

## RESEARCH ARTICLE

# Protease activated receptors (PAR)-1 and -2 mediate cellular effects of factor VII activating protease (FSAP)

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## Abstract

Factor VII activating protease (FSAP) is a circulating serine protease implicated in thrombosis, atherosclerosis, stroke, and cancer. Using an overexpression strategy, we have systematically investigated the role of protease activated receptors (PAR)-1, -2, -3, and -4 on FSAP-mediated signaling in HEK293T and A549 cells. Cleavage of PAR-reporter constructs and MAPK phosphorylation was used to monitor receptor activation. FSAP cleaved PAR-2 and to a lesser degree PAR-1, but not PAR-3 or PAR-4 in both cell types. Robust MAPK activation in response to FSAP was observed after PAR-2, but not PAR-1 overexpression in HEK293T. Recombinant serine protease domain of wild type FSAP, but not the Marburg I isoform of FSAP, could reproduce the effects of plasma purified FSAP. Canonical cleavage of both PARs was suggested by mass spectrometric analysis of synthetic peptide substrates from the N-terminus of PARs and site directed mutagenesis studies. Surprisingly, knockdown of endogenous PAR-1, but not PAR-2, prevented the apoptosis-inhibitory effect of FSAP, suggesting that PAR1 is nevertheless a direct or indirect target in some cell types. This molecular characterization of PAR-1 and -2 as cellular receptors of FSAP will help to define the actions of FSAP in the context of cancer and vascular biology.

## KEYWORDS

apoptosis, FSAP, HABP2, mutations, PARs, vascular

## 1 | INTRODUCTION

Protease activated receptors (PARs) is a family of four G-protein coupled receptors that are activated in an irreversible manner by proteolytic release of a tethered ligand

embedded within their N-terminal extracellular domain. PARs are widely expressed and cleaved by a variety of endogenous and exogenous proteases. The extent of cleavage depends primarily on substrate specificity and enzyme activity, but may be potentiated by exosite interactions or co-receptors

**Abbreviations:** APC, activated protein C; DAPI, 4',6-diamidino-2-phenylindole; FBS, fetal bovine serum; FSAP, factor VII activating protease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HABP2, hyaluronan-binding protein 2; MAPK, mitogen-activated protein kinase; MI, Marburg I; PAR, protease activated receptor; PDGF, platelet-derived growth factor; PFA, paraformaldehyde; SEAP, secreted alkaline phosphatase; SPD, serine protease domain; TFA, trifluoroacetic acid; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; VSMC, vascular smooth muscle cells.

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and regulated by the localization of receptors in the plasma membrane. Cell context-dependent signaling mechanisms that rely on the expression of cofactors, noncanonical cleavage, and their downstream effectors have been described.<sup>1-5</sup>

In the vascular compartment, both PAR-1 and PAR-2 are expressed on endothelial cells (EC), smooth muscle cells, and leukocytes whereas only PAR-1 is expressed on platelets. Canonical cleavage of PAR-1 at Arg41 by thrombin and at a noncanonical site, Arg46, by activated protein C (APC) has been observed.<sup>6</sup> In the case of PAR-2, elastase and plasma kallikrein have both been described to cleave outside the canonical<sup>7</sup> Arg36 position that is targeted by trypsin, factor Xa, and tryptase. PAR-1, PAR-3, and PAR-4 mediate platelet activation, whereas PAR-1 and -2 regulate vascular tone, inflammation, thrombosis, atherosclerosis, and cancer. Effective antagonists with clinical utility have been developed for PAR-1.<sup>6,7</sup> Biased signaling by APC through PAR-1 is responsible for a variety of cyto-protective, neuroprotective, and anti-inflammatory effects and is of considerable therapeutic interest.<sup>2</sup>

Factor VII activating protease (FSAP), encoded by the *hyaluronan binding protein 2 (HABP2)* gene, is a circulating plasma serine protease implicated in thrombosis,<sup>8</sup> vascular biology,<sup>9</sup> inflammation<sup>10</sup> and cancer.<sup>11,12</sup> FSAP has been proposed to mediate some of its effects through PAR signaling eg, the regulation of microvascular permeability.<sup>13</sup> Using a pharmacological approach, PAR-1 was identified as a receptor for FSAP-induced cyto-protective effects in astrocytes and neurons.<sup>14</sup> Consistent with PAR activation, exposure of EC and vascular smooth muscle cells (VSMC) to FSAP induces a transcriptional response related to inflammation, apoptosis and cell growth.<sup>15</sup> A PAR-1-dependent mechanism was evident in VSMC but this was not the case in EC suggesting the involvement of additional receptors.<sup>15</sup> In cancer cells, FSAP has been shown to influence cell migration and colony formation although the mechanisms responsible were not identified.<sup>11</sup> Proteolytic inhibition of platelet-derived growth factor (PDGF)-BB is another possible route of action of FSAP on VSMC.<sup>16</sup> Similarly, modulation of growth factor activity of fibroblast growth factor (FGF2) or the activation of the bradykinin-kallikrein system<sup>17</sup> in EC have been proposed.

In this study, we set out to systematically examine the selectivity and potency of FSAP toward all four human PARs and their role in FSAP signaling in two types of epithelial cells, human embryonic kidney (HEK293T) and human lung carcinoma epithelial (A549) cells. These cancer cell lines were chosen because recent studies suggest a link between expression of FSAP, cell migration, cancer, and metastasis.<sup>11,12</sup> PAR cleavage was assessed by overexpression of the N-terminal secreted alkaline phosphatase (SEAP)-tagged receptors and signaling by MAPK phosphorylation. Small interfering (si)-RNA-mediated knockdown and pharmacological inhibitors were used to assess the role of endogenous PARs.

Mutagenesis experiments and cleavage of synthetic peptides were used to address if FSAP cleaved PARs at their canonical cleavage sites. The role of PARs in the regulation of FSAP-mediated apoptosis inhibition was studied in A549 cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

The purification of FSAP from human plasma has been described.<sup>18</sup> Preparation of recombinant serine protease domain (SPD, amino acids 292-560) of wild type (WT) FSAP as well as Marburg I-FSAP (G534E mutation) will be described in more detail in a separate manuscript. Human plasma thrombin and trypsin was from Sigma Aldrich (Oslo, Norway). Antagonist for PAR-1, vorapaxar, was from Axon Medchem (Groningen, The Netherlands). Antagonists for PAR-2, GB88 and I-191, was a kind gift from David P. Fairlie (University of Queensland, Australia). The pan MMP antagonist, GM6001, was obtained from Millipore (Temecula, CA). Activating peptides for PAR-1/2 (SFLLRN), PAR-2 (SLIGKV), PAR-3 (SFNGGP), and PAR-4 (GYPGQV) were from Bachem (Bubendorf, Switzerland).

### 2.2 | Cell culture

HEK293T cells were a kind gift from Gareth Sullivan (University of Oslo, Oslo, Norway) and cultured in DMEM supplemented with fetal bovine serum (FBS) 10% vol/vol and penicillin (100 U/mL) and streptomycin (100 µg/mL). A549 cells were a kind gift from Alexandre Corthay (Oslo University Hospital, Oslo, Norway) and cultured in the medium described above. HEK293T cells were seeded on fibronectin (10 µg/mL) coated dishes to ensure cell attachment.

### 2.3 | Plasmids and transfection

PAR-1-4 cDNA was cloned into the multiple cloning site of a SEAP/HA tag-vector using XhoI and PacI restriction sites.<sup>19</sup> Point mutations were introduced using Q5 Quickchange site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) and were confirmed by sequencing. HEK293T and A549 cells were seeded on 24-well dishes and were transfected using 1 µL Lipofectamine2000 (Invitrogen, Oslo, Norway) and 0.5µg DNA per well in serum-free DMEM for 3 hours and the cells were used after 24 hours. siRNAs against PAR-1 (Hs\_F2R\_1/SI00031038) and PAR-2 (Hs\_F2LR\_5/SI02757419) (5 nM) were transfected into A549 cells using HiPerFect transfection reagent (Qiagen, Hilden, Germany) in regular growth medium and cells were used after 48 hours.

## 2.4 | SEAP analysis

Cells were serum-starved for 3 hours and stimulated with FSAP (10  $\mu\text{g}/\text{mL}$ ) or other proteases for 2 hours. Supernatants were collected and SEAP was measured using SensoLyte Luminescent Alkaline Phosphatase Assay Kit (Luminometric, AnaSpec, Fremont, CA).

## 2.5 | RNA isolation and qPCR analysis

Total RNA was extracted using total RNA Miniprep Kit from Sigma-Aldrich. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany). For real-time PCR, SensiFast Hi-ROX SYBR Kit (Bioline GmbH, Luckenwalde, Germany) was used and analysis was performed on a ViiA7 Real-Time PCR System (Applied Biosystems). Amplification (cDNA denaturation 95°C for 5 seconds, primer hybridization/elongation 60°C for 20 seconds) plot was monitored over 40 cycles and continuous fluorescence measurement indicated mRNA expression of analyzed genes. Following amplification, the accuracy of amplicons was confirmed by melting curve analysis. Fluorescent threshold cycles (ct) were set and normalized against ct of the reference gene *GAPDH*. Primer sequences are: Hs-*PAR1*(f) 5'-CTCAATGAAACCCTGCTCGAAG, Hs-*PAR1*(r) 5'-ACTGCGGAAGAGCTAAGACATC, Hs-*PAR2*(f) 5'-TGTGCAGAGGTATTGGGTCATC, Hs-*PAR2*(r) 5'-TCTGCTTACGACATACAAAGGG, Hs-*PAR3*(f) 5'-GT CCTCATCTCCCTTCCAACCTC, Hs-*PAR3*(r) 5'-CTCGCC TTAACATACCACAACC, Hs-*PAR4*(f) 5'-CAGAGCAGC CTGAGTGCAG, Hs-*PAR4*(r) 5'-TTGAGGGCGTGCTG TCATC, Hs-*GAPDH*(f) 5'-CCATGTTTCGTCATGGGTGT GA, Hs-*GAPDH*(r) 5'-CATGAGTCCTTCCACGATACCA.

## 2.6 | Peptide cleavage analysis

Synthetic peptides corresponding to N-terminus of human PAR-1 (amino acids 33-62) ATNATLDPRSFLLRNPND KYEPFWEDEEKN and human PAR-2 (amino acids 28-54) GTNRSSKGRSLIGKVDGTSHVTGKGVV were synthesized by commercial vendors. Peptides (10  $\mu\text{M}$ ) were incubated with FSAP or other proteases at 37°C in TBS (50 mM Tris, pH 7.4, 100 mM NaCl, Tween-20 (0.1% wt/vol) and  $\text{CaCl}_2$  (2 mM). Reactions were stopped with final concentration of trifluoroacetic acid (TFA) (0.2% vol/vol). The peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in the reflectron mode using an Ultraflex II (Bruker Daltonics, Bremen, Germany). The peptides were

purified using  $\mu\text{-C18}$  ZipTips (Millipore, Billerica, MA, USA) and directly spotted onto the MALDI target with 0.6  $\mu\text{L}$  matrix (20 mg/mL  $\alpha\text{-cyano-4-hydroxycinnamic acid}$  in 0.25% aqueous TFA/acetonitrile (2:1)). MS spectra were evaluated using the software FlexAnalysis version 2.4. (Bruker Daltonics).

## 2.7 | Western blotting analysis and protein phosphorylation

Cells were starved for 3 hours in serum-free medium and then stimulated for 15 minutes with the appropriate agonist. The experiments were stopped by adding SDS sample buffer containing NaF (10 mM), orthovanadate (1 mM), and pyrophosphate (1 mM), and the samples were processed for Western Blotting. SDS-PAGE was performed and proteins were transferred to PVDF membranes (GE Healthcare, Oslo, Norway). For analysis of Western blotting, ECL prime chemiluminescence (GE Healthcare) was used. Antibodies against p44/p42 (#9102) and P-p44/p42 (#9101) were all obtained from Cell Signaling Technology (Leiden, The Netherlands). Anti-HA tag (ab9110) from Abcam (Cambridge, United Kingdom) was used to detect the overexpression of PARs. Densitometric analysis was performed to calculate relative expression using the ImageJ or Image Lab system (Bio-Rad, Oslo, Norway).

## 2.8 | TUNEL staining

A549 cells were incubated in the presence of FSAP or control HE buffer for 22 hours and further 2 hours with 1  $\mu\text{M}$  staurosporine. Cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) for 25 minutes followed by permeabilization with 0.1% (vol/vol) Triton X-100 and 0.1% (wt/vol) sodium citrate for 5 minutes on ice. Cells were stained using In Situ cell death detection kit, Fluorescein (Roche, Mannheim, Germany) and coverslips were mounted using fluoroshield with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, Oslo, Norway). Apoptotic cells were counted and expressed as a percentage of DAPI-stained cells.

## 2.9 | Immunofluorescent staining

Expression of PAR-1-4 was detected using immunofluorescence staining for the HA tag. Cells were fixed with PFA and permeabilized as described for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Cells were stained with anti-HA and anti-rabbit Alexafluor594 was used for detection.

## 2.10 | Statistical analysis

Statistical significance was analyzed by the one-way ANOVA followed by Tukey's multiple comparison post-test (Graphpad Prism, San Diego, CA). Composite data from all independent replicates or data from a single experiment are shown as indicated in the figure legend. Data are shown as mean  $\pm$  SEM and significance is denoted by \* in case  $P < .05$ .

## 3 | RESULTS

### 3.1 | Cleavage and activation of PARs by FSAP in HEK293 cells

Transcriptional analysis revealed a higher expression of PAR-1, intermediate expression of PAR-2 and PAR-3, and a very low expression of PAR-4 mRNA in HEK293T cells (Supplementary Figure S1A). The functionality of these endogenous receptors was tested with PAR-activating peptides (AP). PAR-1/2-AP increased MAPK phosphorylation in these cells, whereas PAR-4-AP had no effect. Although not statistically significant, effects of a PAR-2-AP suggested a substantial contribution of PAR-2 in the response to the nonselective PAR-1/2-AP. PAR-3-AP had some stimulatory effects, but this was also not significant (Supplementary Figure S1B,C).

To examine the cleavage of PARs by FSAP, we used PAR-1, -2, -3, and -4 constructs with N-terminal SEAP-HA tags, the proteolytic release of which could be detected in a sensitive manner by measuring SEAP activity in the culture supernatant.<sup>19</sup> Analysis of the HA tag by Western blot and immunostaining in HEK293T cells transfected with PARs showed overexpression of all 4 PARs (Supplementary Figure S2A,B). To validate our system, we tested the cleavage of PARs and activation of MAPK with thrombin and trypsin after transfection of cells with PAR-1-4. As expected, thrombin cleaved and activated MAPK phosphorylation in PAR-1, -3, and -4 transfected cells, whereas trypsin cleaved and activated all PAR transfected cells (Supplementary Figure S2C,D). The PAR-1/2 specific AP, SFLLRN (PAR-1/2-AP), increased MAPK phosphorylation approximately threefold in both PAR-1- and PAR-2- transfected cells, whereas the PAR-2 specific AP SLIGKV (PAR-2-AP) only increased MAPK phosphorylation in PAR-2 transfected cells (Supplementary Figure S2E).

Cells transfected with empty vector showed a 1.6-fold increase in MAPK activation with thrombin and a 2.5-fold increase with trypsin. Control transfection of cells altered their responses to PAR-1/2-AP compared to untransfected cells (Figures S1C and S2E). Thus, endogenous PARs are expressed in HEK293T may influence the overall MAPK phosphorylation response originating from the transfected PARs.

These results validate our test system with respect to expression of endogenous as well as overexpression of PAR-SEAP constructs in these cells.

In HEK293T cells transfected with PARs, a concentration dependent analysis showed that plasma-derived FSAP cleaved PAR-2 significantly. No cleavage above background was observed for PAR-1, PAR-3, or PAR-4 (Figure 1A). PAR-2 cleavage by FSAP was time-dependent (Figure 1B).

Heparin is known to function as a cofactor for the cleavage of certain FSAP substrates as described before.<sup>18</sup> Although the cleavage of all four PARs appeared to be higher in the presence of heparin, these increases were not significant (Figure 1C,D and Supplementary Figure S3A,B). Aprotinin, a known inhibitor of FSAP, inhibited PAR-2 cleavage (Figure 1D). Consistent with the cleavage analysis, we observed robust MAPK phosphorylation in PAR-2-transfected cells that was inhibited by aprotinin (Figure 1F). Only a nonsignificant trend toward increased MAPK phosphorylation was observed in PAR-1-transfected cells (Figure 1E). No significant receptor cleavage or MAPK phosphorylation was observed in cells transfected with PAR-3 and PAR-4 under any of the conditions tested (Supplementary Figure S3C,D). Thus, heparin did not serve as a cofactor for FSAP-mediated cleavage of PARs in HEK293T cells and the effect of FSAP on PAR-2 cleavage and activation was dependent on its proteolytic activity. In cells without overexpression of PARs, we did not observe activation of MAPK phosphorylation in response to FSAP (Figure 1E,F).

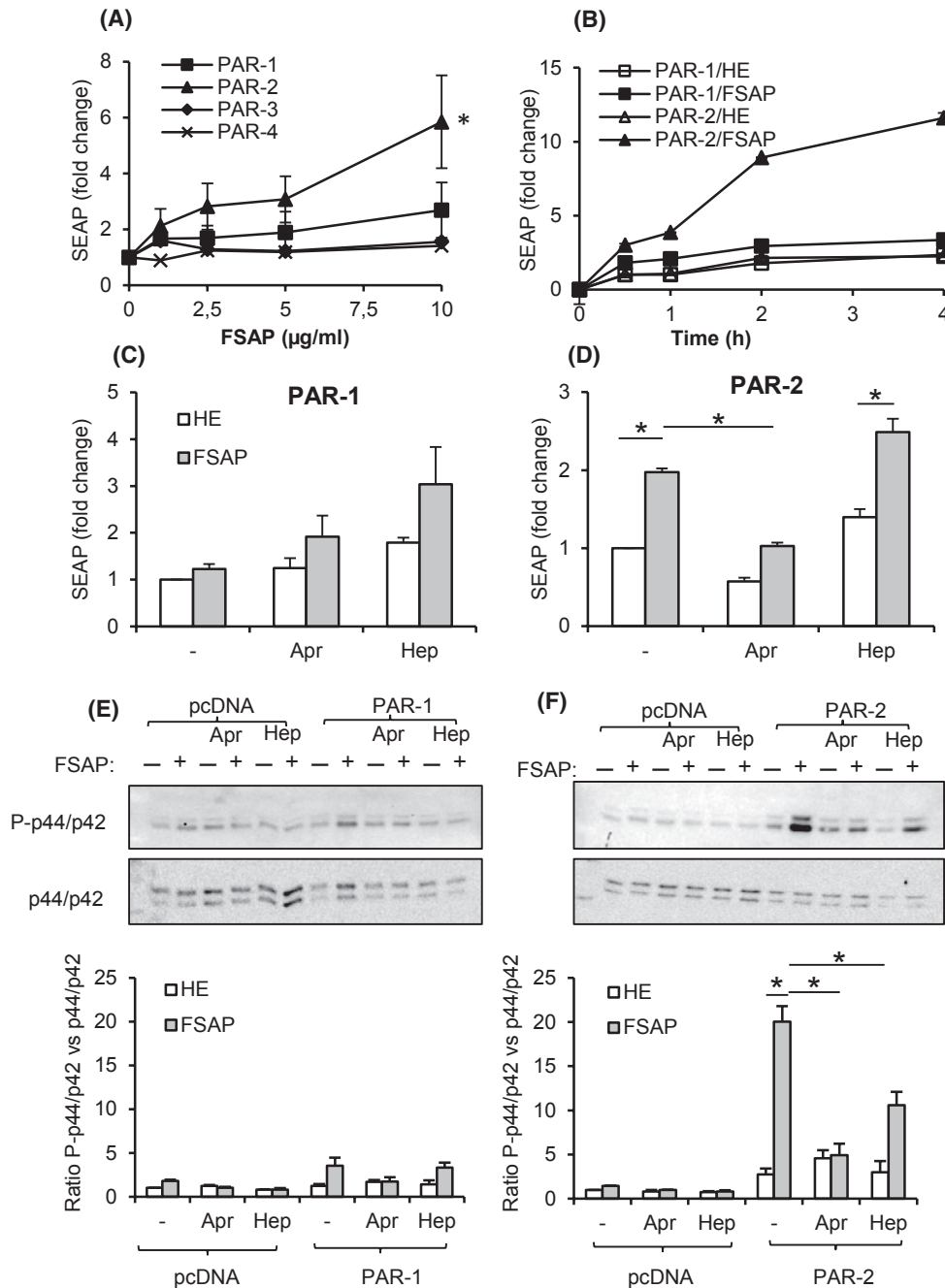
### 3.2 | WT but not the MI recombinant serine protease domain of FSAP activates PAR-2

We next compared the activity of recombinant FSAP wild type (WT)-serine protease domain (SPD) and the proteolytically inactive Marburg I (MI)-FSAP-SPD in activating PARs. In HEK293T cells, WT-FSAP-SPD cleaved PAR-1 and -2 in a concentration-dependent manner (Figure 2A,B). Thus, in contrast to plasma-derived FSAP, WT-SPD could cleave PAR-1. MAPK phosphorylation was stimulated in PAR-2, but not in PAR-1, overexpressing cells (Figure 2C,D). The MI-isoform, which only differs by one amino acid substitution in the serine protease domain, did not cleave PAR-2 or induce any MAPK activation (Figure 2B,D).

### 3.3 | Cleavage and activation of PARs by FSAP in A549 cells

To test whether FSAP could also cleave and activate PARs in another cell system, we used A549 lung epithelial cells. The level of expression of mRNA for endogenous PAR



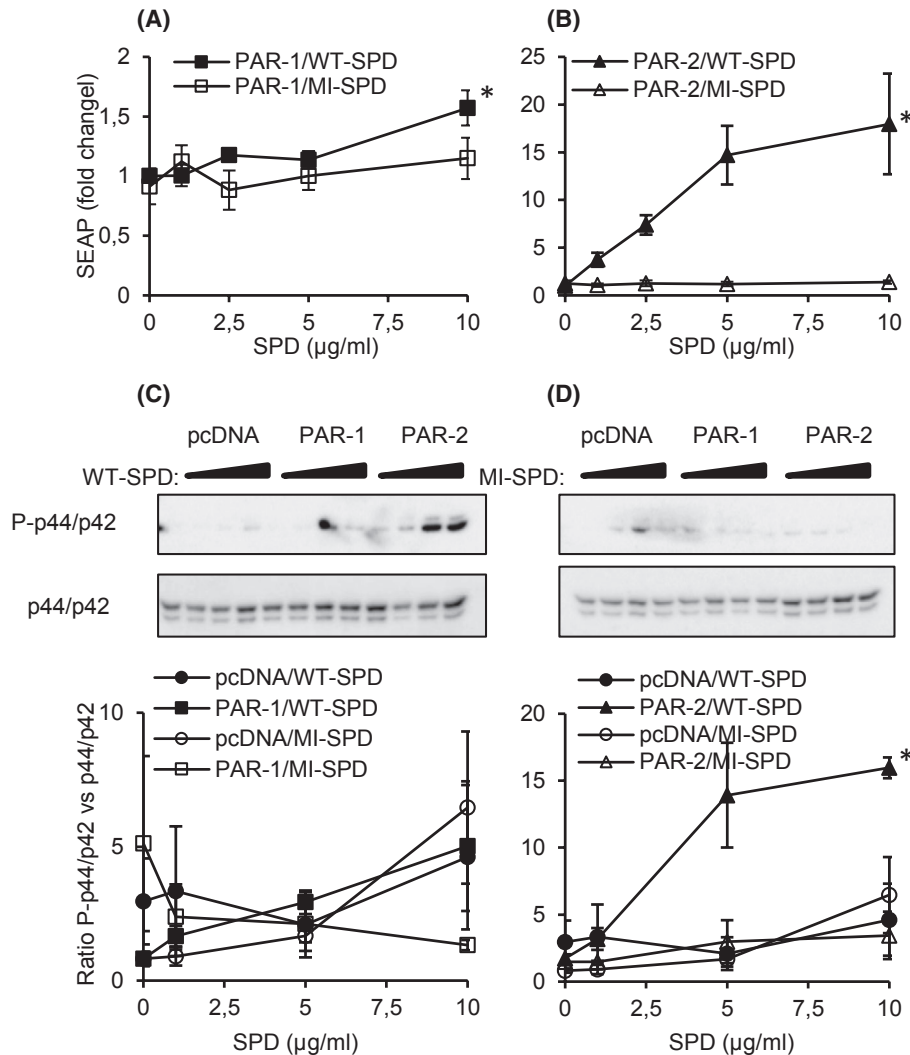


**FIGURE 1** SEAP-PAR-2 overexpressed in HEK293T cells is cleaved by FSAP: A, Cells were transfected with SEAP-PAR-1, -2, -3, or -4 and treated in a concentration-dependent manner with FSAP (0-10 µg/mL) for 2 hours. B, Cells were transfected with SEAP-PAR-1 or -2 and treated with FSAP (10 µg/mL) or control buffer (HE) for various times. C, SEAP-PAR-1 and (D) -2 transfected cells were treated with FSAP or HE buffer for 4 hours in the absence or presence of aprotinin (Apr, 25 µg/mL) or heparin (Hep, 100 µg/mL). In A-D, the amount of SEAP in the cell supernatants was determined. E, F, Cells were transfected and treated as in C and D for 15 minutes and activation of MAPKp44/p42 was determined. Panel B is representative of 2 independent experiments; all other panels represent results from 3 independent experiments; mean ± SEM, \*indicate  $P < .05$

was PAR-1 > PAR-3 > PAR-2 with very little PAR-4 (Supplementary Figure S1A). Although PAR-1/2 and PAR-2 AP increased MAPK phosphorylation, these effects were not significant due to a generally high level of MAPK activation in the basal state in these cells (Supplementary Figure S1B). Control transfection of cells altered their responses to

PAR-1/2-AP compared to untransfected cells (Figures S1D and S4C, F, H).

All SEAP-PAR constructs were expressed in A549 cells although to a lower degree than in HEK293T cells (Supplementary Figure S4A). MAPK was activated by thrombin, trypsin, PAR-1/2-AP, and PAR-2-AP in these



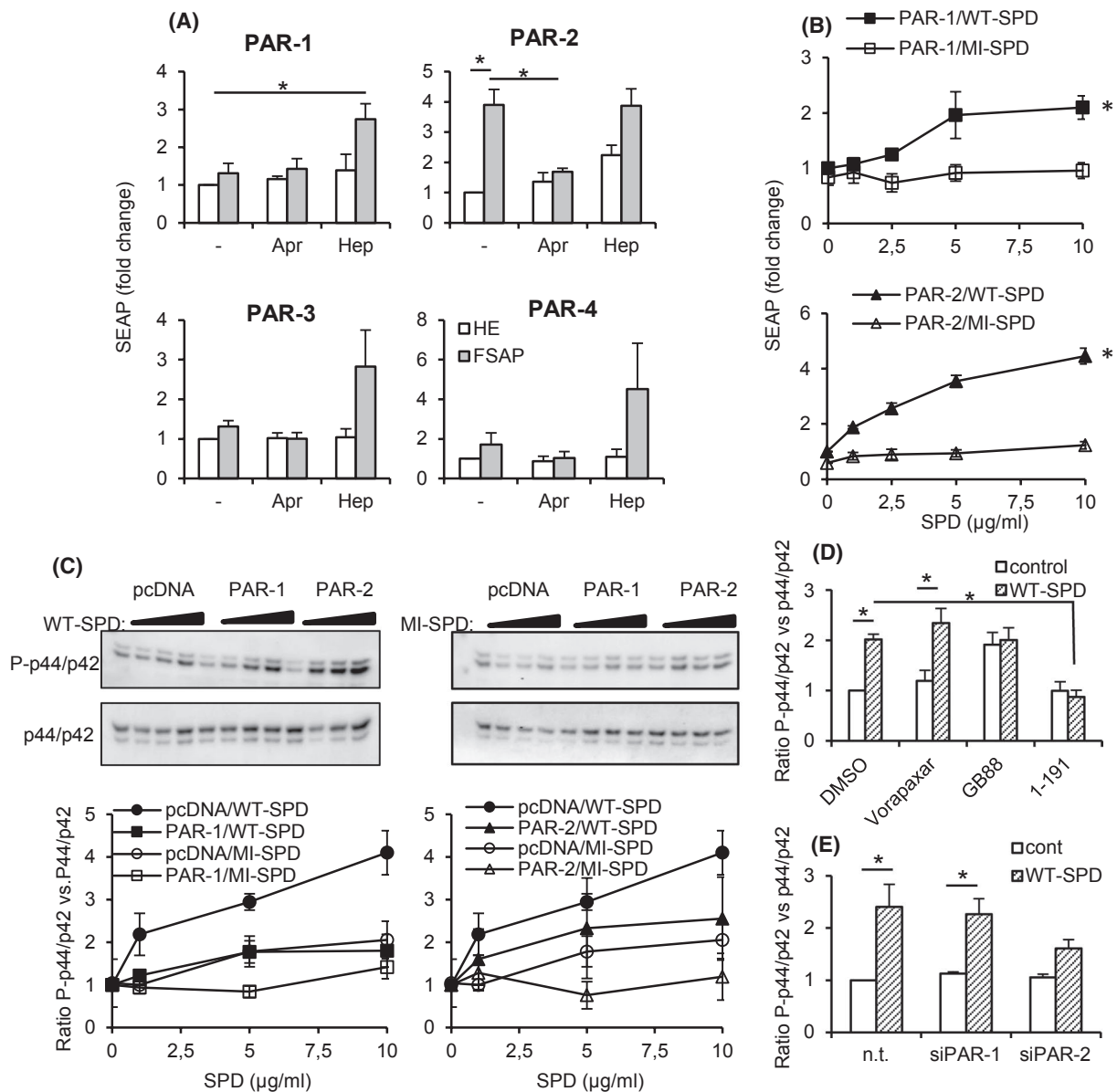
**FIGURE 2** FSAP WT-SPD, but not MI-SPD, activates PAR-2 in HEK293T cells overexpressing SEAP-PARs: A, SEAP-PAR-1 and (B) -2-transfected HEK293T cells were treated with WT-SPD (serine protease domain of FSAP) or MI-SPD (Marburg I isoform) or control buffer for 2 hours. SEAP released into the supernatants was determined. C, D, Cells, transfected as above, were treated with WT-SPD or MI-SPD or control buffer for 15 minutes and the activation of MAPKp44/p42 was determined. Each panel represents results from 3-4 independent experiments; mean  $\pm$  SEM, \* $P < .05$

cells, but PAR overexpression did not lead to an increase in MAPK phosphorylation (Supplementary Figure S4B,C). Like in HEK293T cells, PAR-2 was cleaved by FSAP and its cleavage inhibited by aprotinin (Figure 3A). PAR-1, -3, and -4 were not cleaved in the absence of heparin, but in its presence cleavage was higher and reached significance in the case of PAR-1 only. WT-FSAP-SPD, but not MI-FSAP-SPD, cleaved both PAR-1 and PAR-2 in a concentration-dependent manner (Figure 3B). Activation of MAPK phosphorylation by WT-FSAP-SPD compared to control buffer was higher in cells transfected with empty vector compared to PAR-1 or -2 transfected; MI-FSAP-SPD had no effect (Figure 3C).

The tendency of heparin to promote FSAP-mediated cleavage of PAR-1, raises the possibility that FSAP, in the presence of heparin, may mobilize cell-bound coagulation proteases such as thrombin. The effect of FSAP on PAR-1

transfected cells was not modified in the presence of hirudin (data not shown) indicating that it was not related to the activation of any residual pro-thrombin.

We next tested the involvement of endogenously expressed PARs in FSAP induced MAPK phosphorylation. Two different PAR-2 inhibitors, GB88 and I-191 abolished the activation of MAPK phosphorylation with WT-FSAP-SPD, while the PAR-1 inhibitor vorapaxar had no effect (Figure 3D). The specificity of all PAR inhibitors were evaluated using thrombin, PAR-1/2 and PAR-2 activating peptides (Supplementary Figure S4G,H). GB88 by itself increased MAPK phosphorylation, but this effect was not examined further. Thus, although A549 cells showed a similar pattern of response to FSAP as HEK293T cells with regard to PAR cleavage, activation of MAPK through endogenous PAR2 outweighed effects of transfected SEAP-PAR constructs in these cells.

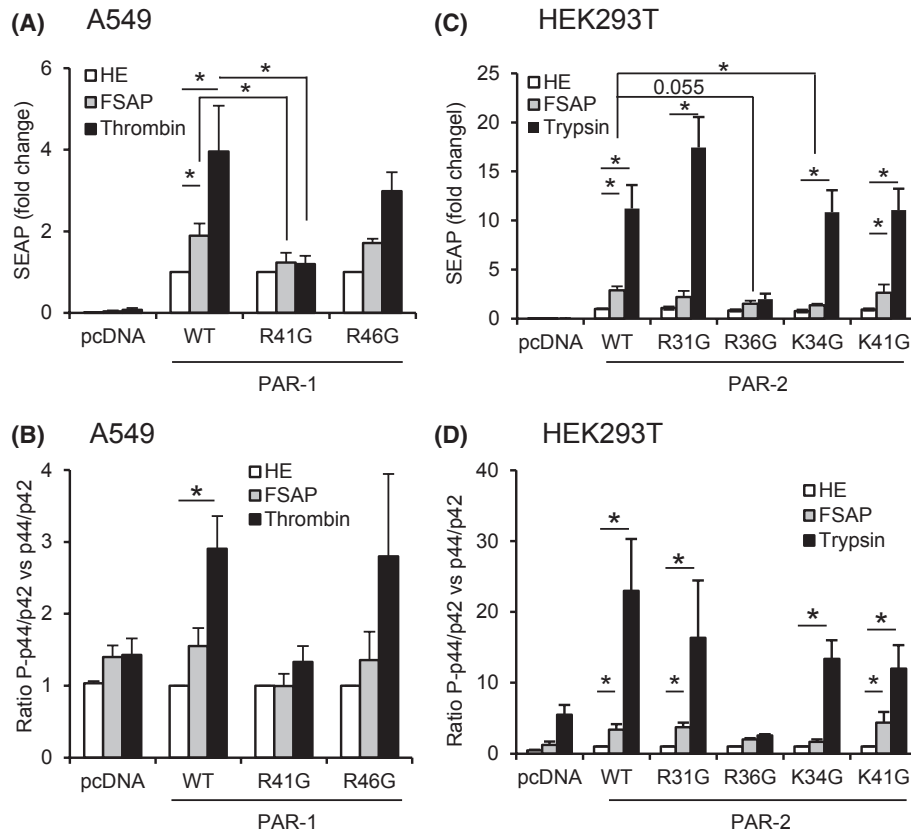


**FIGURE 3** Cleavage of SEAP-PAR-1 and -2 by FSAP in A549 cells: A, Cells were transfected with SEAP-PAR-1 to -4 and treated with FSAP (10 µg/mL) or HE buffer for 4 hours in the absence or presence of aprotinin or heparin. SEAP in supernatants was determined. B, Cells transfected with SEAP-PAR-1 and -2 were treated in a concentration-dependent manner with WT- or MI-SPD for 2 hours and total amount of SEAP in the supernatant was determined. C, Similarly, transfected cells were treated with WT- or MI-SPD for 15 minutes and the activation of MAPK-p44/p42 was determined. D, Cells were treated with WT-SPD or control buffer in the absence or presence of Vorapaxar (1 µM), GB88 (10 µM), I-191 (10 µM) or vehicle for 15 minutes and activation of MAPK-p44/p42 was determined. E, Cells were transfected with siRNA against PAR-1 or PAR-2 or nontargeting siRNA (n.t.) and treated with WT-SPD or control buffer for 15 minutes and activation of MAPK-p44/p42 was determined. Results in panel A were observed in 3 independent experiments and other panels represent results from 3 independent experiments; mean ± SEM, \*indicate  $P < .05$

### 3.4 | Identification of the FSAP-cleavage sites in PARs

Sequence analysis of known FSAP substrates shows that it cleaves after basic amino acids, Arg and Lys, and prefers sites with a cluster of such amino acids.<sup>20</sup> HEK293T cells were used for the PAR-2 experiments and A549 were used

for the PAR-1 experiments in order to identify the cleavage sites in PAR-1 and -2 by site directed mutagenesis. In PAR-1, the thrombin cleavage site, Arg41, and the APC cleavage site, Arg46, were mutated to Gly, and the mutated proteins were expressed in A549 cells. Both thrombin and FSAP cleave and activate WT-PAR-1, but not the canonical cleavage site mutant PAR-1R41G. PAR-1R46G was also cleaved and activated



**FIGURE 4** Identification of key amino acid residues in SEAP-PAR-1 and -2 required for cleavage and activation by FSAP: A, B, A549 cells were transfected with SEAP-PAR-1, -R41G, and -R46G mutant or control transfected cells were treated with FSAP (10  $\mu$ g/mL) or thrombin (1 U/mL) for 2 hours. A, Total amount of SEAP in the supernatant was determined. B, After treatment of cells for 15 minutes the activation of MAPKp44/p42 was determined. C, D, same as above except that SEAP-PAR-2, -K31G, -R36G, -K34G, and K41G mutant or control transfected HEK293T cells were used. Data represent 4-5 independent experiments  $\pm$  SEM, \*indicate  $P < .05$

by FSAP and thrombin, but the increases were not significant. Thus, FSAP cleaves PAR1 at the canonical R41 cleavage site (Figure 4A,B).

While both trypsin and FSAP cleaved and activated WT-PAR-2 as well as the noncanonical K41G cleavage site mutant, neither protease cleaved the canonical R36G mutant in HEK293T cells (Figure 4C,D). The noncanonical site, R31G was cleaved and activated by both FSAP and trypsin. FSAP did not cleave and activate the R34G noncanonical cleavage site mutant, suggesting a broader interface in the interaction between FSAP and the PAR-2. Thus, FSAP also cleaves PAR-2 at the canonical cleavage site.

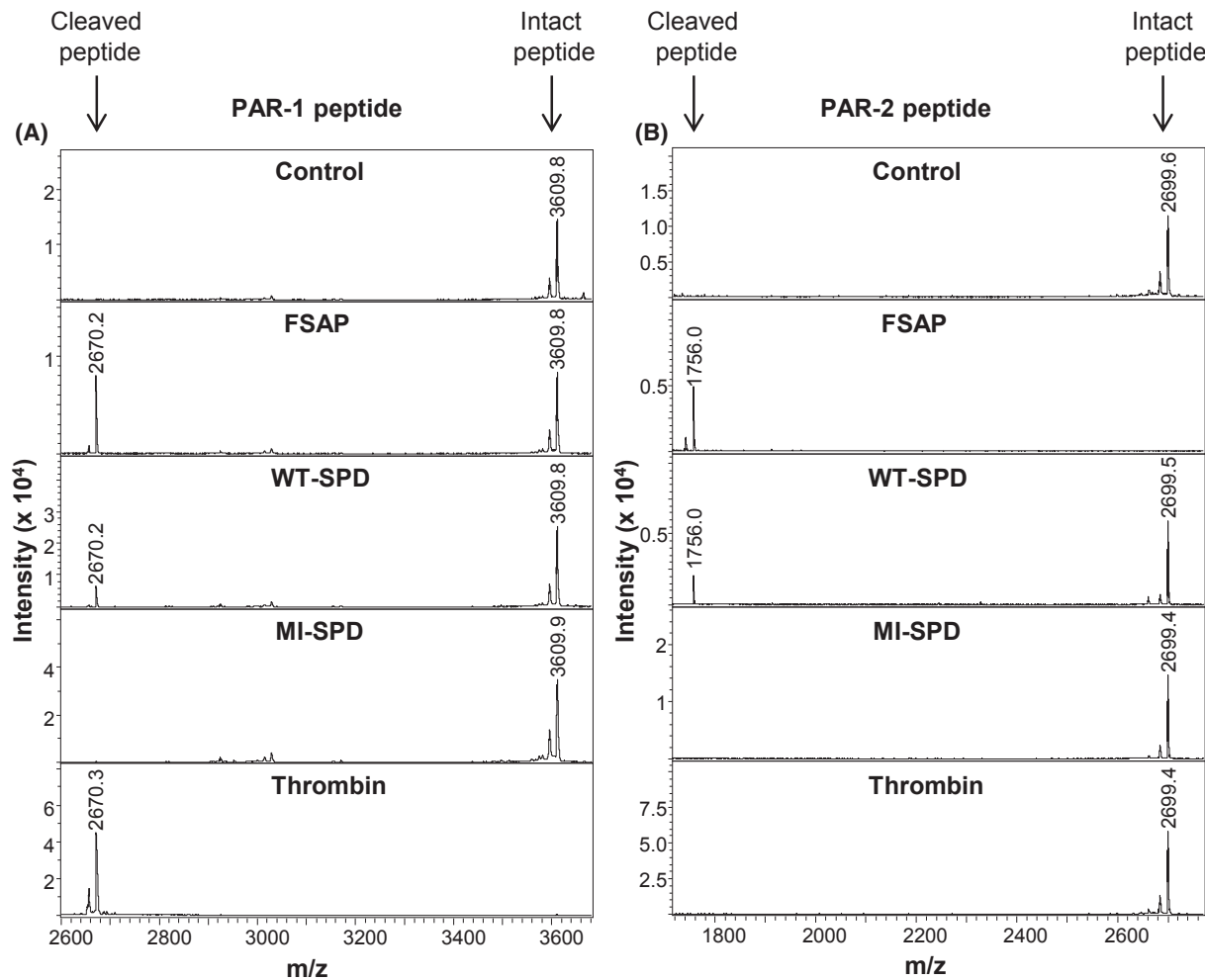
The above approach using specified mutants is based on the presumptions about the nature of PAR cleavage by FSAP. In principle, other basic residues in the N-terminal region of PARs could also be potential FSAP cleavage sites, leading to activation or even inactivation or “disarming” of PARs. To test this in an independent manner, the cleavage of synthetic peptides representing the N-terminal region of PAR-1 (33-62) and PAR-2 (28-54) by FSAP was investigated by mass spectrometry. The mass spectra showed that the PAR-1 peptide was cleaved by thrombin, FSAP and WT-FSAP-SPD, but

not MI-FSAP-SPD, at the canonical thrombin cleavage site. The same pattern was observed for PAR-2 except that this peptide was not cleaved by thrombin as expected (Figure 5). Thus, substrate specificity studies with synthetic peptides confirmed the results obtained by using site directed mutagenesis that the FSAP-mediated cleavage of PAR-1 and PAR-2 is at their canonical cleavage sites.

### 3.5 | FSAP has an anti-apoptotic effect on A549 cells

While the above results point to PAR-2 as a preferred substrate for FSAP on both HEK293T and A549 cells, we have previously observed that FSAP can confer anti-apoptotic properties on neurons and astrocytes<sup>14</sup> via PAR-1. We therefore revisited these results in a model of staurosporine-induced apoptosis. In HEK293T cells, the apoptotic response to staurosporine was not consistent (data not shown). In A549 cells, staurosporine induced apoptosis that was decreased in the presence of FSAP in an aprotinin-sensitive manner (Figure 6A). As expected, the WT-FSAP-SPD also





**FIGURE 5** FSAP and WT-SPD cleave synthetic N-terminal peptides from both PAR-1 and PAR-2: MALDI-MS spectra of (A) PAR-1 and (B) PAR-2 N-terminal peptides, amino acids 33-62, and amino acids 28-54 respectively, were untreated (control) or incubated with FSAP (6  $\mu\text{g}/\text{mL}$ ) for 3 hours, WT-SPD (3  $\mu\text{g}/\text{mL}$ ) for 3 hours, MI-SPD (3  $\mu\text{g}/\text{mL}$ ) for 3 hours, or thrombin (0.5 U/mL) for 1 hour. Positions of the intact and cleaved peptides are indicated by arrows

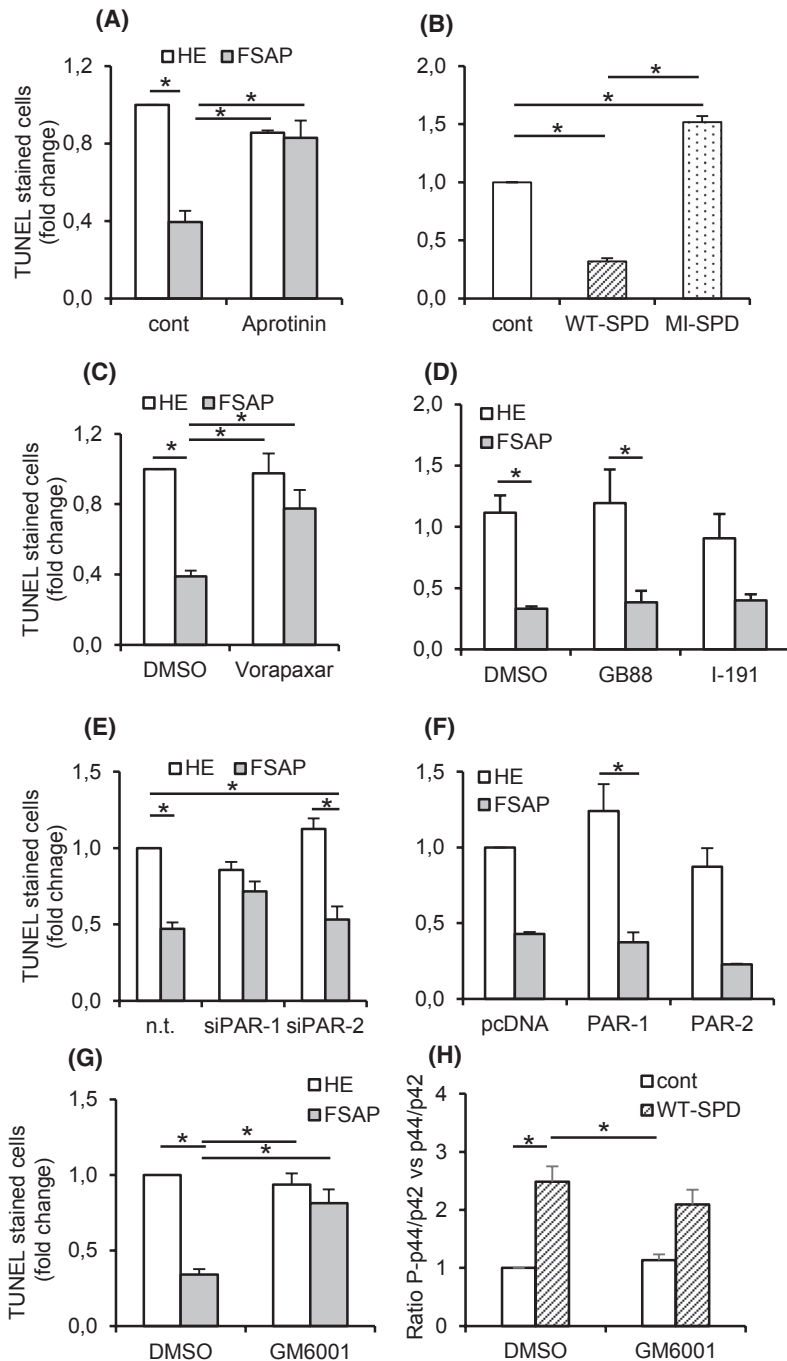
decreased apoptosis, while a slight increase was observed with MI-FSAP-SPD (Figure 6B). Combined, these results show that FSAP protects the cells against apoptosis in a mechanism dependent on its proteolytic activity.

A pharmacological approach was used to evaluate the role of endogenous PARs in regulating the actions of FSAP on apoptosis. Pretreatment of cells with a PAR-1 antagonist, vorapaxar, reversed the anti-apoptotic effect of FSAP (Figure 6C), whereas pretreatment of the cells with PAR-2 antagonists, GB88 and I-191, had no effect (Figure 6D). Gene silencing of PAR-1, but not PAR-2, using siRNA also inhibited the anti-apoptotic effect of FSAP (Figure 6E) providing further verification for the role of endogenous PAR-1 in this process. mRNA levels (Supplementary Figure S4D,E) as well as MAPK phosphorylation after treatment with PAR-1/2-AP and PAR-2-AP (Supplementary Figure S4F) were measured to confirm knockdown of the PAR's. Overexpression of PAR-1 and PAR-2 had little or no additive effect on the anti-apoptotic actions of FSAP (Figure 6F).

On the contrary, the anti-apoptotic effect of FSAP was completely abolished in the presence of the pan-MMP inhibitor GM6001, potentially implicating a role for the ADAM17/TACE pathway downstream of PAR-1.<sup>21</sup> Consistent with a downstream role for MMPs, GM6001 had no impact on FSAP-induced MAPK activation (Figure 6G,H).

## 4 | DISCUSSION

Different disease models in FSAP-knockout mice as well as the association of FSAP-encoding gene polymorphisms with human diseases indicates an important role for FSAP in vascular pathophysiology and cancer. However, molecular details of these effects, particularly relating to cellular receptors, are lacking. In the current study, we provide a detailed molecular characterization of the role of PARs in mediating the cellular effects of FSAP. An overexpression approach in HEK293T and A549 cells showed that FSAP activates



**FIGURE 6** Cytoprotective effect of FSAP in A549 cells as measured using TUNEL staining: Staurosporine (1  $\mu$ M) was used to induced apoptosis which was measured by TUNEL staining. A, Cells were treated with FSAP (10  $\mu$ g/mL) or HE buffer for 16 hours in the absence or presence of aprotinin (25  $\mu$ g/mL) before induction of apoptosis. B, Cells were treated with WT- or MI-SPD (10  $\mu$ g/mL) before induction of apoptosis. C, D, Cells were stimulated with FSAP or HE in the absence of presence of (C) Vorapaxar (1  $\mu$ M), (D) GB88 (10  $\mu$ M) or I-191 (10  $\mu$ M) before induction of apoptosis. E, Cells transfected with control siRNA or that against PAR-1 and PAR-2 were stimulated with FSAP or HE buffer before induction of apoptosis. F, Cells transfected with SEAP-PAR-1 and -2 were treated with FSAP or control HE buffer before induction of apoptosis. G, H, Cells were pretreated with GM6001 (20  $\mu$ M) or DMSO and treated with FSAP or HE buffer (G) or WT-SPD (H) before induction of apoptosis (G) or activation of MAPK (H). In all panels the percentage of apoptotic cells were counted and normalized to wells with staurosporine treatment alone. Each panel represents results from 3–4 independent experiments; mean  $\pm$  SEM, \* $P$  < .05

PAR-2 far more potently than PAR-1, and that it has no activity toward PAR-3 or PAR-4. The effects of FSAP were inhibited by aprotinin and could be replicated with recombinant WT-FSAP-SPD. The inactive MI-FSAP-SPD isoform, which had a single amino acid substitution rendering it catalytically inactive, was inactive against PARs but did show a higher ability to induce apoptosis in A549 cells. Canonical cleavage site mutations in PAR-1 and PAR-2 abolished the effects of FSAP, a finding that was supported by cleavage specificity toward synthetic peptides followed by mass spectrometry analysis.

For these studies, we used plasma-purified FSAP as well as the recombinant SPD domains of WT-FSAP and MI-FSAP. In both cell types, WT-SPD had a stronger effect on PAR-1 than plasma FSAP in both cell types. This may be related to the higher molar concentration of SPD used or could also be due to the fact that the SPD is missing the regulatory domains that further modulates its activity compared to plasma FSAP.

There were differences between the two cell types in relation to how the endogenous and exogenous PARs responded to FSAP. Activation of HEK293T cells with FSAP did not lead to a MAPK phosphorylation response, whereas

overexpression of PAR-2 lead to a strong MAPK phosphorylation. In A549 cells, activation with FSAP lead to a PAR-2 mediated activation of MAPK phosphorylation. Overexpression of PARs made no difference or even decrease the MAPK response. This may be because endogenous receptor signaling is already saturated or the two different pools of receptors are coupled to the signal transduction machinery in different ways. Overexpressed receptors may also inhibit or outcompete endogenous receptors. This could account for the cell-specific differences observed in the study.

The finding that PAR-2 is a preferred FSAP receptor is surprising considering that earlier studies attributed functional effects of FSAP to PAR-1 or PAR-3.<sup>13</sup> It is also surprising that cytoprotective actions of FSAP described in this study were more dependent on PAR-1 than on PAR-2. Engagement of intermediate PAR-activating protease zymogens produced by cells or provided by the culture medium could also result in productive, although indirect, PAR cleavage.<sup>19</sup> We tested the possibility that thrombin was activated in the system, but this was not the case. Thus, although PAR-2 is the preferred substrate for FSAP, both direct and secondary activation of PAR-1 may contribute to responses in cell types in which PAR-1 expression dominates. As the cleavage of only a minor fraction of plasma membrane PARs is sufficient to elicit a cellular response,<sup>19</sup> limited direct PAR-1 cleavage may also be sufficient to account for the functional responses of FSAP. Signaling through PAR-1:PAR-2 heterodimers<sup>3</sup> or the involvement of a FSAP cofactor in some cell types could also help explain the requirement for PAR-1 for some cellular responses. Overexpression does not appear to provide an explanation in our system, as PAR-2 preference was demonstrated for both endogenous and overexpressed receptors. Regardless, our observation suggest that in PAR-2-expressing cells, FSAP is likely to elicit cellular responses similar to those observed with coagulation factor Xa, another PAR-1/PAR-2 agonist.<sup>5</sup> This suggests mechanistic parallels in the modulation of cellular behavior by FSAP and coagulation proteases, which have also been implicated in vascular pathophysiology and cancer.

Both PAR-1 and PAR-2 have been linked to the regulation of apoptosis. Cleavage at the canonical Arg41 in PAR-1 by thrombin was shown to increase apoptosis,<sup>22</sup> whereas cleavage at Arg46 by APC was shown to protect cells against apoptosis.<sup>23,24</sup> In addition, activation of PAR-2 was shown to protect both colonic and lung epithelial cells from apoptosis.<sup>25,26</sup> In previous studies, we have observed that FSAP protected neurons and astrocytes from apoptosis via PAR-1.<sup>14</sup> Here, we show that FSAP protects A549 cells against apoptosis through PAR-1 and downstream MMP's.

A recent study has suggested that lung epithelial cancer cell lines express FSAP and that this FSAP is able to regulate cell migration and promote metastasis.<sup>12</sup> Overexpression of FSAP in thyroid cancer cell lines has also been shown

to promote cell migration and colony formation.<sup>11</sup> Some of these effects described earlier<sup>11</sup> are also elicited by MI-FSAP indicating that nonproteolytic signaling by FSAP is also possible. Tissue injury and inflammation trigger the activation of the zymogen form of FSAP through histones *in vivo*.<sup>27</sup> In blood, this can lead to the inactivation of tissue factor pathway inhibitor that in turn will increase factor VIIa and factor Xa activity<sup>28</sup> that can also contribute to PAR-1 and -2 activation.<sup>5</sup> Thus, activation of PAR-2 and PAR-1 by FSAP may account for some of the previously described actions of FSAP such as vascular remodeling,<sup>9,29</sup> stroke,<sup>14</sup> thrombosis,<sup>8</sup> liver fibrosis,<sup>30</sup> and thyroid cancer<sup>11</sup> (Supplementary Figure S5).

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## CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

## AUTHOR CONTRIBUTIONS

K. Byskov performed most of the experiments and analyzed the data and co-wrote the manuscript with S.M. Kanse. S.M. Le Gall and E. Camerer provided reagents and edited the manuscript. B. Thiede performed the mass spectrometry analysis. S.M. Kanse designed the study, analyzed the data and co-wrote the manuscript together with K. Byskov.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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