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Endothelial-derived microvesicles promote pro-migratory cross-talk with smooth muscle cells by a mechanism requiring tissue factor and PAR2 activation

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Introduction: Microvesicles (MV) released by endothelial cells (EC) following injury or inflammation contain tissue factor (TF) and mediate communication with the underlying smooth muscle cells (SMC). Ser253-phosphorylated TF co-localizes with filamin A at the leading edge of migrating SMC. In this study, the influence of endothelial-derived TF-MV, on human coronary artery SMC (HCASMC) migration was examined.

Methods and Results: MV derived from human coronary artery EC (HCAEC) expressing TF_{wt} accelerated HCASMC migration, but was lower with cytoplasmic domain-deleted TF. Furthermore, incubation with TF_{Asp253}-MV, or expression of TF_{Asp253} in HCASMC, reduced cell migration. Blocking TF-factor VIIa (TF-fVIIa) procoagulant/protease activity, or inhibiting PAR2 signaling on HCASMC, abolished the accelerated migration. Incubation with fVIIa alone increased HCASMC migration, but was significantly enhanced on supplementation with TF. Neither recombinant TF alone, factor Xa, nor PAR2-activating peptide (SLIGKV) influenced cell migration. In other experiments, HCASMC were transfected with peptides corresponding to the cytoplasmic domain of TF prior to stimulation with TF-fVIIa. Cell migration was suppressed only when the peptides were phosphorylated at position of Ser253. Expression of mutant forms of filamin A in HCASMC indicated that the enhancement of migration by TF but not by PDGF-BB, was dependent on the presence of repeat-24 within filamin A. Incubation of HCASMC with TF_{wt}-MV significantly reduced the levels of Smoothelin-B protein, and upregulated FAK expression.

Discussion: In conclusion, Ser253-phosphorylated TF and fVIIa released as MV-cargo by EC, act in conjunction with PAR2 on SMC to promote migration and may be crucial for normal arterial homeostasis as well as, during development of vascular disease.

KEYWORDS

tissue factor, smooth muscle cells, endothelial cells, cell migration, microvesicles, filamin A, protease activated receptor-2, serine phosphorylation

1 Introduction

Vascular wall remodeling is an active and adaptable process that occurs in response to injury, inflammation and stress, in order to maintain the vascular integrity and appropriate blood flow. The regulation of these processes involves a vast number of factors including cytokines, inflammatory molecules, cell-cell interaction, extracellular matrix, extracellular vesicles as well as physical influences including sheer stress and external pressure;

A satisfactory review of these influences is beyond the scope of this study. Inappropriate signals contributed from these factors can result in hypertrophic, hypotrophic and also eutrophic remodeling of the blood vessel leading to multiple vascular disease conditions (1–6). Blood vessels are primarily comprised of endothelial cells (EC) and vascular smooth muscle cells (SMCs) and the interactions between these cell types is essential in the regulation of vascular tone, the response to injury, and contributes to vascular remodeling. Among the mechanisms of cross-talk involved, extracellular vesicles have emerged as important paracrine messengers (1, 6–10). The bidirectional exchanges of cell-derived microvesicles (MV) contribute to the vascular homeostasis and have been identified as major contributors to vascular abnormalities associated with a number of chronic conditions (11–15). Procoagulant microvesicles are a class of vesicles which are often released in response to injury, trauma, cytokines, infection and other inflammatory mediators (8). Among the pro-inflammatory mediators, the expression and release of tissue factor (TF) has been implicated as one that influences cells through coagulation-dependent and coagulation-independent mechanisms (16, 17). The expression of TF has been associated with phenotypic changes in SMCs (18). Such transformation from contractile to synthetic phenotype is associated with both homeostatic repair processes, and progression of vascular deformities (14). TF has been found within hyperplastic intima of the vasculature from atherosclerotic hearts, associated with the non-lipid rich plaques (19). Moreover, TF is reported to contribute to the progress of coronary artery disease (20, 21). Although under normal circumstances EC do not express TF, stimulation with inflammatory cytokines, endotoxins, hypoxia and oxidative stress, can induce the expression and release of TF as procoagulant microvesicles (22–25). In addition to the above, TF is exposed to the bloodstream through injury and trauma to the vasculature and may be released by other cells including macrophages or cancer cells. Microvesicles act as carriers for TF and factor VIIa (fVIIa), and this cargo may be recycled by EC (26, 27). The resultant endothelial-derived microvesicles may therefore contain both endogenous and recycled TF and fVIIa (28), and since TF-MV have been shown to accumulate within the sub-endothelial regions (29), these may come into contact with SMCs (30–34). Therefore any interaction with underlying cells may in turn activate protease activated receptors (PAR), as well as participating in PAR-independent TF-mediated signals resulting in various outcomes as reviewed previously (25). The contribution of PAR2 to phenotypic alterations in SMCs, and the promotion of SMC migration has also been reported (33, 34). Furthermore, the ability of extracellular vesicles as transporters of miRNA between endothelial and SMCs has been reported (35).

The involvement of actin-binding protein filamin A in vascular repair, remodeling and dysfunction has been well documented (36–38), and the contribution to SMC migration has been studied previously (39–42). TF has been shown to co-localize with filamin A at the leading edge of lamellipodia in migrating SMC and epithelial cells (19, 39) and the interaction of TF with filamin A was demonstrated over two decades ago (43, 44). This interaction was shown to be enhanced following the phosphorylation of the cytoplasmic domain of TF (43, 44). We recently showed that the interaction

with filamin A was specifically required the phosphorylation of serine 253 and did not include the phosphorylation of serine 258 (45). In this study, using mutants of TF, peptides corresponding to the cytoplasmic domain of TF, and various antibodies and coagulation factors, we examined the contributions of PAR2 activation and Ser253-phosphorylation within TF, on HCASMC migration. In addition, by expressing mutants of filamin A lacking selected repeat domains in HCASMC, the contribution of the filamin A to TF-induced HCASMC migration was further examined.

2 Material and methods

2.1 Culture of coronary artery endothelial cell and smooth muscle cell

Human coronary artery endothelial cells (HCAEC) were obtained from PromoCell (Heidelberg, Germany) isolated from left and right main coronary arteries, circumflex and anterior coronary arteries and were CD31 and Dil-Ac-LDL uptake positive. The cells were cultured in EC-MV media containing 5% (v/v) FCS and growth supplements (PromoCell) and remained CD31 positive. Human coronary artery smooth muscle cells (HCASMC) were also isolated and provided by PromoCell as smooth muscle α -actin positive, CD31 negative cells. The cells were cultured in SMC-MV2 media containing 5% (v/v) FCS and growth supplements (PromoCell) and were tested for the expression of smoothelin-B which is a marker associated with contractile SMCs from muscular arteries (see discussion for details). Both primary cell types were guaranteed for >15 divisions and were used at around 9 divisions. ECV304 cell line was cultured in M199 medium containing 10% (v/v) FCS.

2.2 Preparation of TF and filamin A mutants

The sequence for full length filamin A was cloned into pcDNA 3 c-myc plasmid and stop codons were engineered into positions Gly2370, Gly2467 and Ala2595 in order to delete the repeat units 22–24, 23–24, or 24 alone. The successful mutations were verified by DNA sequencing (MWG-eurofins, Wolverhampton, UK). The pCMV6-Ac-TF-tGFP plasmid for the expression of full-length wild-type human TF (TF_{WT}-tGFP) was obtained from OriGene (Rockville, USA) and mutations were prepared as previously described (46) to express proteins with Ser253Asp (TF_{Asp253}-tGFP), truncated at Ser241 (TF_{ΔCT}-tGFP), or tGFP alone.

2.3 Transfection of HCAEC and induction and isolation of MV

Human coronary artery endothelial cells (HCAEC) were used throughout the investigation because these cells are the closest representatives to the aims of this investigation. In contrast to

monocytes or tumor cells, these cells do not either constitutively express TF and/or release large amounts of microvesicles spontaneously. Furthermore, unlike tumor cell derived microvesicles, the resultant microvesicles do not contain other material that alter the behavior of vascular cells (e.g., pro-angiogenic mediators). HCAEC have also been shown to express PAR2 on the cell surface (47). HCAEC (5×10^5) and also in some experiments ECV304 cells, were transfected with 0.5 μ g of pCMV6-Ac-TF-tGFP plasmid variants using TransIT-2020 (GeneFlow, Litchfield, UK) according to the manufacturer's instructions and permitted to express the proteins for 48 h prior to use. The expression of TF was previously confirmed by measuring TF mRNA and measuring the total and cell-surface antigen levels by western blot and flow cytometry (46, 47). All cell samples, including the non-transfected cells, were then transferred and adapted to serum-free medium for 1 h. The release of TF-containing microvesicles was induced by incubation with PAR2 activating peptide (PAR2-AP); SLIGKV (20 μ M; Sigma Chemical Company Ltd, Poole, UK). Following 80 min incubation with PAR2-AP, the conditioned media were cleared of any cell debris by centrifuging for 10 min at 2,500 g on a microcentrifuge. The samples (1 ml aliquots) were then placed in 11 \times 34 mm polycarbonate centrifuge tubes (Beckman Coulter, High Wycombe, UK), and the cell-derived microvesicles were sedimented at 100,000 g on a TL-100 ultracentrifuge at 20°C, using a TLA 100.2 rotor (Beckman) for 1 h, as described before (46, 48). Sedimented microvesicles were washed with PBS. The pellet was resuspended in pre-filtered (0.1 μ m) PBS (200 μ l), divided into batches, and frozen at -80°C or used immediately. The specificity of the agonist in the incorporation and release of TF was previously demonstrated and compared to the ability of factor Xa to promote the release of TF-containing microvesicles (46). Additionally we showed that freezing of the microvesicles did not alter the microvesicle size or number as determined by nanoparticle tracking analysis (48), the TF antigen levels (46, 47) or the TF associated thrombin generation and factor Xa-generation. Furthermore, pre-incubation of the microvesicles with antibodies against TF, or immune-purification of the microvesicles using TF antibodies did not alter the size distribution of the microvesicles (Supplementary Figure S1), or the total TF content (48).

Previously, the microvesicles released following PAR2-AP activation were compared to those released following activation with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) (46) and microvesicles released from THP-1 cells, isolated peripheral blood mononuclear cells (PBMC), or HCASMC, in parallel (46, 47). The properties of TF-containing microvesicles from these various sources prepared by different procedures were established previously; in particular the numbers and size distributions of the isolated microvesicles were determined by nanoparticle tracking analysis (NTA) using Nano-Sight LM10 and NTA software (NanoSight Ltd, Amesbury, UK). The Nanosight instrument was calibrated using FluoSpheres[®] carboxylate-modified microspheres with diameters of 0.1 μ m and 1.0 μ m (Invitrogen). Microvesicle samples were diluted 1:5 in 0.1 μ m-filtered PBS and analyzed using the NanoSight LM10 by

tracking particles over 60 s using a camera level of 12 and shutter speed of 21.26 frames/s. A typical NTA trace for microvesicles is included in Supplementary Figure S1. Additionally, size and density of the TF-containing microvesicles were established previously by density gradient centrifugation (48) and the phospholipid content assessed by thin layer chromatography (46). Importantly, the density of the released microvesicles was measured by the concentration of phosphatidylserine, which was determined using the Zymuphen MP-assay kit (Hyphen BioMed/ Quadratech, Epsom, UK) against the standards provided with the kit. This function is the most relevant to this study. The TF content of the microvesicles was also measured by flow cytometry (46–48), TF-specific ELISA (Quantikine, R&D Systems, Abingdon, UK) and western blot (49). Although on the whole the resultant microvesicles were comparable in TF content and microvesicle identity, repeated studies in our laboratory has indicated the release of more consistent TF-containing microvesicles following activation by PAR2-AP and therefore this procedure has been adopted by our laboratory as the most consistent and reproducible procedure for preparing TF-MV (45–49). Previous examination of the microvesicles for exosome markers Tsg101 and CD9 by western blot showed that exosomes were not present, which was likely due to the short incubation times (26, 46, 47). Similarly, exosome marker antibodies (anti-Tsg101 or CD9 antibodies) were not capable pulling down TF-microvesicles (48).

2.4 Transfection of HCASMC

HCASMC were transfected with the pcDNA 3 c-myc constructs using TransIT 2020 reagent to express the wild type or mutant forms of filamin A, lacking repeats domains (22–24, 23–24 and 24 alone). The cells were permitted to express the proteins for 48 h and then employed in migration assays as below. The expression of the proteins was confirmed using a biotin-conjugated mouse anti-c-myc antibody (Miltenyi Biotech Ltd., Woking, UK), probed with HRP-conjugated streptavidin (Cytivia Life Science/ Fisher Scientific UK Ltd., Loughborough, UK) and developed using TMB stabilized substrate (Promega Corp., Southampton, UK). Parallel sets of samples were also examined using a rabbit anti-filamin A antibody (EP2405Y) (Epitomics Inc, Burlingame, CA, USA). The cells were then probed with goat anti-rabbit-alkaline phosphatase antibody (Santa Cruz Biotechnology) and developed using Western Blue substrate (Promega). In some experiments, HCASMC were transfected using TransIT-2020, to express TF-tGFP variants as described for the EC. In other experiments, HCASMC were transfected with a set of peptides corresponding to the cytoplasmic domain of TF with different serine-phosphorylation patterns (50), as stated in the results section. Briefly, the peptides (0.5 μ g) were diluted in PBS (100 μ l) and Chariot reagent (6 μ l; Active Motif, La Hulpe, Belgium) was also diluted in PBS. The two reagents were then mixed and incubated for 30 min. HCASMC were adapted to serum-free medium (400 μ l) for 1 h before and the Chariot-peptide mix (200 μ l) was added to the

cells. The cells were incubated for a further 60 min, the medium replaced and used in the experiments.

2.5 Treatment of MV and HCASMC with antibodies, proteins and other reagents

In some experiments, the isolated MV were pre-incubated for 1 h, with a mouse anti-human-TF antibody, 10H10 (20 µg/ml; BD Bioscience, Wokingham, UK) capable of blocking TF signaling, a mouse anti-human-TF antibody, HTF-1 (20 µg/ml; eBioscience/Thermo Scientific, Warrington, UK) to block TF-fVIIa protease/procoagulant activity, an inhibitory polyclonal rabbit anti-human fVIIa antibody (10 µg/ml; Abcam, Cambridge, UK) or the respective control isotype IgG antibodies (20 µg/ml; New England Biolabs, Hitchin, UK). The MV were then added to the lower chamber in migration assays as below. Alternatively, HCASMC were treated with a mouse anti-human PAR2 antibody, SAM11 (20 µg/ml; Santa Cruz Biotechnology, Heidelberg, Germany), capable of blocking PAR2 signaling, or PAR2-agonist peptide (PAR2-AP) to induce PAR2 signaling. SAM11 antibody was added with the HCASMC in the upper chamber at the start of the experiment and PAR2-AP was added to the lower chamber. PDGF-BB and PDGF-AA (10 ng/ml) were also added to sets of cells, in the bottom chamber as positive and negative controls, respectively. Additionally, to examine the influence of TF alone, and the contributions of other factors, HCASMC were stimulated with combination of recombinant Innovin TF (0–260 pg/ml; Dade Behring, Deerfield, USA), fVIIa (3 nM; Enzyme Research Lab., Swansea, UK), fXa (6 nM; Enzyme Research Lab.) and tissue factor pathway inhibitor (TFPI; 1 nM; American Diagnostica/Axis-Shield Ltd, Dundee, UK). To examine the contribution of activation of FAK, HCASMC were pre-incubated with either FAK inhibitor-14 (0–100 µM; 1,2,4,5-benzenetetraamine tetrahydrochloride; Sigma Chemical Company Ltd, Poole, UK) to inhibit FAK autophosphorylation, or AG82 {2-[(3,4,5-trihydroxyphenyl)methylene]-propanedinitrile}; (0–10 µM; Calbiochem/Merck-Millipore, Gillingham, UK) that prevents receptor-kinase mediated p125FAK tyrosine phosphorylation. Other sets of cells were pre-incubated with calyculin-A (1 nM) in order to prevent TF de-phosphorylation.

2.6 HCASMC migration assays

We have previously shown that exogenous-TF can become incorporated into the recipient cell membrane (26, 28, 47, 51). The HCASMC migration assay relies on the ability of cells to traverse a membrane to the lower chamber using Boyden chambers (8 µm pore size) (VWR International Ltd., Leicestershire, UK). For each assay, identical number of cells (approximately 3×10^4 in 250 µl of complete SMC-MV2 media) were placed in the upper chamber. If required, the cells were supplemented with antibodies directed towards the cellular receptors (e.g., SAM11), or other reagents. Complete media (250 µl) was placed in the lower chamber

containing the test reagent as described above and indicated in the results section. The chambers were incubated at 37°C for 18 h. The cells were then fixed with glutaraldehyde (3% v/v), washed with PBS and then, the cells on the upper side of the chamber were scraped off. To quantify the number of migrating cells, the scraped chambers were stained with crystal violet solution (Active Motif) for 30 min, washed and 10 fields of view were photographed. The cell-associated crystal violet was then released from the cells by incubation with 1% (w/v) SDS (200 µl) for 20 min. The absorptions were then measured at 595 nm and migrated cell numbers were determined from a standard curve prepared using HCASMC. In some experiments, the chambers were washed and stained with haematoxylin for visualization and 10 fields of view were counted manually or subsequently stained with crystal violet as above. For clearer demonstration of HCASMC migration, alternate images have been presented in the results section displaying cells stained with crystal violet at $\times 4$, or at $\times 10$ magnification.

2.7 RNA isolation, RT-PCR and western blot analysis

Total RNA was isolated using the Monarch total RNA extraction kit (New England Biolabs, Hitchin, UK) from 2×10^5 cells and 100 ng of total RNA was used for each reaction. The relative amounts of FAK mRNA was determined using the primer (forward 5'-AATCCTGGAGGAAGAGAAGGC-3'; reverse 5'-TGTTGCTGTCGGATTAGACGC-3') and semi-qualitative analysis carried out in comparison to β -actin (forward 5'-CCAGAGCAAGAGAGGCATCC-3'; reverse 5'-CTGTGGTGGTGAAGCTGTAG-3'). One-step RT-PCR reaction was carried out with 100 ng of isolated total RNA using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Inc., Giles, UK) for 30 cycles for FAK or 18 cycles for β -actin. RT-PCR products were visualized on a 2% (w/v) agarose gel, stained with SYBR Green I (FMC BioProducts, Rockland, USA).

Western blot analysis was carried out to determine the amount of Smoothelin-B protein in the HCASMC. Cells (10^5) were lysed in loading buffer and electrophoresis was carried out on a denaturing 8% (w/v) polyacrylamide gel, transferred to a nitrocellulose membrane and blocked with Tris-buffered saline Tween 0.01% (v/v) (TBST) pH 8. The membranes were probed overnight with a rabbit anti-human Smoothelin antibody (diluted 1:3,000 v/v; Abbeva, Cambridge, UK), or a goat anti-human GAPDH (V18) antibody (diluted 1:3,000 v/v; Santa Cruz Biotechnology, Heidelberg, Germany). The membranes were then washed and developed with goat anti-rabbit, or donkey anti-goat alkaline phosphatase-conjugated antibodies (Santa Cruz), diluted 1:3,000 (v/v), and visualized using the Western Blue stabilized alkaline phosphatase-substrate (Promega).

2.8 Statistical analysis

Presented data include the calculated mean values \pm the calculated standard error of the mean, and the number of experiments is stated in each column. Statistical analysis was

carried out using the GraphPad Prism version 9.0 (GraphPad Software, Boston, Massachusetts USA). Significance was determined using one-way ANOVA (analysis of variance) and Tukey's honesty significance test.

3 Results

3.1 TF-containing MV derived from HCAEC, and recombinant TF-fVIIa enhance HCASMC migration

HCASMC were incubated with MV isolated from HCAEC expressing TF_{WT}-tGFP, TF_{Asp253}-tGFP, TF_{ΔCT}-tGFP, tGFP alone, or from non-transfected cells (control MV). The incorporation of TF-tGFP into the cells and interaction with filamin A (probed with EP2405Y antibody) was monitored qualitatively, by confocal microscopy (Supplementary Figure S2). In addition, the association of TF-tGFP with the c-terminal of filamin A was confirmed *in situ* by proximity ligation assay as before (28, 45), and using a rabbit monoclonal antibody against the c-terminal of filamin A (EP2405Y), in conjunction with first a mouse monoclonal antibody to TF (10H10) and also, with a mouse monoclonal antibody to tGFP protein (2HB, OriGene) (Supplementary Figure S3). The MV were isolated using an established ultracentrifugation procedure, confirmed and quantified as before (48). Selected experiments were repeated using MV derived from transfected ECV304 line. These cells do not express TF under normal conditions but the transfected cells express the recombinant protein efficiently, and the cells release large quantities of MV on PAR2 activation. Typically, the yield of MV ranged between 0.25–0.31 nM and the concentration of TF in the MV from cells that expressed TF-tGFP was between 1 and 3 ng/ml. For the HCASMC migration experiments, 0.5 pg/ml (or equivalent amount of control MV) was placed in the bottom chamber of the Boyden chamber. Additional sets of HCASMC were supplemented with PDGF-BB or PDGF-AA which was added to the bottom chamber, as positive and negative controls, as well as sets of non-treated sample. The reagents were placed in the bottom chamber and the rate of cell migration across the Boyden chamber measured. Incubation of HCASMC with MV containing TF_{WT}-tGFP resulted in increased cell migration compared to cells expressing either tGFP alone, or the control MV. The increases were comparable to but lower than those obtained using the positive control (PDGF-BB) (Figures 1A,B and Supplementary Figure S4). However, the rate of migration was lower with TF_{ΔCT}-tGFP indicating that the cytoplasmic domain of TF is essential for full effectiveness of the signal. Interestingly, incubation of cells with MV containing TF_{Asp253}-tGFP resulted in reduced cell migration to levels comparable to the negative control (PDGF-AA).

In addition, HCASMC were incubated with combinations of recombinant TF, fVIIa, fXa and TFPI. Incubation of cells with the combination of TF and fVIIa resulted in increased cell migration but was not significantly altered by supplementation with fXa (Figures 2A,B). In contrast, neither concentration of

recombinant TF was capable of promoting HCASMC migration in the absence of fVIIa. Furthermore, stimulation with fVIIa alone was less effective in inducing HCASMC migration while fXa alone was only marginally effective. In accordance with previous studies, HCASMC expressed endogenous TF but the levels were low in our study which is also in agreement with the levels reported in healthy vessels (52–55). However, neither the zymogen nor activated fVII/VIIa were detected in HCASMC when examined by western blot (not shown), using a previously verified polyclonal anti-fVIIa antibody (56). Moreover, inclusion of recombinant TFPI reduced the induction of migration. In an attempt to examine the state of HCASMC, the expression of the Smoothelin was examined by western blot analysis. Smoothelin-B was shown to be present in resting HCASMC but not following treatment with TF-fVIIa (Figure 3). However, other markers were not examined in this study. Similarly, the expression of Smoothelin-B was suppressed in HCASMC treated with MV containing TF_{Asp253}-tGFP, or with PDGF-BB, was partially reduced in cells treated with MV containing TF_{ΔCT}-tGFP but was not altered in cells treated with tGFP-MV (Figures 3C,D).

3.2 PAR2 activation is essential for the induction of TF-mediated HCASMC migration

To further examine the underlying mechanisms of induction of HCASMC migration by TF, MV were isolated from HCAEC and ECV304 cell line that were transfected to express TF_{WT}-tGFP, and then pre-incubated with an inhibitory polyclonal antibody against fVIIa (56, 57), a monoclonal anti-TF antibody capable of inhibiting the procoagulant activity (HTF-1) (58), or a monoclonal antibody that blocks TF-mediated signaling (10H10) (59), before adding the MV to the lower chamber. These antibodies are directed to the extracellular domain of TF and fVIIa also associates with the extracellular domain. Alternatively, the HCASMC were pre-incubated with a monoclonal antibody capable of blocking PAR2 activation and signaling (SAM11). Appropriate isotype antibodies were analyzed alongside according to the species of the antibody used. The inclusion of these antibodies did not alter the cell migration by TF-MV, and the isotypes IgG antibodies showed no influence otherwise (Figure 4). The effectiveness of TF-tGFP-containing MV to induce HCASMC migration was largely abolished on blocking PAR2 signaling by pre-incubation of HCASMC with SAM11 (Figures 4A,B and Supplementary Figure S5). Similarly, neutralization of TF-fVIIa protease activity, by pre-incubation with an inhibitory anti-fVIIa antibody, or incubation with HTF-1 antibody significantly reduced the rate of HCASMC migration while, the blocking of TF signaling using 10H10 antibody was ineffective. Stimulation of HCASMC with the PAR2 activating peptide (PAR2-AP, SLIGKV) or PAR1 activating peptide (PAR1-AP, SFLLRN) did not alter the rate of HCASMC migration, while pre-incubation of HCASMC with a monoclonal antibody to block β1-integrin completely abolished any cellular migration (not shown).

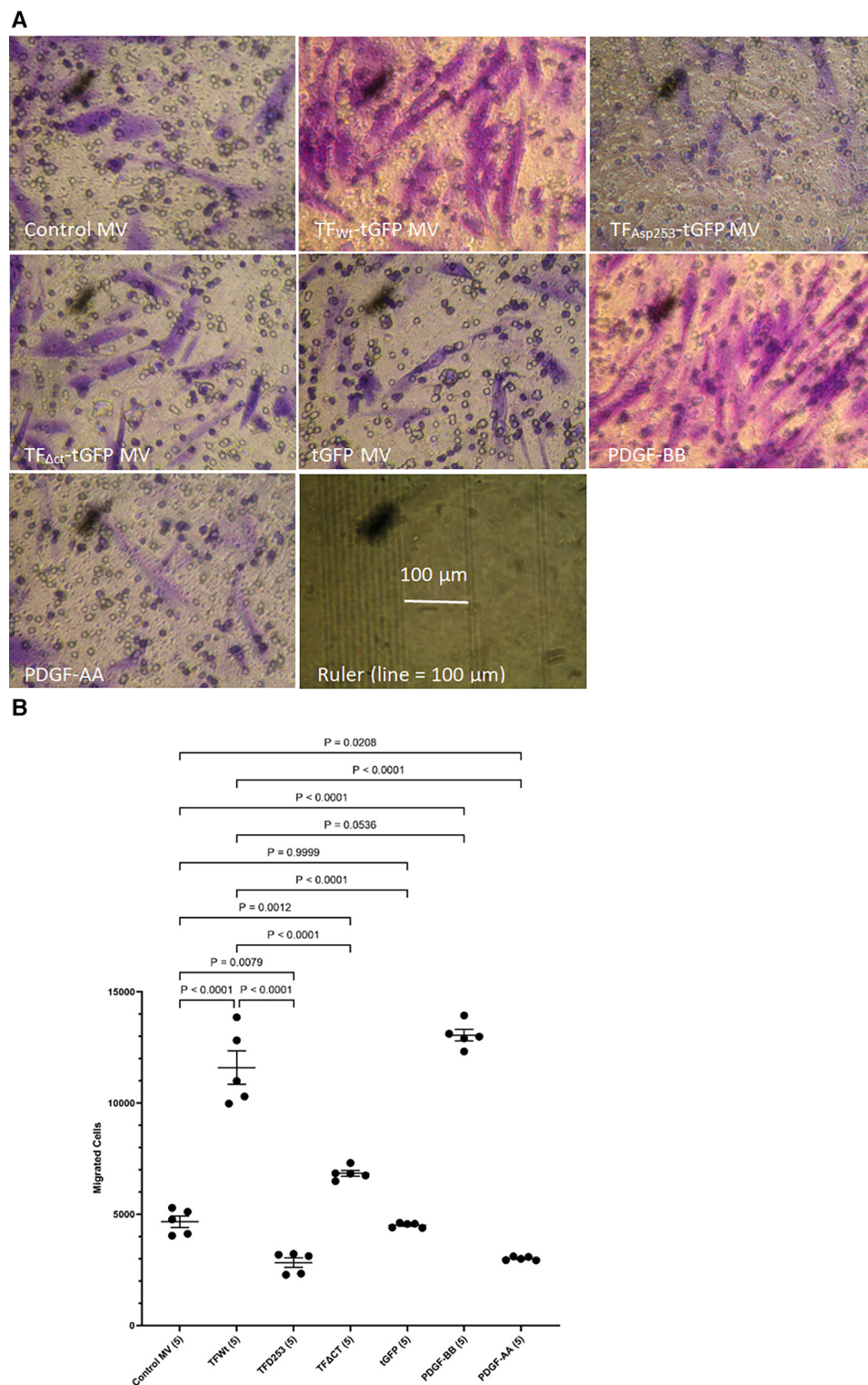


FIGURE 1
 Examination of HCASMC migration in response to TF-containing MV. MV were prepared from HCAEC (5×10^5) transfected to express TF_{WT}-tGFP, TF_{Asp253}-tGFP, TF_{Δct}-tGFP or tGFP alone. HCASMC (3×10^4 in 250 μl of media) were placed in the upper chamber of Boyden chambers. Complete media (250 μl), containing the isolated MV, was placed in the lower chamber and incubated at 37°C for 18 h. Additional sets of cells were incubated with PDGF-BB (10 ng/ml) or PDGF-AA (10 ng/ml) as positive and negative controls as well as non-treated samples. After incubation, the cells were fixed, washed with PBS and the cells on the upper side of the chamber were scraped off. (A) The cells were stained with crystal violet and photographed and (B) the numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values ± the calculated standard error of the mean, from 5 experiments.

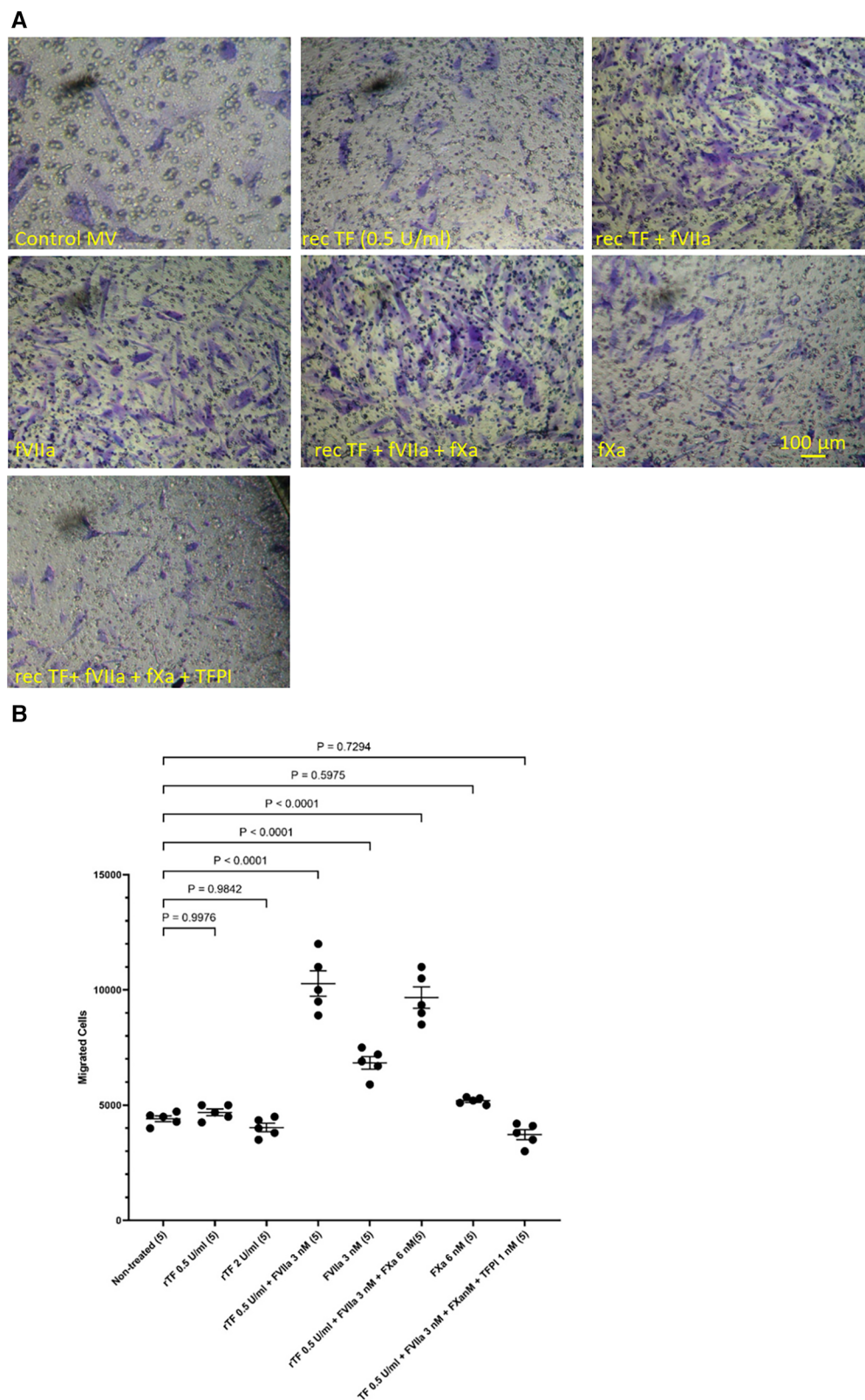


FIGURE 2 Examination of HCASMC migration in response to TF, fVIIa, fXa and TFPI. HCASMC (3×10^4 in 250 μ l of media) were stimulated with combinations of recombinant Innovin TF (0–260 pg/ml), fVIIa (3 nM), fXa (6 nM) and TFPI (1 nM) and incubated at 37°C for 18 h. The cells were then fixed, washed and the cells on the upper side of the chamber were scraped off. (A) The cells were stained with crystal violet and photographed and (B) the numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 5 experiments.

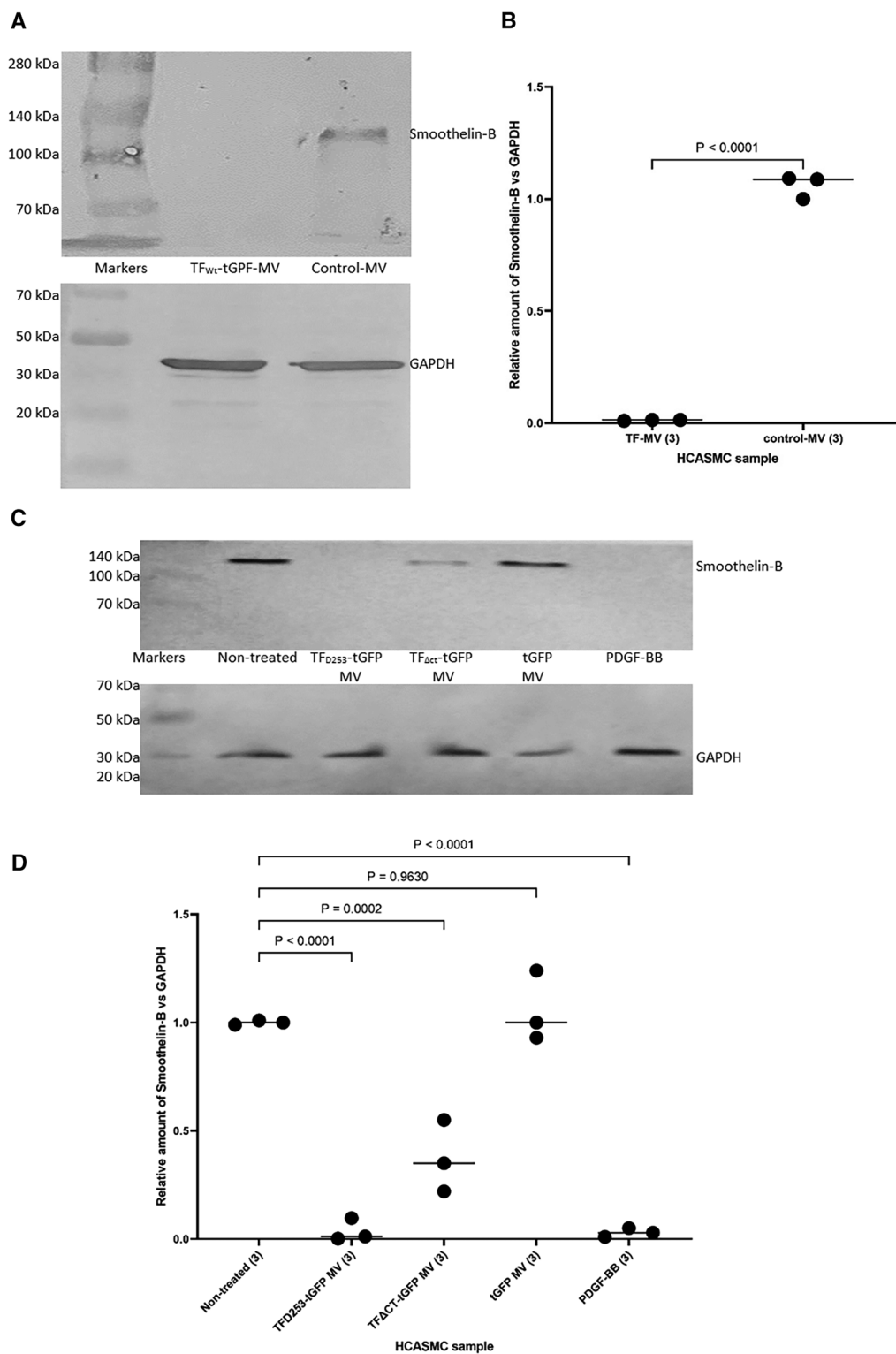


FIGURE 3

The influence of TF-FVIIa on the expression of Smoothelin-B in HCASMC. (A) HCASMC (10^5) were treated with MV prepared from HCAEC (5×10^5) transfected to express TF_{wt}-tGFP, or non-transfected cells. The cells were lysed and the proteins separated by 8% (w/v) polyacrylamide electrophoresis, transferred to nitrocellulose membranes, blocked and probed with a rabbit anti-human Smoothelin antibody (diluted 1:3,000 v/v), or a goat anti-human GAPDH (V18) antibody (diluted 1:3,000 v/v). The membranes were then washed and developed with goat anti-rabbit, or donkey anti-goat alkaline phosphatase-conjugated antibodies diluted 1:3,000 (v/v), and visualized using the Western Blue stabilized alkaline phosphatase-substrate. (B) The density of the Smoothelin-B bands was determined and expressed as a ratio of that of GAPDH in each sample. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 3 experiments. (C) Similarly, HCASMC were treated with MV containing TF_{ASP253}-tGFP, TF_{ACT}-tGFP, tGFP or incubated with PDGF-BB. (D) The expression of Smoothelin-B was then examined as above and the density of the Smoothelin-B bands was expressed as a ratio of that of GAPDH in each sample. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 3 experiments.

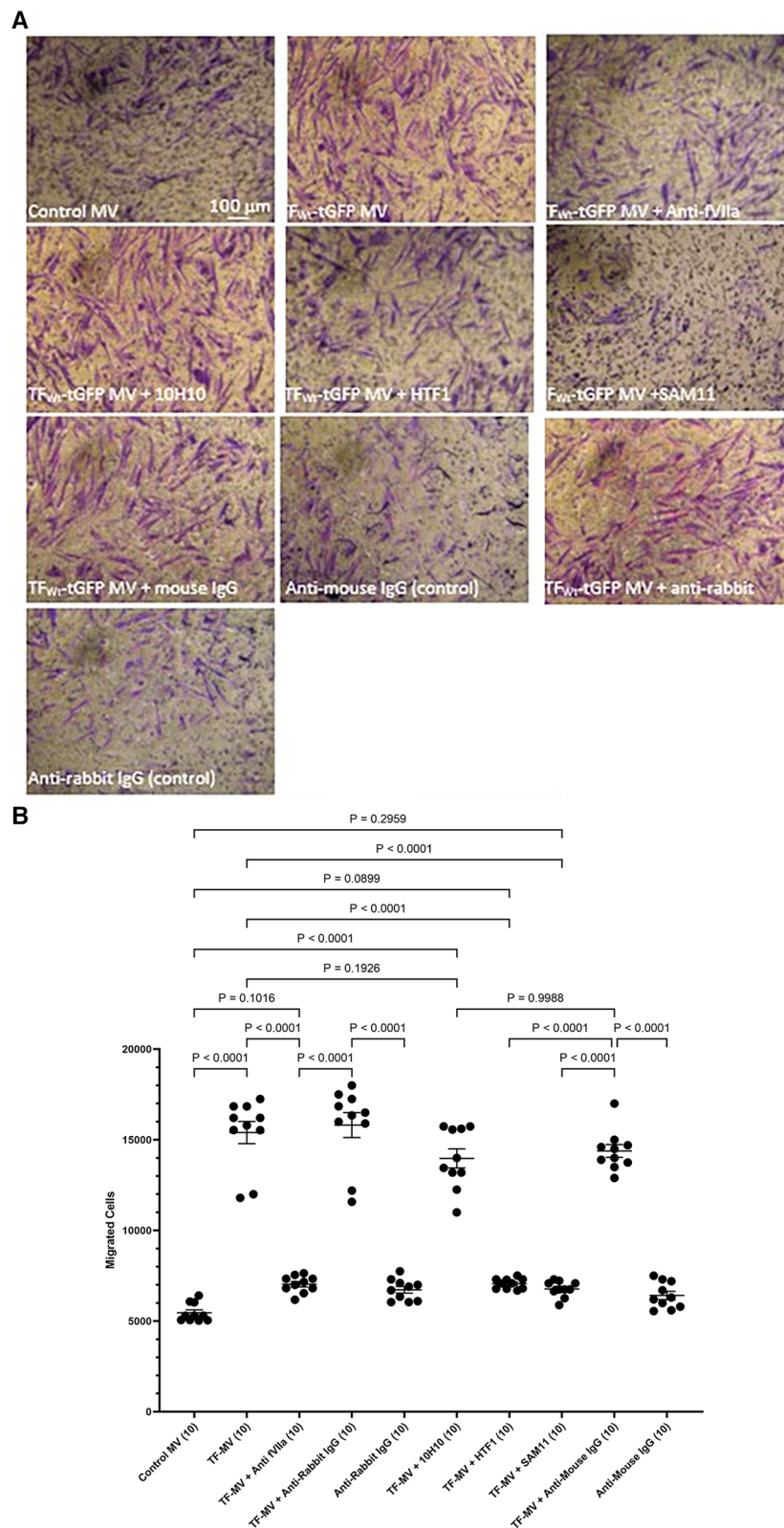


FIGURE 4
 Examination of the pro-migratory mechanism of TF-MV using blocking antibodies. MV were prepared from HCAEC (5×10^5) transfected to express TF_{WT}-tGFP and pre-incubated with mouse anti-human-TF antibodies, 10H10 (20 μg/ml) or HTF1 (20 μg/ml), or an inhibitory polyclonal rabbit anti-human fVIIa antibody (10 μg/ml). Additionally, HCASMC were incubated with an inhibitory mouse anti-human PAR2 antibody, SAM11 (20 μg/ml). HCASMC (3×10^4) were stimulated with MV at 37°C for 18 h. The cells were then fixed, washed and the cells on the upper side of the chamber were scraped off. (A) The cells were stained with crystal violet and photographed and (B) the numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values ± the calculated standard error of the mean, from 5 experiments each examined in duplicate.

3.3 Phosphorylation of Ser253 but not Ser258 is required for the enhanced HCASMC migration

In order to examine the role of TF phosphorylation, HCASMC were transfected with peptides corresponding to the 18 amino acids of the cytoplasmic domain of TF and the rate of migration examined as above. The transfection of a similar FITC-labelled peptide into the cells was confirmed by flow cytometry which indicated up to 85% transfection rate in both washed and unwashed cells and mean fluorescence intensity of up to 3 orders of magnitude (Supplementary Figure S6). Additionally, transfection of the peptides does not influence cell viability (46, 50). Transfection with either form of the peptide, incorporating a phosphorylated-Ser253 (pSer253 or pSer253/pSer258) resulted in significant reduction in the number of migrating cells in response to TF-fVIIa (Figures 5A,B). In contrast, the peptide incorporating phospho-Ser258 (pSer258) and the non-phosphorylated form were largely ineffective.

3.4 Induction of HCASMC migration is mediated through binding to filamin A

Phosphorylation of Ser253 is known to be essential for the interaction of TF with the repeat domain 22–24 within filamin A (43, 45). Additionally, TF is known to colocalize with filamin A at the leading edges of migrating SMCs (19). To further assess the contribution of filamin A to TF-mediated cell migration, HCASMC were transfected to express three truncated forms of filamin A without the repeat domains 22–24, 23–24 and 24 alone. The expression of the filamin A variants (filamin A_{WT}, filamin A_{Δ22–24}, filamin A_{Δ23–24} and filamin A_{Δ24}) was confirmed by western blot, probed using an antibody against the c-myc and anti-filamin A (Supplementary Figure S7). Moreover, the lack of any detrimental outcome on cell viability was established by determining the cell numbers in the transfected and control cells. HCASMC migration across Boyden chamber membranes was then stimulated with the HCAEC-derived MV containing TF_{WT}-tGFP, or devoid of TF (control MV). Removal of either set of repeat domains (22–24, 23–24 or 24 alone) resulted in significantly reduced HCASMC migration (Figures 6A,B). Next, whether repeat-24 is required specifically for TF-induced migration, or also affects other/non-specific migration mechanisms/processes, was examined. Expression of either filamin A_{WT} or filamin A_{Δ24} resulted in similar rates of migration in unstimulated HCASMC, cells stimulated with control MV, or in response to PDGF-BB (10 ng/ml) (Figures 6A,C). These were in contrast to the difference in migration observed on stimulation with TF-MV. Consequently, this study indicates that repeat-24 is likely to participate in TF-specific promotion of migration in HCASMC.

3.5 De-phosphorylation of TF at Ser253 permits HCASMC migration

While the incorporation of TF within the focal adhesion points may establish the direction of cell migration (44), focal adhesion

disassembly is vital for trailing edge retraction. In the above experiments the stimulation of HCASMC with MV carrying TF_{Asp253}-tGFP resulted in the retardation of cell migration. In addition, transfection of HCASMC with phospho-Ser253 peptide prevented the stimulation with MV carrying TF_{WT}-tGFP. To examine the involvement of TF-de-phosphorylation in chemotaxis, first PP2A phosphatase activity was inhibited in HCASMC (46). Pre-incubation of HCASMC with calyculin-A (1 nM) prior to stimulation with MV carrying TF_{WT}-tGFP severely suppressed TF-induced cell migration (Figures 7A,B). However, cell migration in response to PDGF-BB was also affected by inclusion of calyculin-A. Therefore, no definitive conclusions were derived from these experiments. To further explore the possibility that the TF-de-phosphorylation is required for migration, HCASMC were transfected to express TF_{WT}-tGFP, TF_{Asp253}-tGFP, or tGFP alone and the rate of cell migration in response to fVIIa, examined. Since unlike TF_{WT}-tGFP, TF_{Asp253}-tGFP retains the ability to interact with filamin A, the observed reduction in the rate of HCASMC migration suggests that TF-de-phosphorylation is likely to be essential as part of the chemoattractant or migratory mechanism (Figures 7C,D).

3.6 Induction of HCASMC migration requires focal adhesion kinase activity but is independent of receptor-kinase activity

The above data suggest that HCASMC migration in response to TF may be distinct to that induced by growth factor engagement. To further decipher the mechanisms involved in TF-mediated cell migration and to differentiate between the TF-induced and growth factor/cytokine-promoted cell migration, HCASMC were pre-incubated with either FAK inhibitor-14 (0–100 μM; 1,2,4,5-benzenetetraamine tetrahydrochloride) to inhibit FAK autophosphorylation, or AG82 that prevents receptor-kinase mediated p125FAK tyrosine phosphorylation. Preincubation of cells with FAK inhibitor-14 inhibited the TF-MV mediated SMC migration at both concentrations used. However, pre-incubation of the cells with AG82 (up to 10 μM) did not interfere with TF-fVIIa induced cell migration (Figures 8A,B) but reduced response to PDGF-BB (not shown). Furthermore, examination of the expression of FAK mRNA, in HCASMC following incubation with TF_{WT}-tGFP containing MV, indicated the upregulation of FAK expression at 18 h post-treatment (Figures 8C,D), but not on incubation with control MV.

4 Discussion

The importance of the communication between vascular endothelial and SMCs in the promotion and regulation of repair, following injury or trauma to the blood vessel has been established (11–15). This crosstalk has also been implicated in vascular remodeling through induction of synthetic SMC leading to increased proliferation and migration (6, 13, 14). Additionally, the contributions of cell-derived microvesicles (MV) to these

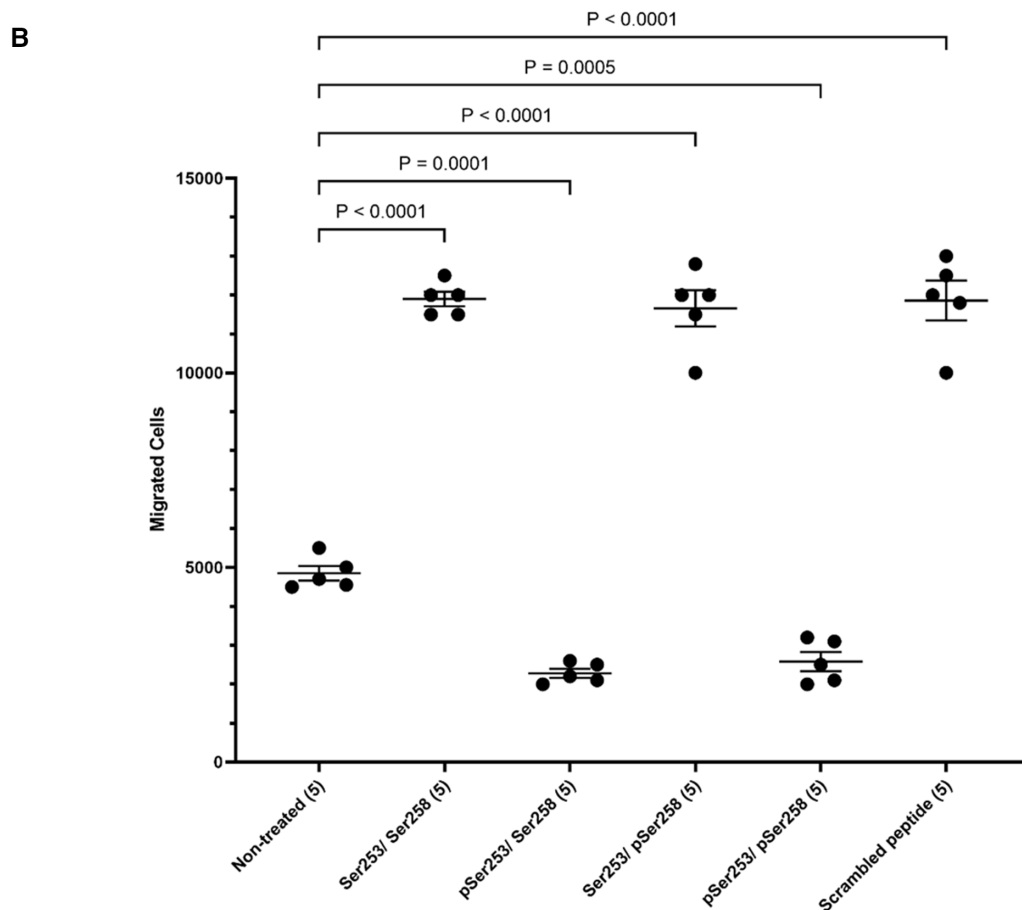
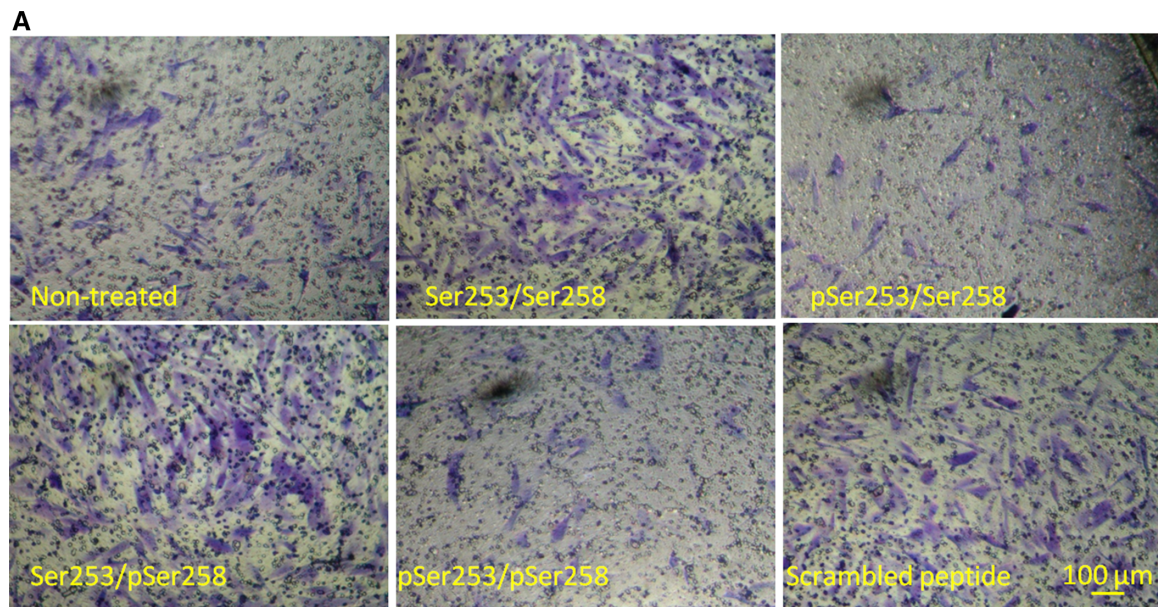


FIGURE 5
 Examination of the influence of TF phosphorylation on HCASMC migration. HCASMC were transfected with a set of peptides corresponding to the cytoplasmic domain of TF with different serine-phosphorylation patterns at the three serine residues. Migration was induced in the transfected HCASMC with TF-MV at 37°C for 18 h. The cells were then fixed, washed and the cells on the upper side of the chamber were scraped off. (A) The cells were separately stained with crystal violet and (B) the numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values ± the calculated standard error of the mean, from 5 experiments.

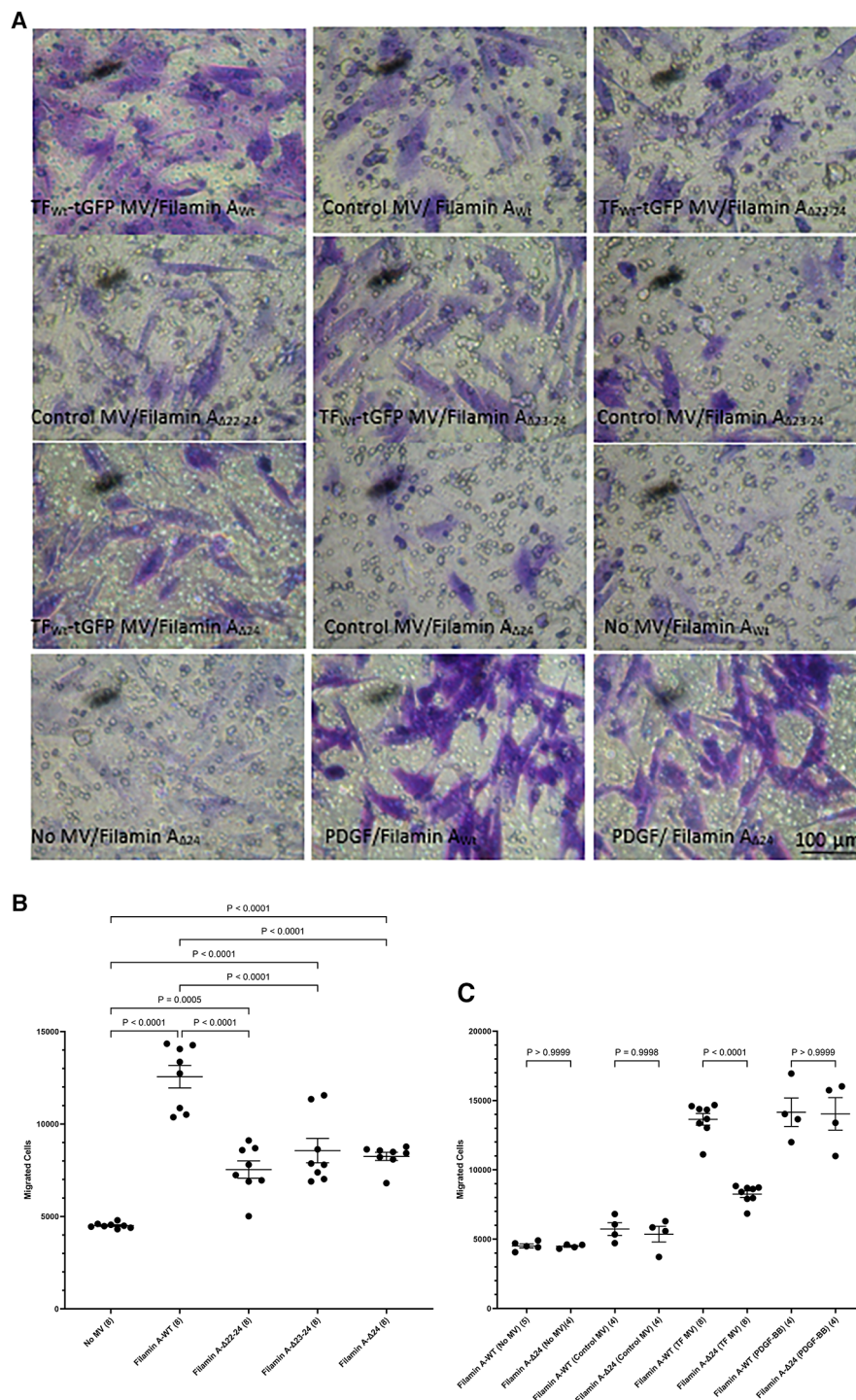


FIGURE 6

Examination of the requirement for filamin A repeats 22–24 for TF-induced HCASMC migration. HCASMC were transfected with the pcDNA 3 c-myc constructs to express the wild type or mutant forms of filamin A, lacking repeats domains (22–24, 23–24 and 24 alone) and permitted to express the proteins for 48 h. Cells (3×10^4 in 250 μ l of media) were placed in the upper chamber of Boyden chambers. Complete media (250 μ l), including MV containing TF_{WT}-tGFP, was placed in the lower chambers and incubated at 37°C for 18 h. Additional sets of cells were incubated with PDGF-BB (10 ng/ml) control MV (from non-transfected HCAEC), as well as sets of non-treated sample. The cells were then fixed, washed and the cells on the upper side of the chamber were scraped off. (A) The cells were stained with crystal violet and photographed. (B) The number of migrating cells were determined in HCASMC, in response to TF_{WT}-tGFP containing MV, and compared to control MV. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 4 experiments, each examined in duplicate. (C) The migration of HCASMC, expressing wild type filamin A, and filamin A lacking repeat-24, in response to TF_{WT}-tGFP containing MV, control MV, PDGF-BB (10 ng/ml), or without any supplement were determined. The rates of migration were measured by staining with crystal violet which was then eluted and absorptions measured at 595 nm. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 8 experiments for the samples treated with TF-MV, and 4 experiments for all other samples, as shown on each column.

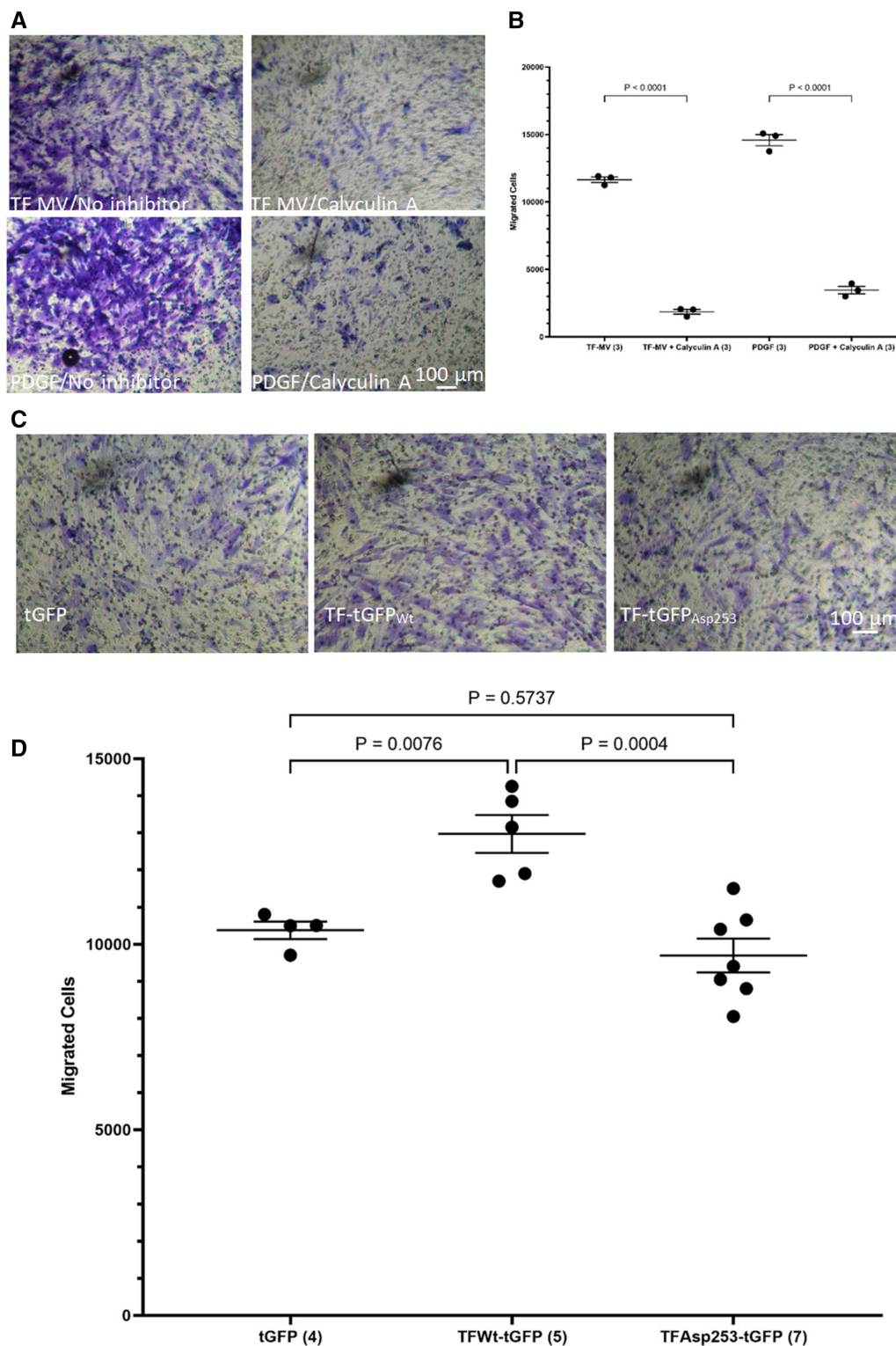
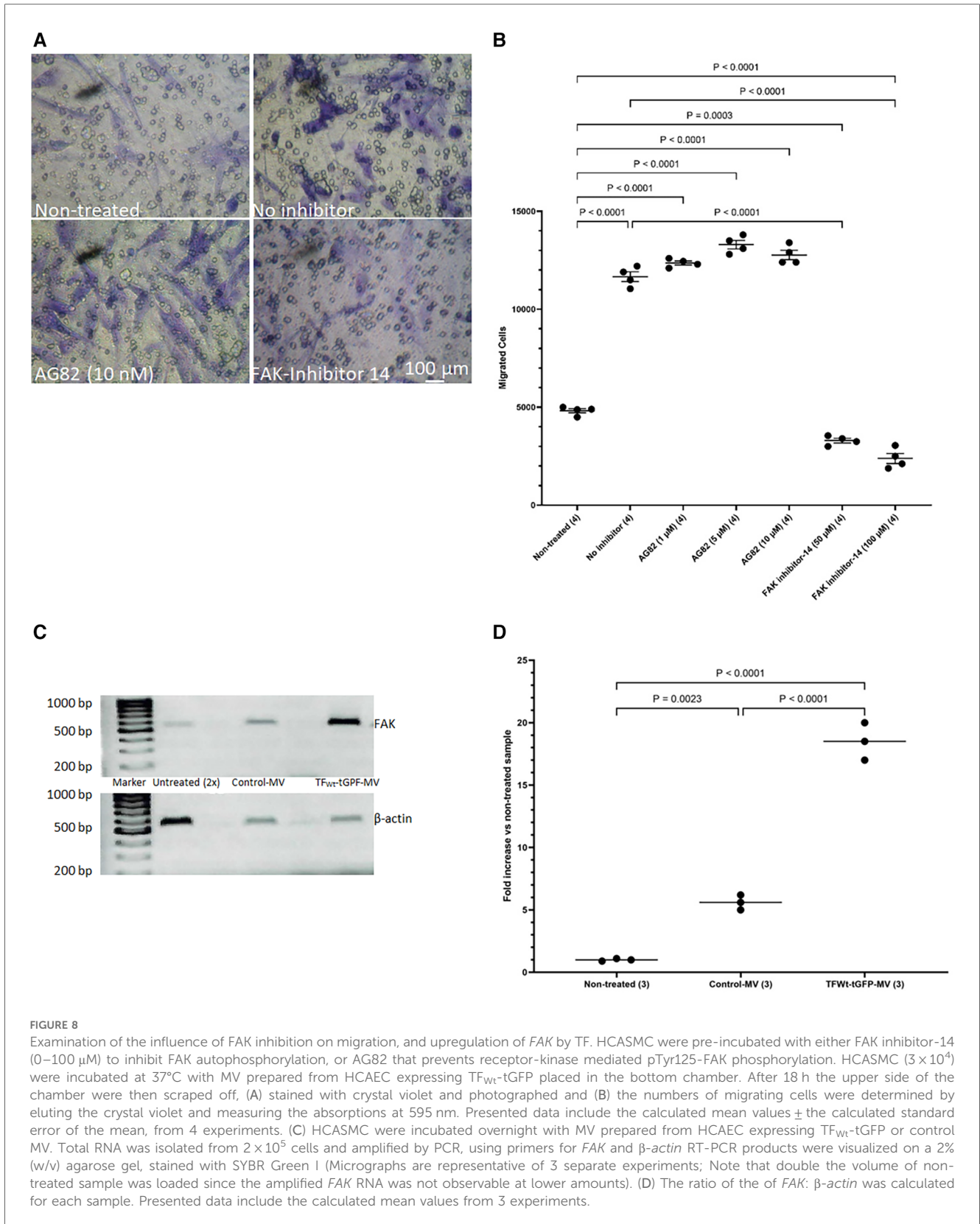


FIGURE 7

Examination of the influence of TF de-phosphorylation on HCASMC migration. HCASMC were treated with calyculin-A (1 nM) and cell migration stimulated with MV containing TF_{wt}-tGFP or PDGF-BB (10 ng/ml) at 37°C for 18 h. The cells on the upper side of the chamber were scraped off, stained with crystal violet and (A) photographed. (B) The numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values from 3 experiments. HCASMC were transfected to express TF_{wt}-tGFP, TF_{Asp253}-tGFP, or tGFP alone. Cell migration was induced by placing fVIIa (3 nM) in complete media (250 μ l), in the lower chamber and incubated at 37°C for 18 h. The cells were then fixed, washed and the cells on the upper side of the chamber were then scraped off. (C) The cells were stained with crystal violet and photographed and (D) the numbers of migrating cells were determined by eluting the crystal violet and measuring the absorptions at 595 nm. Presented data include the calculated mean values \pm the calculated standard error of the mean, from 4–7 experiments as shown at the base of each column.



processes has been highlighted recently and a subject of interest (1, 6, 8, 12, 15, 18). The endothelial layer is the site of early damage during disease and consequently, the arising inflammatory signals relay instructions to the underlying cells

within the intima. TF-containing microvesicles are released early as a means of initiating the coagulation mechanism following injury (1) but also act as means of promoting cellular processes leading to repair. However, prolonged exposure to TF has been

implicated in a number of conditions, including vascular diseases (22, 23). Different influences of TF-containing microvesicles derived from SMCs, on endothelial dysfunction have previously been reported (12, 15). As stated above, injury and inflammation stimulate vascular SMCs to switch into a de-differentiated synthetic phenotype. The increased rate of synthetic VSMC migration promotes vascular repair, but inappropriate migration/proliferation can also lead to vascular deformity. This study aimed to decipher some of the influences exerted by endothelial TF-MV on SMC mobility.

Our data highlights the potential of MV as carriers for TF, transferring this cargo to VSMC to promote TF-dependent cell migration. This mechanism involves PAR2 activation which appears to be mainly activated by fVIIa-TF complex, rather than by fXa, and agrees with previous reports (33, 34). Addition of fVIIa alone induced a lower rate of cell migration since HCASMC were shown to express low levels of TF (50, 52–54). Interestingly, in the absence of exogenous TF, direct activation of PAR2 was not sufficient to accelerate cell migration suggesting the low-level engagement of cellular TF. Inhibition of protease activity of TF-fVIIa complex, either by pre-incubation of MV with HTF-1 antibody, or an inhibitory anti-fVIIa antibody reduced the rate of HCASMC migration to that observed in non-treated cells. Similarly, blocking PAR2 activation/signaling by pre-incubation of HCASMC with SAM11 antibody reduced the rate of HCASMC migration. The interaction of fVIIa with TF appears to promote the co-localization with PAR2 (28) resulting in the activation of PAR2 (60) and in turn induces a diverse number of downstream signaling mechanisms (33, 34, 61, 62). The majority of the reports also implicate the activation of RhoA as an essential step in the promotion of cell migration (63–65). This ability of RhoA to promote cytoskeletal reorganization points to a chemotactic function for PAR2 signaling. However, feedback mechanisms from PAR2 activation are also known to induce the phosphorylation of the cytoplasmic domain of TF at serine 253, by protein kinase C (46, 66) which as stated above, also promote signaling mechanisms by interaction with β 1-integrin and through binding to filamin-A. However, blocking of the exosite on TF, using the 10H10 antibody had no influence on cell migration. Also unsurprisingly, inhibition of β 1-integrin using the AIIB2 antibody completely abolished HCASMC adhesion/migration and was attributed to the disruption of the adhesion complex and possibly independent of TF. Therefore, while it is likely that the interaction of TF with β 1-integrin contributes to cell migration by disrupting cellular adhesion to the extracellular matrix (67), the direct outcomes of signaling arising from this complex formation could not be established and was not pursued.

In contrast, deletion of the cytoplasmic domain leaving a procoagulant active TF significantly reduced the rate of HCASMC migration although the levels were above those observed in the non-treated control cells. Moreover, treatment of cells with recombinant TF was only capable of inducing migration when in conjunction with exogenous fVIIa. Together, these data suggest that the cytoplasmic domain of TF may act as a guide, determining the direction of the cell migration towards the point of contact with MV. Therefore, in agreement with

previous studies (19, 43) both the cytoplasmic domain of TF and PAR2 activation appear to be required for the efficient induction of HCASMC migration. In this model, the PAR2 signaling provides the components for chemokinesis while the interaction of TF with filamin-A permits directional chemotaxis.

As stated before, TF has been shown to co-localize with filamin A at the leading edge of lamellipodia in migrating SMC (19, 39). Furthermore, the interaction of TF with filamin A is enhanced following the phosphorylation of the cytoplasmic domain of TF (43, 44), and is specifically mediated through phosphorylation of serine 253 (45). To further explore the role of TF phosphorylation, peptides corresponding to the cytoplasmic domain of TF, which contained different phosphorylation patterns were used as competitors. The ability of TF-MV to induce HCASMC migration was completely abolished in cells transfected with peptides when phosphorylated at position of serine 253, and was irrespective of the state of serine 258. These data agree with previous studies identifying the phosphorylation of serine 253 as an essential step in recruitment by filamin A (43, 45), permitting the association of TF with filamin A at the leading edge of cells (19, 39). Furthermore, expression of mutant form of filamin A devoid of repeat-24 alone, was sufficient to disrupt the response of HCASMC to TF-MV, identifying this domain as responsible for TF-mediated HCASMC migration. Interestingly, deletion of repeat-24 did not influence the migratory response of HCASMC towards PDGF-BB. Moreover, inhibition of FAK phosphorylation through growth factor receptor kinase activity did not influence TF-induced HCASMC migration. Therefore, the interaction with repeat-24 of filamin A may be a specific mechanism, used by a few unconventional receptors, including TF. The proximity of the repeat-24 domains in filamin A dimers may also explain the previous observations of TF dimerization on cell surface (68).

Stimulation of HCASMC with MV containing aspartate 253-substituted TF (TF_{Asp253}-tGFP) to mimic phospho-serine 253 resulted in the retardation of cell migration. This was also confirmed by the reduction in the rate of HCASMC migration, on expression of TF_{Asp253}-tGFP, when compared to cells expressing the wild type TF. One possible explanation may be that while the phosphorylation of TF at the leading-edge of the cell promotes its recruitment within the focal adhesion points, de-phosphorylation of TF at the trailing-edge, may be essential for the disengagement and release. Increased RhoA activity is known to occur following PAR2 activation, and by TF-fVIIa complex (19, 63, 64). Combined RhoA and ROCK activity is associated with the tail retraction of migrating cells (69), while RhoA-signaling via formin mDia 1 leads to lamellipodia formation (70–72). It has therefore been suggested that too much RhoA activity at the leading edge can inhibit lamellipodia extensions (73, 74). Additionally, membrane blebbing has been associated with directional migration during development (69). It is possible that the interaction of TF with filamin A alters the membrane-to-cortex attachment, subsequently encouraging a bleb-initiated direction for the migrating cells (69, 73). In contrast, prolonged TF activity as observed with TF_{Asp253}, would significantly increase the rate of MV blebbing, and reduce the directionality of cell migration (69, 75). A better understanding the function of TF in VSMC migration

would require detailed investigations on the influence of MV release from the cell on its migration, the differential regulation of RhoA by PAR2, and the possible function of TF de-phosphorylation in the dismantling of the focal adhesion complexes.

The above observations, together with upregulation of *FAK* mRNA expression by TF-MV suggests the enhanced migration may include the transformation of the cell phenotype. We previously showed that the expression of TF_{Asp253} mutants in EC, improves cell viability (76) while expression of TF_{Ala253} to prevent phosphorylation, induced cell apoptosis. These processes involve the activation of Src but are independent of FAK phosphorylation (77). Other TF-induced phenotypic alterations have also been associated with FAK recruitment (78, 79). Therefore, alterations in one specific marker, expressed only in contractile HCASMC phenotype were explored. Smoothelin-A and -B are specific markers of SMCs and have been reported to differentiate between contractile or synthetic phenotypes (80, 81). Smoothelin-B is specifically expressed in the vascular SMCs, particularly associated with muscular arteries, is lower in elastic arteries, and is absent in the capillaries, pericytic venules and small veins (82). Importantly, Smoothelin-B is rapidly downregulated following vascular damage and/or compromise. The expression of Smoothelin-B is then restored upon successful completion of remodeling to healthy tissue. However, the levels of smoothelin-B have been shown to remain low during chronic conditions for example, the course of atherosclerosis, or the development of restenosis (83–87). In our study, incubation of HCASMC with MV containing TF-tGFP or TF_{Asp253}-tGFP almost completely eliminated the expression of vascular-specific Smoothelin-B while the truncated TF_{ΔCT}-tGFP was partially effective (Figure 3). These reductions were similar to those observed on incubation of HCASMC with PDGF-BB which is also known to suppress the expression of Smoothelin-B (88). The gene for Smoothelin can be differentially expressed as two separate transcripts (A & B). These are also regulated through separate repressor regions but the exact mechanisms are unclear (89). Therefore, while our data suggests a change in the cell morphology, a comprehensive study is required to establish the influence of TF-mediated signaling on VSMC phenotype markers. Therefore, we have refrained from speculating about cell morphological changes which are beyond the remit of the study.

In conclusion, this study has shown that Ser253-phosphorylated TF and fVIIa released as MV-cargo by EC in response to inflammation or injury, can act as messengers for rapid promotion of VSMC migration. The underlying mechanism requires both the activation of PAR2 by TF-fVIIa, as well as the interaction of TF with filamin A, involving repeat-24 on the latter protein. These pathways are initiated following incorporation of TF into the cell membrane but subsequently diverge. This mechanism ensures a rapid and proportional VSMC response, gauged to the level of inflammation conveyed by the endothelium, and appropriate for restoring normal arterial integrity/function. However, prolonged inflammatory response by the endothelium could contribute to the development of vascular abnormalities associated with chronic diseases.

Data availability statement

The original contributions presented in the study are included in the article/**supplementary materials**, further inquiries can be directed to the corresponding author.

Author contributions

SF: Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. CE: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2024.1365008/full#supplementary-material>

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