide (SLIGRL) and demonstrated that these molecules increased cellular migration as well (Fig. 1, A and B).

Boydren chamber filter migration assay. We compared this new coverslip migration assay to the Boyden chamber (4, 45) (Supplemental Fig. S1F). Cell culture inserts (8 μm pore size) were placed in a 24-well culture dish in which 0.8 ml of prewarmed 2% FBS culture medium of DMEM (GIBCO, Grand Island, NY) containing 10% FBS, 100 nM FVIIa, and 20 nM SLIGRL or 20 nM trypsin (Supplemental Fig. S1B, iv). PCMVECs were then induced to migrate outward from the coverslip to the culture dish (Supplemental Fig. S1B, iii). After 72-h incubation the original cell-covered coverslips were removed. Cells remaining in the culture dish are migrated cells (Supplemental Fig. S1B, iv). FBS was used as a mediator to stimulate cellular migration (Supplemental Fig. S1D). In FVIIa-induced pCMVEC migration measurements, 50 μl of Matrigel containing 2.5 μM fibronectin was pasted on the bottom edge of each well of a 12-well culture dish to form a gradient chemotactractant force to direct cellular migration outward from the coverslip.

Boydren chamber filter migration assay. We compared this new coverslip migration assay to the Boyden chamber (4, 45) (Supplemental Fig. S1F). Cell culture inserts (8 μm pore size) were placed in a 24-well culture dish in which 0.8 ml of prewarmed 2% FBS culture medium (DMEM) were preseeded to each well. Then 2× 10⁴ TF-transfected pCMVECs were seeded to each well and stimulated with 100 nM FVIIa or 10% FBS in DMEM medium for 6 h. The cells inside the insert were removed by scratching up with cotton swabs. Migrated cells on the lower (outside) face of the insert membrane were rinsed by PBS and 50% methanol, respectively. To fix the cells to the membrane, the membranes were incubated in cold (4°C) 100% methanol for 10 min. Subsequently, Giemsa stain was applied to stain the membrane overnight. The migrated cells were counted in each viewing field by microscopy.

Fig. 1. Factor (F)VIIa and protease-activated receptor (PAR2) agonists induced porcine cerebral microvascular endothelial cell (pCMVEC) migration. A: migration of tissue factor (TF)-transfected pCMVECs were induced with increasing FVIIa concentrations and analyzed with the coverslip migration assay. B: migration of TF-transfected pCMVECs stimulated with fibronectin (2.5 μM) in the absence or presence of FVIIa (100 nM), SLIGRL (20 μM), and trypsin (Tryp: 20 μM). Values are means ± SE of 3 separate experiments, each conducted in quadruplicate. *Significant increase in migration of cells by ~2.5-fold above the fibronectin control (P < 0.05).

FVIIa/TF activates PAR2. Expressed Flag-PAR2-DA [NH₂ terminus-linked Flag, and COOH terminus-linked hematoxylin (HA)] was digested by VIla by ELISA assay in a Flag antibody-coated plate (Sigma). Flag-PAR2-HA/pcDNA3 was transient-cotransfected with or without TF/pcDNA3.1 in pCMVECs, respectively. Two days later, the cells were lysed with lysis buffer containing 1% Triton X-100, 150 mM NaCl, 150 mM KCl, 5 mM MgCl₂, 25 mM Tris-HCl, pH 7.4, and protease inhibitors cocktail. Then, 150 μg of the cell lysate per each well were loaded on FLAG-coated plate and reacted for 120 min at room temperature. After three-repeated washings with PBS, the bound Flag-PAR2-DA was then digested with 100 nM FVIIa or 20 nM trypsin in PBS buffer for 60 min. PBS alone was used as control. FVIIa or trypsin was again washed twice with PBS. The integrity (uncleaved inactivated form) of PAR2 was detected by reacting with HA-FITC antibody (Santa Cruz Biotechnology) for another 60 min. After three repeated washings with PBS, the activated PAR2 no longer bound to the Flag-coated plate resulting in decreased HA binding, measured using a fluorometer at 485/535 nm.
10^6 cells/well TF-transfected pCMVECs were seeded at 4 h before stimulation and treated with mitomycin-C for 30 min. The cells were then stimulated with 10% FBS in DMEM medium for 48-h incubation. The surviving cells were stained with MTT and quantitated with the use of optical density at 545 nm in a spectrophotometric reader (51).

Gene transfection and expression knockdown by shRNA. Short-hairpin RNAs (shRNA) (29) were employed to knock down PAR2, RhoA, and cortactin gene expression. pCMVEC were transfected with 4 μg corresponding plasmid [such as: NS-pSM2c (non-specific sequence shRNA, NS shRNA, from OpenBiosystems), PAR2-pSM2c (PAR2 shRNA), RhoA-pSM2c (RhoA shRNA), cortactin-pLKO.1 (cortactin shRNA), hTF-pCdNA3.1, or h-T-Ct-hTF-pCdNA3.1] by TransIT-TKO transfection reagent (Mirus, Madison, WI). Briefly, 70% confluent pCMVECs in six-well culture dish were cultured per well in complete DMEM. Next, 4 μg plasmid was mixed with 10 μL TKO reagent in 50 μL DMEM and kept for 20 min at room temperature. Finally, the mixture was dropped into the corresponding well. Then 4 h later the cells were exchanged with fresh complete DMEM. Finally, 48–72 h later the cells were used for subsequent experiments, such as migration assay, gene induction, immunofluorescence stain, tube formation assay. Gene expression knockdown by shRNA ranged between 70 and 80%.

Gene expression semiquantification by RT-PCR. pCMVECs transfected with TF and corresponding shRNA were prestarved with complete DMEM. After 20–30% confluence the cells were stimulated with 100 nM FVIIa and incubated in a 37°C incubator with 5% CO2 for 25 min and then reacted with 2% paraformaldehyde for 20 min. After being starved overnight, the cells were stimulated with 100 nM FVIIa or 20 μM SLIGRL for 10 min (for cortactin phosphorylation analysis) or 12 h (for cortactin protein analysis) being starved overnight and then stimulated with 100 nM FVIIa and incubated in a 37°C incubator with 5% CO2 for 20–30 min. The stimulated cells were then harvested for gene expression analysis by RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the product manual. cDNA reverse transcription and RT-PCR were performed as previously described (51). For RT-PCR amplification, primers for porcine HIF were forward: 5'-TCTTACATGTCCGCAGT-3' and reverse: 5'-TCTCACTTCTCAGCCCAC-3'; for porcine cortactin were forward: 5'-GGCAATGGTGGTTCAG-3' and reverse: 5'-ATGCCATACCCTTCTG-3'; for pig RhoA were forward: 5'-AAGGACCAGTTCAGAGGT-3' and reverse: 5'-CCTCCTAGGGTGTCACCAC-3'; for porcine actin were forward: 5'-GGTCACACTCAGACTCTC-3' and reverse: 5'-TTGCTACTTCTGTTG-3'; for porcine Dia2 were forward: 5'-AGGATGTTATTAGTGTAAC-3' and reverse: 5'-AGGCTCTAGAGGCCATCTAC-3'; for porcine Dia1 were forward: 5'-GAAAGGAAGTGTGGCTCTGC-3' and reverse: 5'-GAAAGGAAGTGTGGCTCTGC-3'; for mouse Dia2 were forward: 5'-ATGTGCGTGACCGAATT-3' and reverse: 5'-CCCTATTGCGAAGATGTGT-3'; for mouse cortactin were forward: 5'-ACTTCTACGAGGCACGAGAT-3' and reverse: 5'-GAAAAGAATCTGCTTGCCG-3'. QuantumRNA (Ambion, Austin, TX) universal 18S standard primers were used as internal standard references.

Western blot analysis. pCMVECs were prestarved for 4 h and then stimulated with 100 nM FVIIa or 20 μM SLIGRL for 10 min (for cortactin phosphorylation analysis) or 12 h (for cortactin protein expression determination). The stimulated cells were subsequently harvested and lysed with lysis buffer containing the protein phosphatase inhibitor, vanadate, for Western blot analysis as previously described (51). The 30 μg cellular lysate was loaded in each well on a 10% SDS-PAGE gel for Western blot analysis. Phosphorylated cortactin was probed by rabbit polyclonal phosphocorticatin antibody at Tyr421 (Cell Signaling, MA). The cortactin protein level was analyzed by mouse monoclonal cortactin (A-4) antibody (Santa Cruz Biotechnology). β-actin was utilized as an endogenous protein expression reference probe by a mouse monoclonal antibody (C4) (Santa Cruz Biotechnology).

Immunofluorescence stain. pCMVECs were seeded onto a 12-well culture dish with a circle coverslip and then transfected with the appropriate plasmid TF-pCdNA3, ΔC-TF-pCdNA3, or shRNA plasmid as required. The final confluency rate was kept ~20–30%. After being starved overnight, the cells were stimulated with 100 nM FVIIa for 20 min. For immunofluorescence stain, the cells were fixed with 4% (wt/vol) paraformaldehyde buffer for 20 min. After incubation with blocking buffer (5% fetal calf serum and 5% goat serum) for 40 min, the cells were permeabilized by adding Triton X-100 to 0.1% for 25 min and then reacted with 2 μg/ml Alexa Fluor 555-conjugated monoclonal cortactin antibody for 120 min. β-actin was probed with mouse monoclonal antibody (C4) and visualized with goat anti-mouse antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA). To localize nuclei, the cells were stained with 50 ng/ml DAPI for 10 min. The fluorescence-labeled cells were analyzed with Zeiss LSM 510 META laser scanning fluorescence confocal microscopy (Zeiss, Heidelberg, Germany).

Lamellipodia quantification. Lamellipodia were analyzed by using Photoshop. The lamellipodia area was highlighted by lasso tool, and the digital area data was obtained as pixels from the histogram function. Each group data was counted from 20–50 random cells from 5–10 random fluorescence-stained images. To transfer the pixel units to micrometer-squared units, we measured 20 random CMVECs nuclei to get an average of 6,556 pixel, which equals 28.26 μm² (calculated based upon the average nuclear diameter of 6 μm).

Endothelial tube formation assay. Cells (8 × 10⁶) of TF transfected pCMVECs were seeded onto each well of a Matrigel precoated 24-well culture dish. Gene silencing within these cells was performed by transfection with NS shRNA, PAR2 shRNA, RhoA shRNA, or cortactin shRNA. Cells were serum starved overnight and then stimulated with 100 nM FVIIa and incubated in a 37°C incubator with 5% CO2 for 20 min and fixed with 70% ethanol. Random images (5–10 per each sample) were captured under a phase contrast microscope at ×10 magnification. The numbers of tube structures (closed endothelial circles) were counted in every 100 cells.

Statistical analysis. Data were analyzed by a one-way ANOVA factoring for treatments, followed by the Newman-Keuls comparison among means test. Statistical significance was set as P < 0.05. Values are presented as means ± SE.

RESULTS

Cell migration assay. TF was overexpressed in the pCMVEC cells through the use of a pcDNA3.1 plasmid construct (Supplemental Fig. S1A). A cell migration assay was developed (see MATERIALS AND METHODS) in which cells plated on a coverslip (Supplemental Fig. S1B,i) are transferred to a 12-well plate (Supplemental Fig. S1B,ii), and agents were tested to induce migration of the cells from the coverslip to the titer plate (Supplemental Fig. S1B, iii). The migrated cells are clearly separated from nonmigrated cells by removing the coverslip (Supplemental Fig. S1B, iv) and estimating cell number using an MTT stain assay. Proliferative effects are eliminated using the antiproliferative mitomycin C (10 μg/ml) (Supplemental Fig. S1C). The system was validated initially using various concentrations of FBS at three time points (Supplemental Fig. S1D). Cells migrated outward from the coverslip as a function of time and reached a maximal rate of migration in 10% FBS (Supplemental Fig. S1D). Cell migration rate was linearly correlated with cell density (Supplemental Fig. S1E). This (simpler) new cell migration assay was further validated by comparing it with the Boyden chamber assay; results for both assays were comparable, albeit the Boyden chamber was less sensitive (Supplemental Fig. S1F), as previously reported (40); the new cell migration assay was utilized thereafter.

FVIIa dose-dependently stimulated cellular migration with maximum efficiency reached at 100 nM FVIIa (Fig. 1A). From here onward, 100 nM FVIIa was used; [cell migration to FVIIa was also detected using the Boyden chamber assay (Supplemental Fig. S1F)]. Fibronectin was also utilized to direct cellular movement from the coverslip; addition of FVIIa,
PAR2 agonist SLIGRL, or trypsin significantly increased migration of cells by ~2.5-fold above the fibronectin control (Fig. 1B).

**FVIIa/TF directly activates PAR2.** PAR2 has been reported to be involved in numerous FVIIa/TF-induced cellular responses, such as inflammation, atherogenesis, angiogenesis, tissue repair, tumor growth, and metastasis (37). The requirement for TF in the activation of PAR2 by FVIIa/TF was verified utilizing an ELISA assay. Inactivated PAR2 was detected in a Flag-coated plate with a dual-tagged construct of PAR2 [Flag-PAR2-HA/pcDNA3 (see MATERIALS AND METHODS)]-expressed pCMVEC cells. Interestingly, both FVIIa and trypsin cleaved flag-bound PAR2 in cells expressing both TF and Flag-PAR2-HA (Fig. 2). However, FVIIa was not able to activate PAR2 in TF nonexpressing cells compared with trypsin, which does not require TF (Fig. 2). Hence TF is required for PAR2 activation by FVIIa.

**FVIIa/TF regulates RhoA and cortactin.** In the context of cell migration we evaluated the expression of genes potentially affected by PAR2 activation in TF-overexpressing pCMVECs. These genes include the proangiogenic gene transcription factor, HIF (6), actin cytoskeleton polymerization and rearrangement promoter, cortactin (11), stress fiber regulator, RhoA (41), and Dia1 and Dia2 (41). Stimulation of cells with FVIIa/TF (1 h) induced expression of cortactin, and RhoA expression but not HIF, Dia1, nor Dia2 (Fig. 3A). We then attempted to determine the sequential order of activation of PAR2, RhoA, and cortactin on FVIIa/TF-induced gene expression, using established corresponding shRNAs (Fig. 3B). PAR2 silencing decreased FVIIa-induced cortactin and RhoA expression (Fig. 3C). RhoA gene silencing diminished cortactin expression, while the cortactin shRNA knockdown only significantly downregulated cortactin expression but did not affect RhoA mRNA levels (Fig. 3C). A nonspecific sequence shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.

**Cortactin activation by FVIIa/TF results in pCMVEC lamellipodia formation.** Activation of cortactin was determined by analyzing phosphocortactin at Tyr421 (25) (Fig. 3E). Stimulation of TF-overexpressing cells with FVIIa and SLIGRL for 10 min induced an increase in cortactin phosphorylation suggesting again a requirement for TF. Once again, RhoA knockdown abolished this effect, indicative of RhoA dependence.

Inactivated cortactin is localized throughout the cytoplasm, but once activated through phosphorylation, cortactin will target reactive sites in the cells, such as actin. The cortactin-assisted Arp2/3-nucleated actin branching is most prominent in the actin cortex, at the cell periphery to involve cellular movement processes, such as lamellipodia formation (48, 49). Under basal conditions, cortactin and β-actin primarily localized at the cytoplasm and perinuclear region of TF-overexpressing pCMVECs (Fig. 4A). Following stimulation with FVIIa (20 min), cortactin translocated to the cell periphery and colocalized with β-actin [at lamellipodia (see arrows in Fig. 4A)] PAR2 knockdown abrogated these effects. The COOH-terminal fragment of TF is implicated in FVIIa/TF-induced angiogenesis (43). We tested whether the COOH-terminal tail of TF participates in FVIIa-induced cortactin activation. For this purpose, we transfected a TF truncated of its COOH-terminal domain (ΔC-TF-pcDNA3) into pCMVECs and determined cellular localization of cortactin (Fig. 4B). The ΔC-TF-expressing pCMVECs did not affect FVIIa-induced cortactin translocation. Colocalization with β-actin, or lamellipodia formation (Fig. 4B); PAR2 silencing abrogated these effects. We also quantified the lamellipodia and determined that in the control untreated cells, ~0.5 lamellipodia were present per cell, while when challenged with FVIIa the number of lamellipodia tripled to 1.7 lamellipodia per cell (Fig. 4C). PAR2 silencing abrogated this increase in lamellipodia. The area of the lamellipodia in the control cells was ~20 μm²/cell and increased 3.5-fold to 70–75 μm²/cell when challenged with FVIIa; PAR2 silencing abrogated this increase. These data confirm that lamellipodia formation are dependent on PAR2 activation.

**FVIIa/TF-induced pCMVEC migration and tube formation.** Based on the evidence presented above, we determined the role of RhoA, cortactin and ΔC-TF on FVIIa/TF-induced endothelial migration. FVIIa-induced pCMVEC migration was abolished by silencing of RhoA and cortactin, but not by ΔC-TF (Fig. 5A). Since cell migration is required for endothelium to form tube-like structures, reminiscent of angiogenesis, we investigated the effects of FVIIa and trypsin and the respective roles of RhoA and cortactin on tube formations of TF-overexpressing pCMVECs placed in Matrigel. Tube-like structures were increased by FVIIa, SLIGRL, and trypsin in control TF-overexpressing pCMVECs but not in PAR2 shRNA-, RhoA shRNA-, and cortactin shRNA-treated cells (Fig. 5, B and C); hence FVIIa/TF-induced pCMVEC tube formation is PAR2, RhoA, and cortactin dependent.

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**Fig. 2.** TF is necessary for FVIIa-induced cleavage (activation) of PAR2. Flag-PAR2-HA was digested by FVIIa and followed by an ELISA assay on a FLAG Ab-coated plate. Experiments were performed on Flag-PAR2-HA/pcDNA3 transiently cotransfected pCMVECs with (white bars) or without (black bars) TF/pcDNA3; details of procedures are presented in MATERIALS AND METHODS. Bound Flag-PAR2-HA molecules were then digested with FVIIa (100 nM) or trypsin (20 nM) as indicated; PBS was used as control. Uncleaved Flag-PAR2-HA was detected by reacting with HA-FITC antibody, and measurement was performed in a fluorometer at 485/535 nm. Values are means ± SE of 4–6 samples per group. *P < 0.05 compared with the values without asterisks.

**Fig. 3.** A, shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA levels (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.

**Fig. 4.** A, PAR2 silencing decreased FVIIa-induced cortactin and RhoA expression (Fig. 3C). RhoA gene silencing diminished cortactin expression, while the cortactin shRNA knockdown only significantly downregulated cortactin expression but did not affect RhoA mRNA levels (Fig. 3C). A nonspecific sequence shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.

**Fig. 5.** A, PAR2 silencing decreased FVIIa-induced cortactin and RhoA expression (Fig. 3C). RhoA gene silencing diminished cortactin expression, while the cortactin shRNA knockdown only significantly downregulated cortactin expression but did not affect RhoA mRNA levels (Fig. 3C). A nonspecific sequence shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.

**Fig. 6.** A, PAR2 silencing decreased FVIIa-induced cortactin and RhoA expression (Fig. 3C). RhoA gene silencing diminished cortactin expression, while the cortactin shRNA knockdown only significantly downregulated cortactin expression but did not affect RhoA mRNA levels (Fig. 3C). A nonspecific sequence shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.

**Fig. 7.** A, PAR2 silencing decreased FVIIa-induced cortactin and RhoA expression (Fig. 3C). RhoA gene silencing diminished cortactin expression, while the cortactin shRNA knockdown only significantly downregulated cortactin expression but did not affect RhoA mRNA levels (Fig. 3C). A nonspecific sequence shRNA (NS shRNA) did not affect FVIIa and SLIGRL (PAR2-activating peptide) induction of cortactin and RhoA mRNA (Fig. 3C). Concordant expression of cortactin protein induced by FVIIa/TF (for 8 h) were observed on Western blot analysis of TF-expressing pCMVECs (Fig. 3D). Collectively, data suggest that FVIIa/TF-elicited PAR2 activation induces sequentially RhoA and cortactin.
Fig. 3. FVIIa/TF regulates cortactin, and RhoA expression. 

A: mRNA expression of HIF, cortactin, RhoA, Dia1, and Dia2 (by RT-PCR) in TF-transfected pCMVECs before (control) or after stimulation with 100 nM FVIIa for 60 min. HIF, cortactin, RhoA, Dia1, and Dia2 gene expression were detected by RT-PCR. B: effects of PAR2, RhoA, and cortactin shRNA gene silencing determined by RT-PCR in TF-transfected pCMVECs. Nonspecific (NS)-shRNA transfected cells were used as a transfection control. Gene expression was estimated densitometrically (right). C: effects of PAR2, RhoA, and cortactin silencing (using corresponding shRNA) on FVIIa-induced gene induction; the positive control was the nonspecific sequence shRNA (NS shRNA). For A–C, QuantumRNA Universal 18S (Ambion, Austin, Texas) expression was used as internal standard. D: cortactin protein expression in TF-transfected pCMVECs stimulated with FVIIa (100 nM) or SLIGRL (20 μM) for 8 h. β-actin was utilized as a control for total protein level. Densitometric analyses are a compilation of results from 3 independent samples. E: cortactin activation in pCMVECs was detected by determining its phosphorylation (top) using a specific polyclonal phospho-cortactin (Tyr421) antibody. Calculated values were normalized to corresponding cortactin levels. For A–E, the data presented in adjacent bar graphs to the right of bands are values of 3–5 independent experiments. *P < 0.01 compared with corresponding values without asterisks.
DISCUSSION

FVIIa/TF-induced angiogenesis occurs through activation of PAR2, which, in turn, induces mitogenesis and neovascularization (9, 10). Previously, angiogenic aspects of FVIIa/TF and PAR2 have suggested roles for DNA primerase 1 (13) and the tie2 receptor (51). Angiogenesis involves cell migration. However, the mechanisms of endothelial cell migration evoked by FVIIa/TF and PAR2 are not well understood. Results in the

Fig. 4. Cortactin activation and lamellipodia formation by FVIIa/TF in pCMVEC. pCMVEC were transfected with full-length TF (wTF; A) or COOH-terminal truncated TF (ΔC-TF; B), with or without PAR2 silencing (using shRNA PAR2). Twenty minutes after stimulation with FVIIa (100 nM) both brightfield microscopy and immunofluorescence microscopy were performed for cortactin and β-actin; nuclei were counterstained by DAPI. Nonstimulated (control) and AF555-stained samples were the negative controls. Note accumulation of cortactin on the extending cellular periphery (lamellipodia and protrusion formations) as indicated by arrows. C: number and area of the lamellipodia were quantified per cell as described in MATERIALS AND METHODS. The data is presented as means ± SE (n = 20–50 cells). *P < 0.05 calculated using a one-way ANOVA (H ratio is 33.6 for number/cell; H ratio is 29.4 for area/cell analysis) to both the control group and shRNA-treated groups.
present study reveal an unprecedented role for RhoA and cortactin in response to PAR2 activation in the process of lamellipodia formation and cell migration.

We hereby show RhoA to be the GTPase responsible for cortactin activation in endothelial cell-mediated migration in response to FVIIa/TF/PAR2 stimulation. Through the regulation of actin polymerization, Rho GTPases affect cellular polarity and mobility processes, such as exocytosis, endocytosis, phagocytosis, endoplasmic reticulum transport, and plasma membrane lamellipodia, ruffles, and filopodia (41). Among them, RhoA, Rac1, and Cdc42 are well known as the most important members of the GTPase family to stimulate actin filament formation via the ERK/WASP/WAVE/Arp2/3 complex pathway (41). Rac1 and Cdc42 have been previously well characterized in FVIIa/TF-induced signaling (11). Rho plays a key role in locomotion through effecting actin polymerization and is suggested to play a role in detachment of the cell, which occurs through interaction via Dia1 (18, 23, 36). Dia1 and Dia2 were reported as downstream proteins of Cdc42 and RhoA to contribute to actin nucleation (41). More recently, Dia2 was shown to be a mediator of actin filopodia in Cdc42 and Rif-induced filopodia (34, 35). But in response to PAR2 stimulation, Dia1 and Dia2 are not implicated; Dias are more closely linked to the processes of vesicle formation, such as exocytosis and endocytosis (41). A critical role for cortactin in FVIIa/TF-induced endothelial cell migration was also observed. Cortactin is a monomeric protein located in the cytoplasm. Cortactin activation through phosphorylation by Src initiates interaction with WASP and N-WASP to activate the Arp2/3 complex (28), which, in turn, promotes polymerization and rearrangement of the actin cytoskeleton around the cellular periphery, which leads to lamellipodia formation, an important process of cellular migration (41). FVIIa/TF increased cortactin gene and protein expression and its phosphorylation (Fig. 4) but also stimulated its relocalization from the cytoplasm to the plasma membrane to participate in cellular extension (lamellipodia and formation of protrusions; Fig. 4A).

An important feature of this study applies to the role of the COOH-terminal cytoplasmic domain of TF in FVIIa-induced migration. TF structure is functionally divided into three domains: extracellular domain, transmembrane domain, and cytoplasmic domain (21 amino acids). The extracellular domain is the FVIIa binding site, and currently it is assumed to be

Fig. 5. FVIIa-induced pCMVEC migration and tube formation. A: pCMVECs cotransfected with wTF or ΔC-TF and NS shRNA, PAR2 shRNA, RhoA shRNA, or cortactin shRNA were stimulated with FVIIa (100 nM) and cell migration was measured using the coverslip migration assay (see Supplemental Fig. S1). Cell migration was calculated as a relative fold change from nonstimulated (control) samples. Data are presented as means ± SE of 3–6 separate experiments. *P < 0.05 compared with values without asterisks. B: for tube formation, pCMVECs were cotransfected with TF and NS shRNA, PAR2 shRNA, RhoA shRNA, or cortactin shRNA as indicated. After starvation, the pCMVECs were seeded on a Matrigel-coated 24-well culture dish and then stimulated with FVIIa (100 nM) or SLIGRL (20 μM) for 6 h in a 37°C incubator. Random images (5–10 per sample) were captured with a phase contrast microscope at ×10 magnification. Examples of tube-like structures are depicted by arrows. Numbers of tube-like structures formed (representing early vessel formation) were counted for every 100 cells in each image. The panels were typical representative images of 3–4 independent samples each conducted in 5–10 images. Compiled data for the 3–4 samples are presented in C as means ± SE. *P < 0.01 compared with values without asterisks.

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involved in functional signaling (5). The transmembrane domain is the hydrophobic area to cross the membrane, and the cytoplasmic domain is thought to be involved in signal transduction (5). The cytoplasmic domain of TF was shown to be mandatory and necessary for FVIIa-induced porcine aortic endothelial migration toward platelet-derived growth factor (43), VEGF production for angiogenesis in melanoma cells (1), and breast carcinoma cell migration (19). This is in contrast to FVIIa-induced cortactin activation and lamellipodia-like cellular membrane extension, which was detected even in ΔC-TF transfected pCMVECs (Fig. 4); this suggests that the cytoplasmic domain of TF does not contribute to cortactin upregulation and cellular extension induced by FVIIa, but does infer that other TF domains are involved in this endothelial cell migration pathway, as TF is required for FVIIa actions. Interestingly, the lack of the cytoplasmic domain of TF has been described as not interfering with FVIIa-induced lamin-5 activity (46), which is believed to mediate the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. The signaling from the cytoplasmic domain of TF has been suggested to occur through the Rac and p38 kinase pathway (33). Whereas, ERK/MAP kinase activities are independent of the TF cytoplasmic domain (30, 44) and are reported to mediate cortactin regulation in vascular smooth muscle cell lamellipodia formation (15), Arp2/3 complex activity (28). Collectively, the data support our results that FVIIa-induced cortactin activation and cell lamellipodial extension is independent of the cytoplasmic domain of TF; on the other hand, ERK/MAP kinase may be involved in this process (30, 44).

We simplified the cell migration assay by developing a coverslip migration assay. It retains much of the advantages found in the scratch wound-healing assay (low cost and simplicity), while adding the advantages of reproducibility and ease of scale up. By creating a clear migration border and facilitating isolation of original cells from migrating ones, it offers an accurate alternative to determine cellular migration rates. In addition, because migrated cells can be analyzed by MTT, and therefore read by a colorimetric reader (ELISA), results can be rapidly obtained and tabulated. Most other migration assay methods [scratch wound and healing (14, 50), microcarrier bead migration assay (20), omnidirectional migration assay (12), phagokinetic track motility assay (2), Teflon fence assay (38), and under-agarose migration (12), phagokinetic track motility and healing (14, 50), microcarrier bead migration assay (20),] have been suggested to occur through the Rac and p38 kinase cascade. Cell Motil Cytoskeleton 11: 395–404, 1977.

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