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Tissue Factor: Factor Viia Complex Regulates The Angiogenic Factors In Extra Villous Trophoblast Cell Lines

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Abstract

Background: *Angiogenesis is crucial during placental development and function, and its dysregulation contributes different pathologies. Tissue Factor (TF) forms a complex with Factor VIIa (FVIIa), activating Protease-Activated Receptor 2 (PAR2), which influences pro-angiogenic responses. This study investigates TF: FVIIa-PAR2 signaling in HTR-8/SVneo cells, an in vitro model for villous trophoblast angiogenesis.*

Methods: *HTR-8/SVneo cells were treated with FVIIa and a PAR2 activating peptide (PAR2 AP), SLIGKV-NH2. Total RNA was extracted, reverse transcribed into complementary DNA (cDNA), and reverse transcriptase polymerase chain reaction (RT-PCR) was performed to assess gene expression of Vascular Endothelial Growth Factor (VEGF), Placenta Growth Factor (PGF), and Platelet-Derived Growth Factor (PDGF). Beta-actin (β-actin) served as the internal reference.*

Results: *FVIIa treatment significantly upregulated VEGF mRNA by approximately 0.1-fold ($p<0.05$), PGF mRNA by approximately 0.4-fold ($p<0.05$), and PDGF mRNA by approximately 0.4-fold ($p<0.05$) compared to untreated control. PAR2 AP similarly increased VEGF mRNA by approximately 0.1-fold ($p>0.05$), PGF mRNA by approximately 0.8-fold ($p<0.01$), and PDGF mRNA by approximately 0.9-fold ($p<0.01$) compared to control.*

Conclusion: *TF: FVIIa-PAR2 signaling potently upregulates key pro-angiogenic factors in HTR-8/SVneo cells, highlighting its critical role in placental angiogenesis and potential implications for pregnancy complications.*

Keywords: *Trophoblast, Angiogenesis, Tissue factor, PAR2, VEGF.*

INTRODUCTION

Angiogenesis, defined as the intricate process of new blood vessel formation from pre-existing vasculature, is a fundamental biological phenomenon essential for numerous physiological processes, including embryonic development, wound healing, and the cyclical changes of the menstrual cycle [1]. However, the delicate balance of angiogenic regulation can be disrupted, leading to dysregulated angiogenesis that underlies the pathogenesis of a wide array of diseases, such as various cancers, diabetic retinopathy, and macular degeneration [2]. A comprehensive understanding of the precise molecular mechanisms governing angiogenesis and its dysregulation is therefore of paramount importance for the development of targeted therapeutic strategies across these diverse clinical conditions.

Tissue Factor (TF), a 47 kDa transmembrane glycoprotein, is widely recognized as the primary cellular initiator of the extrinsic coagulation cascade. Upon vascular injury, TF becomes exposed to circulating blood, where it binds to Factor VIIa (FVIIa), a serine protease, to form the TF:FVIIa complex. This complex then catalytically activates Factor X (FX) and Factor IX (FIX), initiating a cascade that culminates in thrombin generation and subsequent fibrin formation, which is crucial for hemostasis [3]. Beyond its well-established role in blood coagulation, the TF:FVIIa complex also possesses significant non-coagulant cell signaling properties that are independent of its procoagulant function. This signaling capacity plays a pivotal role in various cellular processes, including inflammation, cell proliferation, cell survival, and notably, angiogenesis [4]. The ability of TF:FVIIa to elicit cellular responses through these non-coagulant pathways is a critical area of investigation. This distinction between the procoagulant and non-coagulant signaling roles of TF:FVIIa is particularly relevant for therapeutic

interventions. Focusing on the non-coagulant signaling pathways offers a promising avenue for inhibiting pathological angiogenesis without incurring the increased risk of bleeding associated with broad inhibition of the TF coagulation pathway [5]. This selective targeting represents a significant advancement in the development of anti-angiogenic therapies as well.

The TF:FVIIa complex directly cleaves and activates Protease-Activated Receptor 2 (PAR2), a G protein-coupled receptor. This proteolytic cleavage exposes a tethered ligand that then interacts with and activates the receptor [6]. A key characteristic distinguishing PAR2 from other protease-activated receptors (PARs), such as PAR1, is that PAR2 is not activated by thrombin, making it a distinct and specific signaling node in the context of coagulation factor-mediated cellular responses [7]. Activation of PAR2 by TF:FVIIa triggers a complex array of intracellular signaling cascades. These include canonical G protein-coupled pathways, as well as G protein-independent pathways mediated by the recruitment of intracellular adaptor proteins like β-arrestin [8]. This intricate signaling network is known to induce the expression of a diverse set of pro-angiogenic and immune-modulatory cytokines, chemokines, and growth factors [9]. The TF cytoplasmic domain also plays an important role in controlling PAR2 signaling and subsequent Vascular Endothelial Growth Factor (VEGF) expression, with its phosphorylation status correlating with VEGF levels in cancer [10]. Furthermore, interactions with integrins, particularly β1 and αvβ3, are crucial for TF:FVIIa-PAR2 signaling and the subsequent internalization of the complex, which influences downstream signaling events [11]. The consistent emphasis across multiple studies on PAR2, rather than PAR1, as the primary mediator of TF:FVIIa-driven angiogenesis underscores a highly specific and conserved mechanism. This specificity in receptor activation reinforces the potential for developing highly targeted therapeutic strategies that can precisely modulate angiogenic processes.

HTR-8/SVneo cells are an immortalized human extravillous trophoblast cell line, originally derived from first-trimester placental explants. These cells exhibit a variety of characteristic markers of invasive trophoblast cells in situ, including insulin-like growth factor (IGF)-II, proliferating cell nuclear antigen (PCNA), and a distinct set of integrins (alpha 1, alpha 3, alpha 5) [12, 13]. They possess a high propensity to form spheroid bodies, indicative of self-renewal capacity, and are capable of undergoing epithelial-to-mesenchymal transition (EMT), a process vital for their invasive potential during placental development. HTR-8/SVneo cells are widely utilized as a robust in vitro model to investigate trophoblast biology, placental function, and the mechanisms underlying various placental pathologies. This includes conditions such as preeclampsia, which is characterized by insufficient trophoblast invasion and angiogenesis, and gestational trophoblastic disease, involving excessive invasion and angiogenesis [14]. Importantly, TF and TF:FVIIa-dependent integrin regulation have been shown to be expressed and active in HTR-8/SVneo cells, influencing their migration and invasion. It is specifically demonstrated that FVIIa treatment led to a dose-dependent upregulation of integrins in HTR-8/SVneo cells, without witnessing cytotoxicity or apoptosis [13]. The selection of HTR-8/SVneo cells for this study provides a unique biological context, allowing for the investigation of TF:FVIIa signaling within a model directly relevant to placental development and its associated pathologies. This approach contributes specific knowledge to the understanding of placental angiogenesis, which is distinct from the more commonly studied cancer angiogenesis models, thereby adding significant depth and specificity to the research's implications for reproductive health.

Based on the established roles of TF: FVIIa-PAR2 signaling in angiogenesis and the characteristics of HTR-8/SVneo cells, it is hypothesized that TF: FVIIa-PAR2 signaling dysregulates angiogenesis in HTR-8/SVneo cells by upregulating key pro-angiogenic growth factors. This study aims to characterize the expression of pro-angiogenic genes, specifically Vascular Endothelial Growth Factor (VEGF), Placenta Growth Factor (PGF), and Platelet-Derived Growth Factor (PDGF), in HTR-8/SVneo cells following stimulation with FVIIa and a specific PAR2 activating peptide, and to elucidate the role of PAR2 in mediating these observed pro-angiogenic effects in HTR-8/SVneo cells.

MATERIALS AND METHODS

MATERIALS

Recombinant human FVIIa was obtained from Merck (Germany). The PAR2 activating peptide (PAR2 AP), SLIGKV-NH2, a known and specific agonist for PAR2 (Ki = 9.64 μM and IC_{50} = 10.6 μM), was obtained from Sigma (United States). The cDNA reverse transcription kit and PCR master mix were from Takara (Japan). All other general reagents, including trypsin-EDTA solution for cell detachment, were procured from Himedia

(India).

CELL CULTURE

HTR-8/SVneo cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2. For experimental treatments, HTR-8/SVneo cells were cultured to approximately 70-80% confluence in 6-well plates. Prior to treatment, cells were serum-starved for 12-16 hours in DMEM containing 0.5% FBS to minimize interference from growth factors present in serum. Following serum starvation, cells were treated with recombinant human FVIIa (10 nM) and PAR2 AP (10.6 µM) for 24 hours.

RNA ISOLATION AND CDNA SYNTHESIS

Total RNA was extracted from treated and control HTR-8/SVneo cells using Trizol reagent according to the manufacturer's instructions. RNA quantity and purity were assessed using a spectrophotometer (NanoDrop One, Thermo Scientific), with A260/A280 ratios between 1.8-2.0 considered acceptable. Complementary DNA (cDNA) was synthesized from approximately 2 µg of total RNA using a high-capacity cDNA reverse transcription kit (Takara, Japan) with random primers, following the manufacturer's recommended protocol.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) FOR GENE EXPRESSION ANALYSIS

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to quantify the mRNA expression levels of target genes. Reactions were carried out using a thermocycler. Each reaction contained cDNA template, gene-specific forward and reverse primers, and PCR master mix. The thermocycling conditions typically included an initial denaturation step (95°C for 4 min), followed by 35 cycles of denaturation (94°C for 1 min) and annealing (58°C for 45 sec) and extension (72°C for 1 min). The PCR products were then loaded onto a 1% agarose gel for electrophoresis and visualized using a LAS500 Chemidoc system (GE Healthcare, United States) for documentation.

The target genes investigated were VEGF, PGF, and PDGF. β-actin was used as the endogenous reference gene for normalization to account for variations in mRNA input and reverse transcription efficiency. Relative gene expression was determined by analyzing the band intensities. The primer sequences used for amplification are detailed in Table 1.

Table 1: Primer Sequences for Gene Expression Analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)
VEGF	TTGCCTTGCTGCTCTACCTC	AGCTGCGCTGATAGACATCC	117
PGF	ACTCTTCAGTGACTCCTGCT	TGGCTGGCTTCTCTCTTTCT	121
PDGF	CCTTCCAAAACCTGCTTCCTTC	CCGGCGGATTCATTTGGTTT	137
β-actin	CTTGCGGTATCCACGAGAC	GCGCCATACAGAGCAGAA	132

STATISTICAL ANALYSIS

All experiments were performed in triplicate and repeated independently at least three times. Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 10.0). Differences between groups were assessed using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (e.g., Tukey's multiple comparisons test) for multiple group comparisons. A two-tailed Student's t-test was used for comparisons between two groups. A p-value of <0.05 was considered statistically significant.

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RESULTS

VEGF GENE EXPRESSION UPREGULATED BY FVIIA AND PAR2 AP

Reverse transcriptase PCR analysis revealed that both FVIIa and the PAR2 AP significantly upregulated the gene expression of VEGF in HTR-8/SVneo cells. Following FVIIa treatment, VEGF mRNA levels were increased by approximately 0.1-fold compared to untreated control cells as depicted in figure 1. Stimulation with PAR2 AP also resulted in an increase of VEGF mRNA by approximately 0.1-fold ($p>0.05$). The consistent upregulation of VEGF by FVIIa and a direct PAR2 agonist strongly indicates that the TF:FVIIa complex may signals through PAR2 to promote the expression of this major pro-angiogenic factor. This finding establishes a direct link between the activation of the TF:FVIIa-PAR2 signaling pathway and the transcriptional regulation of a key mediator of angiogenesis.

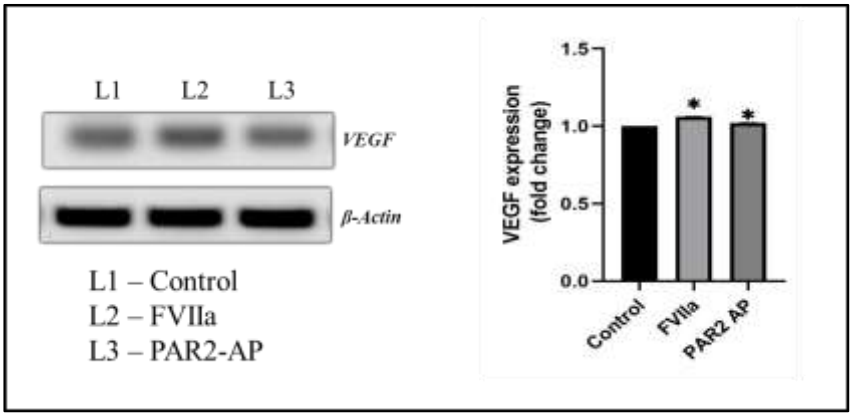

Figure 1. Vascular Endothelial Growth Factor (VEGF) gene expression in HTR-8/SVneo cells. Cells were treated with FVIIa or PAR2 AP. Data are presented as fold change relative to control. *$p<0.05$.

PGF GENE EXPRESSION INCREASED BY FVIIA AND PAR2 AP

In addition to VEGF, the gene expression of Placental Growth Factor (PGF), another important member of the VEGF family, was also significantly upregulated in HTR-8/SVneo cells following treatment. As indicated in figure 2, FVIIa stimulation led to an increase in PGF mRNA levels by approximately 0.3-fold ($p<0.05$), while PAR2 AP treatment resulted in an increase of approximately 0.8-fold ($p<0.01$) compared to control cells. While PGF is recognized as part of the VEGF family and TF: FVIIa-PAR2 signaling is known to upregulate a broad range of pro-angiogenic proteins, demonstrating its upregulation in HTR-8/SVneo cells represents a specific contribution to understanding the breadth of pro-angiogenic factors influenced by the TF: FVIIa-PAR2 pathway within the context of trophoblast biology. This highlights the comprehensive influence of this signaling axis on the angiogenic profile of placental cells.

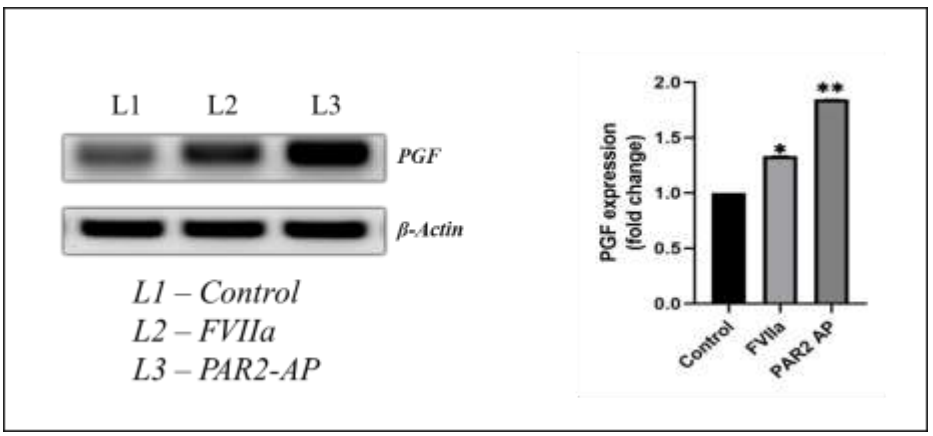

Figure 2. Placenta Growth Factor (PGF) gene expression in HTR-8/SVneo cells. Cells were treated with FVIIa or PAR2 AP. Data are presented as fold change relative to control. *$p<0.05$, **$p<0.01$.

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PDGF GENE EXPRESSION ELEVATED BY FVIIA AND PAR2 AP

Furthermore, the gene expression of Platelet-Derived Growth Factor (PDGF) was also significantly increased in HTR-8/SVneo cells upon exposure to FVIIa and PAR2 AP. As demonstrated in figure 3, FVIIa treatment resulted in an increase in PDGF mRNA by approximately 0.4-fold ($p<0.05$), and PAR2 AP treatment induced an increase of approximately 0.9-fold ($p<0.01$). The upregulation of PDGF is particularly noteworthy given its known synergistic relationship with PAR2-dependent angiogenesis.[11] This observation suggests a multi-pronged pro-angiogenic effect of TF:FVIIa-PAR2 signaling. The pathway might not only directly stimulate angiogenesis through factors like VEGF and PGF but also create a more permissive or amplified angiogenic environment by upregulating factors such as PDGF that are known to synergize with its own effects. This indicates a deeper level of complexity in the pathway's influence on the angiogenic microenvironment, potentially involving positive feedback loops or complementary signaling.

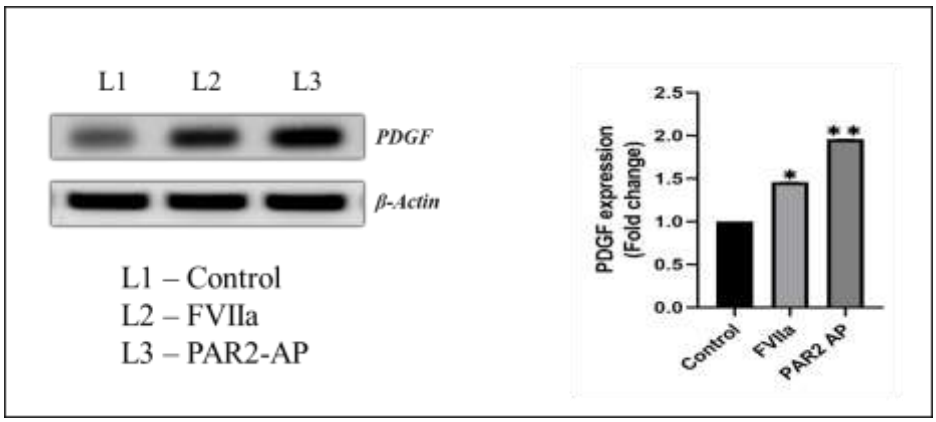

Figure 3. Platelet-Derived Growth Factor (PDGF) gene expression in HTR-8/SVneo cells. Cells were treated with FVIIa or PAR2 AP. Data are presented as fold change relative to control. *$p<0.05$, **$p<0.01$.

DISCUSSION

The present study demonstrates that both FVIIa and PAR2 AP significantly upregulate the gene expression of Vascular Endothelial Growth Factor (VEGF), Placenta Growth Factor (PGF), and Platelet-Derived Growth Factor (PDGF) in HTR-8/SVneo cells. These findings strongly underscore the direct and potent role of TF: FVIIa-PAR2 signaling in promoting angiogenesis. This observed effect is consistent with its established pro-angiogenic functions in various other cell types, particularly within the context of tumor angiogenesis [15]. The upregulation of these critical angiogenic factors in HTR-8/SVneo cells specifically extends the known pro-angiogenic role of TF: FVIIa-PAR2 signaling from cancer models to the distinct context of trophoblast biology. This expansion of understanding suggests a conserved mechanism of angiogenic regulation by this pathway across different physiological and pathological settings. The consistency of this signaling axis across diverse cellular environments reinforces its fundamental importance in controlling vascular development and remodeling.

The observed upregulation of VEGF, PGF, and PDGF in response to FVIIa and PAR2 AP highlights the central role of PAR2 in mediating these pro-angiogenic effects. PAR2 activation by FVIIa, which occurs through proteolytic cleavage of the receptor's N-terminus, initiates a cascade of intracellular signaling events [16]. These include canonical G protein-coupled pathways and G protein-independent pathways, notably involving β-arrestin recruitment [8]. Downstream of PAR2 activation, pathways such as the Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) pathway are known to be engaged, leading to transcriptional changes that promote angiogenesis. For instance, VEGF is a major transcriptional target, and its expression is tightly controlled by the TF cytoplasmic domain, with phosphorylation of this domain correlating with VEGF levels [17, 18]. The finding that a direct PAR2 agonist, SLIGKV-NH2, elicits similar pro-angiogenic effects to FVIIa provides robust support for the conclusion that PAR2 is the primary receptor mediating these specific angiogenic responses of the TF:FVIIa complex in HTR-8/SVneo cells. This helps to distinguish the PAR2-dependent effects from other potential TF-FVIIa interactions, such as integrin binding, that might not directly involve PAR2 cleavage. The observed upregulation of PDGF, particularly considering its known synergistic relationship with PAR2-dependent angiogenesis, suggests a complex interplay of growth factors [19].

This indicates that TF:FVIIa-PAR2 signaling might not only directly stimulate angiogenesis but also contribute to a more permissive or amplified angiogenic environment by upregulating factors that synergize with its own effects, thereby creating a more robust pro-angiogenic response.

The current findings in HTR-8/SVneo cells align remarkably well with existing literature concerning TF:FVIIa-PAR2 signaling in various contexts, particularly in cancer angiogenesis. Previous studies have consistently demonstrated that TF:FVIIa-PAR2 signaling upregulates a range of pro-angiogenic factors, including VEGF, Interleukin 8 (IL-8), Fibroblast Growth Factor 2 (FGF2), and C-X-C Motif Chemokine Ligand 1 (CXCL1), in tumor cells [5]. The observed increase in VEGF, PGF (a VEGF family member), and PDGF in trophoblasts mirrors these established responses, indicating a conserved molecular mechanism. The role of integrins, such as $\alpha v\beta 3$ and $\beta 1$, in mediating TF:FVIIa signaling and angiogenesis has also been extensively documented [11]. These adhesion molecules are known to interact with TF:FVIIa and influence downstream signaling, including the internalization of the complex, which is crucial for prolonged MAPK activation downstream of PAR2. Gundappa et al. (2022) specifically showed that FVIIa treatment upregulates integrins in HTR-8/SVneo cells, influencing their migration [13]. Furthermore, a consistent finding across many studies is that PAR2, and not PAR1, is crucial for TF-dependent angiogenesis. The congruence of our findings in HTR-8/SVneo cells with these established mechanisms in cancer models suggests a universal role for this non-coagulant pathway in regulating angiogenesis across different pathological states. This strengthens the argument for targeting this pathway for broader therapeutic applications, as the fundamental signaling components and their pro-angiogenic outcomes appear to be conserved across diverse biological systems.

Given that HTR-8/SVneo cells serve as an in vitro model for extra villous trophoblasts, the findings of this study carry significant implications for understanding normal placental development and the pathogenesis of placental complications. Normal placental development necessitates tightly regulated angiogenesis to ensure adequate fetal growth and efficient maternal-fetal exchange. Dysregulation of this process can lead to severe pregnancy complications [20]. For instance, conditions like preeclampsia are characterized by insufficient trophoblast invasion and impaired angiogenesis, leading to inadequate placental perfusion [21]. Conversely, gestational trophoblastic diseases involve excessive trophoblast proliferation and invasion, often accompanied by aberrant angiogenesis [22]. The demonstration that TF:FVIIa-PAR2 signaling potently upregulates pro-angiogenic factors in trophoblasts suggests that aberrant activation or dysregulation of this pathway could directly contribute to these pathologies by tipping the angiogenic balance. This establishes TF:FVIIa-PAR2 mediated angiogenesis as a critical regulatory pathway for placental development and function. Understanding how this pathway is modulated in health and disease could offer novel therapeutic targets for managing pregnancy complications, potentially by selectively modulating trophoblast angiogenic capacity.

LIMITATIONS AND FUTURE DIRECTIONS

This study, while providing significant mechanistic insights, has certain limitations inherent to its in-vitro design. The focus on gene expression provides valuable information on transcriptional regulation, but it does not directly assess protein expression levels or functional angiogenic outcomes. Future research should therefore investigate the protein levels of VEGF, PGF, and PDGF using techniques such as Western blotting or Enzyme-Linked Immunosorbent Assay (ELISA). Furthermore, functional angiogenic assays, including in vitro tube formation assays, cell migration assays, and invasion assays, are essential to confirm the biological impact of the observed gene expression changes in HTR-8/SVneo cells.

Further exploration of the specific downstream signaling molecules activated by PAR2 in HTR-8/SVneo cells, beyond the general MAPK/ERK pathway, is warranted. Investigating the involvement of pathways such as Phosphatidylinositol 3-Kinase (PI3K)/AKT Serine/Threonine Kinase (AKT)/Mammalian Target of Rapamycin (mTOR), which are known to be activated by TF and influence cell proliferation and survival [7], could provide a more comprehensive understanding of the signaling network. Ultimately, validating these in vitro findings in relevant in vivo models of placental dysfunction would be crucial to translate these molecular insights into potential therapeutic strategies for pregnancy complications.

CONCLUSION

This research elucidates a critical role for Tissue Factor: Factor VIIa-Protease-Activated Receptor 2 (TF: FVIIa-PAR2) signaling in the dysregulation of angiogenesis in HTR-8/SVneo cells. The findings unequivocally

demonstrate that activation of this pathway, either by FVIIa or a specific PAR2 activating peptide, leads to a significant upregulation of key pro-angiogenic growth factors, namely VEGF, PGF, and PDGF. This study extends the understanding of TF: FVIIa-PAR2 signaling, previously characterized in contexts such as cancer, to the unique biological environment of human extra villous trophoblasts. The observed pro-angiogenic effects highlight the potential involvement of this pathway in the intricate process of placental development and its associated pathologies. Understanding the mechanisms by which TF: FVIIa-PAR2 signaling modulates angiogenesis in trophoblasts offers novel avenues for exploring therapeutic interventions aimed at restoring angiogenic balance in conditions like preeclampsia and other pregnancy complications.

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