Filamin-A is required for the incorporation of tissue factor into cell-derived microvesicles

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Tissue factor incorporation into MVs

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Summary

We previously reported that the incorporation of tissue factor (TF) into cell-derived microvesicles (MVs) is regulated by the phosphorylation of the cytoplasmic domain of TF. Since the cytoskeletal protein filamin-A is known to bind to the cytoplasmic domain of TF in a phosphorylation-dependent manner, the involvement of filamin-A in the incorporation of TF into MVs was examined. Endothelial cells were transfected to express TF, whereas MDA-MB-231 cells were used to examine endogenously expressed TF. MV release was induced by activating protease-activated receptor-2 (PAR2). Partial suppression of filamin-A expression using two different filamin-A siRNA sequences resulted in significant reductions in the incorporation of TF antigen into MVs as determined by TF-ELISA and western blot analysis, and was reflected in reduced thrombin-generation and FXa-generation capacities of these MVs. Deletion of the cytoplasmic domain of TF also resulted in reduced incorporation of TF into MVs, whereas the suppression of filamin-A expression had no additional effect on the incorporation of truncated TF into MVs. Partial suppression of filamin-A expression had no effect on the number and size distribution of the released MVs. However, >90 % suppression of filamin-A expression resulted in increased MV release, possibly as a result of increased instability of the plasma membrane and underlying cytoskeleton. In conclusion, the presence of filamin-A appears to be essential for the incorporation of TF into MVs following PAR2 activation, but is not required for the process of MV formation and release following PAR2 activation.

Key words: Tissue factor, filamin-A, microvesicles, microparticles, endothelial cells

Introduction

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein which acts as the initiator of the extrinsic coagulation cascade by binding to coagulation factor VII/FVIIa. Various cells release TF-positive microvesicles (MVs), including endothelial cells (1,2), monocytes (3), smooth muscle cells (4) and cancer cells (5). TF has a small cytoplasmic domain which has no intrinsic kinase activity and which is not required for the procoagulant activity of TF (6). However, serine residues 253 and 258 within the cytoplasmic domain of TF are phosphorylated following the activation of protein kinase C (7). Recently we have shown that the incorporation of TF into MVs is regulated by the phosphorylation of serine residues 253 and 258 following PAR2 activation (8), but the underlying mechanism for this phosphorylation-dependent incorporation of TF into MVs is not known.

The release of MVs from cells is an active process that is induced by increases in intracellular calcium levels, which result in the loss of phospholipid asymmetry within the cell membrane and reorganisation of the cytoskeleton (9,10). The cytoskeletal protein filamin-A maintains the stability and structure of the actin cytoskeleton and also binds to and regulates the function and cellular localisation of many transmembrane and cytosolic proteins (11). Filamin-A is a 280 kDa actin-binding protein consisting of an N-terminal actin-binding domain and 24 Ig-like repeats (12). Filamin-A has been shown to bind to TF through a direct interaction between the cytoplasmic domain of TF and repeats 22-24 within the C-terminus of filamin-A (13). Furthermore, the phosphorylation of TF at serine residues 253 and 258 has been shown to enhance the binding of TF to filamin-A (13). Since the phosphorylation of TF at serine 253 also promotes the incorporation of TF into MVs (8), this study examined whether filamin-A is required for the incorporation of TF into MVs.

Materials and Methods

Cell culture, transient transfection and preparation of the truncated TF plasmid construct

Human coronary artery endothelial cells (HCAEC) and human dermal blood endothelial cells (HDBEC) (PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium MV (Promocell) containing 5% (v/v) foetal calf serum (FCS), 0.4 % (v/v) endothelial cell growth supplement, epidermal growth factor (10 ng/ml), heparin (90 µg/ml) and hydrocortisone (1 µg/ml) (PromoCell). The MDA-MB-231 breast cancer cell line was cultured in DMEM (Sigma Chemical Company Ltd, Poole, UK) containing 10% (v/v) FCS. Cells were maintained at 37°C under 5% (v/v) CO_2. Endothelial cells were transfected with 1 µg of pCMV-XL5-TF in order to express TF (OriGene, Rockville, USA) using Lipofectin (Invitrogen, Paisley, UK). Following transfection, the cells were incubated for 48 h at 37°C to allow the expression of TF. A truncated form of TF lacking the cytoplasmic domain was prepared by site-directed mutagenesis of pCMV-XL5-TF using the Q5 site-directed mutagenesis kit (New England Biolabs, Hertfordshire, UK) and the following primers to convert serine residue 241 to a stop codon:

Truncated TF forward primer:

5’-TTGTCATCATCCTGGCTATATAACTACACAAGTGTAGAAAGGCAGGAG-3’

Truncated TF reverse primer:

5’-TTGTGTAGTTATATAGCCAGGATGATGACAAGGATGATGACCACAAATAC-3’

MV isolation and analysis

Cells previously adapted to low serum were washed with PBS and placed in serum-free medium. MVs were isolated from the media by ultracentrifugation as described previously

(14). The concentrations of phosphatidylserine-positive MVs were determined using the Zymuphen MV determination kit (Hyphen BioMed/Quadratech Ltd, Epsom, UK). The TF antigen content of the MV samples was measured using a TF-ELISA (Affinity Biologicals, Ancaster, Canada). The TF content of the MVs was also examined by SDS-PAGE and western blot analysis of MV samples by loading volumes of MV samples adjusted to the number of cells that the MVs were isolated from. TF activity of the MVs was measured using a two-stage chromogenic thrombin generation assay as described previously (15) with a standard curve prepared alongside using lipidated recombinant TF (American Diagnostica, Stamford, USA). TF-dependent thrombin generation was assessed by pre-incubation of MVs with a mouse anti-TF inhibitory antibody (25 μg/ml) (4509, American Diagnostica) or normal mouse IgG (25 μg/ml) (Cell Signaling Technology). The TF activity of the MV samples was also assessed by FXa generation using the Actichrome TF activity kit (American Diagnostica) with a standard curve prepared alongside using lipidated recombinant TF. The number of cells/well from which the MVs were isolated from was determined using crystal violet staining as described previously (14). The TF antigen content, TF activity and MV densities were normalised to cell numbers and then standardised to TF antigen, TF activity or MV density per million cells.

Nanoparticle tracking analysis

The numbers and size distributions of the isolated MVs were determined by nanoparticle tracking analysis (NTA) using NanoSight LM10 and NTA software (NanoSight Ltd, Amesbury, UK). The Nanosight instrument was calibrated using FluoSpheres® carboxylate-modified microspheres with diameters of 0.1 μm and 1.0 μm (Invitrogen). MV samples were diluted 1:5 in 0.1 μm-filtered PBS and analysed using the NanoSight LM10 by tracking particles over 60 s using a camera level of 12 and shutter speed of 21.26 frames/s. Data were

analysed using the NTA software to determine the concentration and size distribution of the MVs.

SDS-PAGE and western blotting

Protein samples (10 μg) were separated by 10 or 12% (w/v) SDS-PAGE and proteins transferred onto nitrocellulose membranes as described previously (14). The membranes were probed with a rabbit anti-filamin-A antibody (EP2405Y) (Epitomics Inc, CA, USA), a rabbit anti-TF antibody (FL-295) (Santa Cruz Biotechnology), a mouse anti-PAR2 antibody (SAM11) (Santa Cruz Biotechnology) or an anti-GAPDH antibody (Santa Cruz Biotechnology) diluted 1:1000 in TBST followed by horse radish peroxidase (HRP)-conjugated secondary antibodies as described previously (14).

siRNA-mediated knock-down of filamin-A expression

Cells (5×10^4) were seeded out into 12-well plates and transfected with 80 nM of human filamin-A siRNA or control siRNA (Santa Cruz Biotechnology) using Lipofectin. The cells were transfected for 5 h and then incubated at 37ºC for 48 h prior to experiments. The efficiency of siRNA transfection was assessed using a fluorescein-conjugated control siRNA and the percentage of fluorescent cells determined by flow cytometry. Cell viability was assessed using an MTS-based assay (Promega) (14). The filamin-A siRNA from Santa Cruz Biotechnology was a pool of three different siRNA duplexes, the sequences of which are shown below:

sc-35374A: Sense: 5’-CCAUCACUGACAACAAAGAtt-3’

Antisense: 5’-UCUUUGUUGUCAGUGAUGGtt-3’

sc-35374B: Sense: 5'-CUGCAGAGUUUAUCAUUGAtt-3'

Antisense: 5'-UCAAUGAUAAACUCUGCAGtt-3'

sc-35374C: Sense: 5'-GCUACCUCAUCUCCAUCAAtt-3'

Antisense: 5'-UUGAUGGAGAUGAGGUAGCtt-3'

The data obtained with the filamin-A siRNA from Santa Cruz Biotechnology, was confirmed using Silencer Select pre-designed filamin-A siRNA (ID: s5276) from Life Technologies, the sequences of which are shown below:

Sense: 5'-CCAACAAGCCCAACAAGUUtt-3'
Antisense: 5'-AACUUGUUGGGCUUGUUGGtg-3'

Cells were transfected with SilencerSelect siRNA by reverse transfection as follows. Filamin-A siRNA or control siRNA diluted in serum-free media were placed into the wells of 12-well plates. Lipofectamine RNAiMAX diluted in serum-free media was added to the diluted siRNA and incubated at room temperature for 20 min. Cells (6×10^4) were then seeded out into the wells to give final siRNA concentrations of 0.5-2 nM. Cells were incubated at 37ºC for 48 h prior to experiments to allow suppression of filamin-A expression.

Flow cytometric analysis of cell surface TF and preparation of plasma membrane proteins

Cells (10^5) in 6-well plates were detached and resuspended in PBS (0.1 ml). The cells were incubated with an anti-TF Alexa Fluor 488-conjugated antibody for 1 h at 4°C. The cells were then washed with PBS and examined on a FACsCalibur flow cytometer (Becton Dickinson) alongside non-labelled cells. Plasma membrane TF, total TF and total membrane

TF were prepared from MDA-MB-231 cells using the Minute plasma membrane protein isolation kit according to the manufacturers' instructions (Invent Biotechnologies Inc). Samples (6 µg of protein) were separated by SDS-PAGE followed by western blot analysis using a rabbit anti-TF antibody (FL-295).

Statistical analysis

Data represent the calculated mean values from the number of experiments stated in the figure legends ± the calculated standard error of the mean. The data were analysed using the statistical package for the social sciences (SPSS). Significance was determined using one-way ANOVA and the Tukey's honestly significance test and values of $p<0.05$ were considered to be significant.

Results

MDA-MB-231 cells release TF-positive MVs in response to PAR2 activation

We have previously shown that endothelial cells release TF-positive MVs following the activation of protease-activated receptor 2 (PAR2) using a PAR2-agonist peptide (PAR2-AP) or FXa (8). In this investigation, MDA-MB-231 cells were used to examine the release of TF-positive MVs from cells that constitutively express TF. MDA-MB-231 cells constitutively released TF-positive MVs into the culture media and activation of these cells with PAR2-AP (SLIGRL; 20 µM) resulted in increased release of TF-positive MVs, peaking at 30 min post-activation (Supplemental Figures 1A-C). Activation of MDA-MB-231 cells with PAR2-AP (20 µM), FXa (10 nM) or a protease-activated receptor 1 agonist peptide (PAR1-AP) (TFLLR; 20 µM) resulted in increased MV release (Supplemental Figure 1D). Incubation of cells with PAR2-AP or FXa resulted in increased incorporation of TF into MVs, whereas PAR1-AP or scrambled peptide had no effect on levels of TF incorporated into the MVs

(Supplemental Figure 1E). PAR2-AP was therefore used throughout this study as a stimulus that induces both the release of MVs and the active incorporation of TF into MVs.

Optimisation of siRNA-mediated suppression of filamin-A expression

Two sets of filamin-A-specific siRNA were used to suppress the expression of filamin-A. The transfection efficiency of an FITC-conjugated control siRNA was determined to be approximately 92% by flow cytometry (not shown). Transfection of cells with filamin-A siRNA (80 nM; Santa Cruz Biotechnology) resulted in approximately 50 % reductions in filamin-A expression in endothelial cells and MDA-MB-231 cells at 48 h post-transfection, but had no detectable influence on the expression of TF (Supplemental Figures 2A-C). Reverse transfection of MDA-MB-231 cells with 0.5-2 nM of SilencerSelect filamin-A siRNA (Life Technologies) resulted in 57-77% decreases in filamin-A expression and did not reduce TF expression (Supplemental Figures 2 D&E). Cell morphology and cell viability were not altered following transfection of cells with filamin-A siRNA (Supplemental Figures 3 A-C) and suppression of filamin-A expression did not appear to alter the gross structure of the actin cytoskeleton (Supplemental Figure 4).

Suppression of filamin-A expression reduces the incorporation of TF into MVs

Suppression of filamin-A expression in endothelial cells using a specific siRNA (80 nM) significantly reduced the incorporation of TF into MVs, compared to MVs from endothelial cells transfected with the control siRNA as measured by ELISA (Figures 1A&B). Similarly, the incorporation of TF antigen into MVs was reduced following the suppression of filamin-A expression in MDA-MB-231 cells (Figure 1C). Decreased incorporation of TF into MVs was also observed in MDA-MB-231 cells following transfection with a different filamin-A siRNA (SilencerSelect; 0.5-2 nM) compared to control cells (Figure 1D). Western blot

analysis showed that the suppression of filamin-A expression in MDA-MB-231 cells to 55% ± 8.25% (SEM n=4, p=<004 vs. control siRNA) of control cells, resulted in the reduction of the incorporation of TF into released MVs to 52% ± 2.83% (SEM n=3, p=<0.005 vs. control siRNA) of those detected in MVs derived from control cells (Figure 1E). Since PAR2 does not bind to filamin-A, the level of MV-associated PAR2 was also measured as a loading control, and shown to be unaffected by filamin-A suppression (Figure 1E). In addition, deletion of the cytoplasmic domain of TF significantly reduced the incorporation of TF into MVs, but this was not further affected by the suppression of filamin-A expression (Figure 1F). Levels of MVs released by cells following PAR2 activation were not altered by filamin-A suppression or the expression of the truncated form of TF (not shown). The reductions in the amount of TF antigen in the MVs were also reflected in the decreased thrombin-generation (Figures 2A-C) and FXa-generation (Figure 2D) capacity of MVs derived from cells with suppression of filamin-A expression. Pre-incubation of MVs with an inhibitory anti-TF antibody suppressed thrombin generation capacity by 77.2% ± 12.3% (SD, n=3) compared to MVs incubated with a control IgG, indicating that the thrombin-generation activity was mostly TF-dependent. No significant difference was observed between the cell surface expression of TF in MDA-MB-231 cells with suppression of filamin-A expression compared to control cells (Figure 3A). However, activation of cells with PAR2-AP resulted in a 56% increase in plasma membrane-associated TF and a 60% increase in the amount of TF in MVs, whereas total cellular TF expression remained unaltered (Figure 3B).

The effect of the suppression of filamin-A expression on MV release

The release of phosphatidylserine-positive MVs from endothelial cells and MDA-MB-231 cells as measured using the Zymuphen assay was not significantly reduced by the suppression of filamin-A expression (Figures 4A-D). Total numbers and size distributions of the released

MVs as determined using nanoparticle tracking analysis, were also not significantly affected by the suppression of filamin-A expression (Figure 4E). The size distribution of all MV samples ranged from 60-500 nm in diameter with two main populations observed, the major population with a peak size of 100 nm and the smaller population with a peak size of 300-400 nm (Supplemental Figure 5). Analysis of MV samples isolated from control cells and not subjected to ultracentrifugation revealed a similar size distribution compared to MVs isolated by ultracentrifugation (Supplemental Figure 5D), and no particles were detected in the 0.1 μm-filtered PBS used for resuspending MVs (Supplemental Figure 5E). The exosome marker Tsg101 was barely detectable in HDBEC-derived MV preparations, even when concentrated, but was present in concentrated MV samples derived from MDA-MB-231 cells (Supplemental Figure 6). Transfection of MDA-MB-231 cells with 10 nM of SilencerSelect filamin-A siRNA resulted in >90% decreases in filamin-A expression compared to control cells (Figure 5A) and this substantial knock-down of filamin-A expression resulted in significant increases in MV release (Figure 5B) and reduced cell viability by 20% (not shown).

Discussion

We previously reported that the incorporation of TF into MVs is promoted by the phosphorylation of serine 253 within the cytoplasmic domain of TF (8). Since filamin-A binds to the cytoplasmic domain of TF in a phosphorylation-dependent manner (13), this study examined the role of filamin-A in the incorporation of TF into MVs. Suppression of filamin-A expression significantly reduced the amount of TF antigen and associated TF activity incorporated into MVs derived from both endothelial cells and MDA-MB-231 cells (Figures 1 & 2), indicating that filamin-A is required for the incorporation of TF into MVs. Filamin-A binds and regulates the cellular distribution and trafficking of a number of

transmembrane and cytosolic proteins. For example, filamin-A localises and stabilises dopamine receptors at the cell surface (16,17), controls the translocation of proteins to lipid rafts (18) and regulates receptor endocytosis (19) and recycling (20). Furthermore, it has been demonstrated that proteins are selectively incorporated into MVs, resulting in the enrichment of MVs with specific antigens (21). Since filamin-A binds to the cytoplasmic domain of TF (13), filamin-A may serve to localise TF to specific sites on the cell surface for subsequent incorporation and release into MVs in response to PAR2 activation. Filamin-A and TF have been shown to co-localise in lipid rafts during cell migration (22), and it has been suggested that MVs originate from lipid rafts (3,23). It is therefore possible that filamin-A regulates the accumulation of TF into lipid rafts for subsequent incorporation into MVs. Interestingly, the suppression of filamin-A expression did not alter levels of cell surface TF expression in non-activated cells (Figure 3A), indicating that filamin-A is not required for the normal turnover of TF at the cell surface. However, in agreement with a previous report (24), the activation of PAR2 resulted in the translocation of TF to the plasma membrane (Figure 3B), a process which is believed to precede TF release into MVs (25). Filamin-A has been shown to regulate the transport of receptors to the cell surface, such as the transport of GPIbα from the endoplasmic reticulum to the surface of platelets (26,27). Therefore, the possible role of filamin-A in PAR2-induced translocation of TF to the cell surface for subsequent incorporation of TF into MVs requires further investigation.

Interestingly, deletion of the cytoplasmic domain of TF also resulted in reduced incorporation of TF into MVs, and the suppression of filamin-A expression had no further effect on the incorporation of cytoplasmic domain-deleted TF (Figure 1F). This shows that both the cytoplasmic domain of TF and filamin-A are required for the incorporation of TF into MVs. This appears to contradict the study by Schecter et al (2000) which showed that smooth

muscle cells release truncated and wild-type TF into MVs at similar levels (4). However, this discrepancy may be due to differences between the TF constructs used in these two studies. In the study by Schecter et al, both the cytoplasmic and transmembrane domains of TF were replaced with the C-terminus of the GPI-anchored protein decay-accelerating factor, resulting in the extracellular domain of TF being anchored to the cell surface through a GPI-link. GPI-anchored proteins are known to localise to cholesterol-rich microdomains and lipid rafts (28,29). Since MVs are thought to originate from lipid rafts (3,23), the GPI-anchored form of TF is likely to be preferentially targeted to sites of MV release and incorporated into MVs at similar levels to wild-type TF. In fact, it has been shown that decay-accelerating factor is spontaneously released by cells under normal culture conditions (30). In contrast, in our study only the cytoplasmic domain and not the transmembrane domain of TF was deleted to permit the incorporation of TF into the cell membrane. The interaction between the cytoplasmic domain of TF and filamin-A may regulate the proper cellular localisation of TF for subsequent incorporation into MVs. Therefore, we propose that TF lacking the cytoplasmic domain cannot properly localise to be incorporated into MVs following PAR2 activation.

Partial suppression of filamin-A expression had no significant influence on the number or size distribution of the released MVs (Figure 4 & Supplemental Figure 5), indicating that filamin-A is not involved in PAR2-mediated MV formation and release. However, >90 % suppression of filamin-A expression resulted in significantly increased MV release (Figure 5). The formation and release of MVs involves the reorganisation and degradation of the cytoskeleton (10), leading to the dissociation of the plasma membrane from the peripheral cytoskeleton. A major role of filamin-A is to maintain the integrity of the cytoskeleton (11), and a complete lack of filamin-A expression, as seen with the M2 melanoma cell line, results in the instability of the peripheral cytoskeleton and spontaneous blebbing of the plasma

membrane (31). Similarly, high levels of MV release observed in cells with low levels of filamin-A expression are likely to be due to the de-stabilisation of the cell membrane and the underlying cytoskeleton, resulting in increased MV release. Consequently, in this study, a suitable range of filamin-A siRNA concentrations was used to demonstrate that partial suppression of filamin-A expression results in the reduced incorporation of TF into MVs, but without affecting MV release.

This is the first study to demonstrate that filamin-A is required for the incorporation of TF into MVs following PAR2 activation. Importantly, this seems to be independent of the process of MV release in response to PAR2 activation, and suggests a mechanism by which TF is actively incorporated into MVs. Since the N-terminal actin-binding domain of filamin-A binds directly to actin, it is possible that the filamin-A-dependent incorporation of TF into MVs is mediated by filamin-A linking TF to the actin cytoskeleton. Additionally, filamin-A may act as a scaffolding protein mediating the activation of signalling proteins (11) necessary for the incorporation of TF into MVs. Furthermore, while PAR2 activation was used as a stimulus to induce MV formation and release, the protein composition of MVs is known to be dependent on the activating stimulus (32). Therefore, whether stimuli such as inflammatory cytokines and thrombin which induce endothelial cells to release MVs (9,21) also modulate the incorporation of TF into MVs through a filamin-A-dependent mechanism remains to be elucidated. In conclusion, this study proposes a novel mechanism by which TF is actively incorporated into MVs by a process requiring filamin-A, and is independent of the process of MV formation and release.

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Figure 1. Suppression of filamin-A expression reduces the incorporation of TF into MVs. HDBEC (A), HCAEC (B) transfected to express wild-type TF, or MDA-MB-231 cells (C) were transfected with 80 nM of filamin-A siRNA or control siRNA. Cells were activated with PAR2-AP (20 μM) and MVs isolated at 90 min (A & B) and at 30 min (C). TF antigen in the MV samples was determined using a TF-ELISA. (HDBEC n=4; HCAEC and MDA-MB-231 n=3.*=p<0.05 vs. control siRNA). D&E) MDA-MB-231 cells transfected with SilencerSelect filamin-A siRNA (0.5-2 nM) or control siRNA (2 nM) were activated with PAR2-AP for 30 min and MVs isolated. The TF antigen content of the MVs was determined using D) a TF-ELISA (n=5, *=p<0.05 vs. no siRNA) or E) western blot analysis (n=3). F) HDBEC transfected to express truncated TF together with filamin-A or control siRNA were activated with PAR2-AP for 90 min and the TF content of the MVs determined using a TF-ELISA. (n=3) *=p<0.043 and **=p<0.047 vs. wild-type TF with control siRNA.

Figure 2. Suppression of filamin-A expression reduces the TF activity of MVs. HDBEC (A), HCAEC (B) transfected to express wild-type TF, and MDA-MB-231 cells (C) were transfected with filamin-A siRNA or control siRNA (80 nM) and activated with PAR2-AP. MVs were isolated at 90 min (A & B) and at 30 min (C) post-activation. TF activity of the MV samples was measured using a chromogenic thrombin-generation assay (n=4). D) TF

activities of MDA-MB-231-derived MVs were also determined using a FXa-generation assay (n=3). *=p<0.05 vs. control siRNA.

Figure 3. Cell surface expression of TF in MDA-MB-231 cells with suppression of filamin-A expression and examination of levels of TF in plasma membrane fractions following PAR2 activation. A) MDA-MB-231 cells were transfected with 1 nM of filamin-A siRNA (Life Technologies) or 1 nM of control siRNA and incubated for 48 h to allow for the suppression of filamin-A expression. Cells were then labelled with an anti-TF Alexa Fluor 488-conjugated antibody for 1 h at 4˚C and analysed by flow cytometry. Black filled in = no anti-TF Alexa Fluor488-conjugated antibody, light grey line = control siRNA, dark grey line = filamin-A siRNA. Data are representative of three experiments. B) MDA-MB-231 cells were activated with PAR2-AP (20 μM) for 30 min. MVs were then isolated from the culture media by ultracentrifugation and cells were lysed. Plasma membrane, total cell lysate and total membrane fractions were prepared using the plasma membrane isolation kit. Equal quantities of protein (6 μg) for each sample were separated by SDS-PAGE and membranes were probed using a rabbit anti-TF antibody.

Figure 4. Suppression of filamin-A expression does not alter MV release. HDBEC (A), HCAEC (B) and MDA-MB-231 cells (C) were transfected with filamin-A siRNA or control siRNA (80 nM) and activated with PAR2-AP. The concentrations of MVs released at 90 min (A&B) and at 30 min (C) post-activation were measured using the Zymuphen MV density assay (n=3). (D) MDA-MB-231 cells transfected with SilencerSelect filamin-A siRNA (0.5-2 nM) or control siRNA were activated with PAR2-AP for 30 min. MVs were isolated from the media and measured using the Zymuphen assay (n=5). E) HDBEC co-transfected with pCMV-XL5-TF and control or filamin-A siRNA were activated with PAR2-AP for 90 min,

together with a non-activated sample. MVs were isolated and numbers of MVs were analysed by NTA (n=3).

Figure 5. The effect of >90 % suppression of filamin-A expression on the release of MVs from MDA-MB-231 cells. A) Cells were reverse transfected with 10-75 nM of filamin-A siRNA (Life Technologies) or 75 nM control siRNA using Lipofectamine RNAi MAX. Cells were incubated for 48 h and then lysed in Laemmli's buffer. 10 μg of protein for each sample was separated by 12 % SDS-PAGE and analysed by western blot for filamin-A, TF and GAPDH. Molecular weight markers (M) are shown in kDa. B) MDA-MB-231 cells were reverse transfected with 10 nM of filamin-A siRNA (Life Technologies) or 10 nM control siRNA using Lipofectamine RNAi MAX. Cells were incubated for 48 h and then adapted to serum-free media and activated with PAR2-AP (20 μM) for 30 min. MVs were isolated from the media by ultracentrifugation and the MV concentration of the samples analysed using the Zymuphen MV assay. n = 5, * = $p<0.002$ vs. control siRNA.