SPOTLIGHT REVIEW ARTICLE

Hemostasis and endothelial functionality: the double face of coagulation factors

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Received: October 2, 2023.
Accepted: February 15, 2024.
Early view: February 29, 2024.

https://doi.org/10.3324/haematol.2022.282272

Abstract

Hemostasis is a sophisticated sequence of events aimed at repairing vessel injury. This process occurs in combination with angiogenesis, which leads to new blood vessel formation, helping in wound repair and facilitating tissue healing. The fine mechanisms that regulate hemostasis and angiogenesis are well described, but for a long time, coagulation factors (CF) have been considered merely players in the coagulation cascade. However, evidence from several experiments highlights the crucial functions of these CF in regulating endothelial functionality, especially in the angiogenic process. Some of these CF (e.g., thrombin and tissue factor) have been widely investigated and have been described as triggering intracellular signaling related to endothelial cell (EC) functionality. For others (e.g., factor VIII and thrombomodulin), potential receptors and molecular mechanisms have not been fully elucidated but some data show their potential to induce EC response. This review focuses on the emerging roles of selected CF in regulating EC functions, highlighting in particular their ability to activate signaling pathways involved in angiogenesis, migration, proliferation and endothelial barrier stability.

Introduction

Coagulation factors (CF) are a well-known class of proteins essential for hemostasis. They can be divided into pro- and anti-CF according to their function in promoting or arresting coagulation, respectively. Pro-CF work together in a cascading sequence, initiating a series of enzymatic reactions that lead to the formation of a blood clot; this process is counterbalanced by anti-CF for a correct homeostasis. CF participate in the regulation of the clotting process by interacting with platelets, vessel walls, and other proteins to form a stable clot, thus preventing excessive bleeding when blood vessels are injured.[1]

Beyond hemostasis, angiogenesis is a physiological mechanism involved in the repair of vessel injury. The vascular sprouting and the new blood vessel formation are required for wound closure and co-operate with the hemostatic system maintaining blood flow and regulating platelet adherence and fibrin deposition. During vascular injury, initial vessel constriction occurs to control blood flow and to reduce hemorrhage, followed by sub-endothelial matrix exposure where platelets can adhere.[2] Vascular endothelial cells are first required to bind and anchor the clot and then, from the clot margins, they invade the fibrin structure to form a new vessel wall. Many proteins released by endothelial cells (EC) and present in the blood are required to finely control this process. Indeed, by adhering to EC, platelets regulate angiogenesis releasing pro-angiogenic molecules, like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), angiopoietin-1 (Ang1), insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), platelet-derived growth factor (PDGF), and sphingosine 1-phosphate. On the other hand, platelets can also release proteins that inhibit vessel formation, such as endostatin, thrombospondin-1 (TSP-1), plasminogen activator inhibitor-1 (PAI-1), and angiostatin.[3] Recent evidence supports the notion that angiogenesis and broader EC functions can be influenced by some specific CF. These factors have been shown to have a variety of roles, encompassing both pro- and anti-angiogenic features, and influencing EC permeability. Their multifaceted action extends beyond simple hemostatic roles, contributing to the intricate modulation of angiogenic processes and the regulation of EC barrier function.[2]

Thus, studying the crossroads between hemostasis and angiogenesis would promote better understanding of vascular dynamics. This review aims to explore this intricate interplay, deciphering the dual facets of some CF, not merely as hemostatic agents, but also as regulators of EC functionality.

The role of several coagulation factors on endothelial cell functionality

Thrombin

Thrombin, a multifaceted enzyme pivotal in hemostasis, plays a critical role in forming clots by cleaving fibrinogen into fibrin, generating the structural backbone of clots that entrap platelets and blood cells to arrest blood flow from injured vessels. It amplifies the coagulation cascade by activating the platelets, further helping clot formation at injury sites. Its influence extends beyond coagulation, triggering inflammation and specific signaling pathways through G protein-coupled receptors (GPCR), particularly protease-activated receptor 1 (PAR1), across various cell types.[1]

Interestingly, thrombin has been demonstrated to impact on EC in several different ways, including increasing their permeability. Indeed, thrombin disrupts the endothelial barrier, influencing the organization and disassembly of cell-cell adhesion proteins.[4] By binding to GPCR, thrombin decreases cyclic adenosine 3′,5′-monophosphate (cAMP) and increases Ca^{2+} levels through specific secondary mediators, resulting in endothelial cytoskeleton rearrangement and regulation of Ras homologous (Rho) family GTPases, ultimately leading to increased permeability.[5] Recent phospho-proteomic analyses of thrombin-stimulated EC unveiled a novel non-canonical thrombin-mediated pathway through p38 mitogen-activated protein (MAP) kinase, promoting EC barrier disruption.[6] Furthermore, corroborating this hypothesis, some studies indicate that thrombin induces capillary tube regression akin to other pro-inflammatory agents and is responsible for endothelial dysfunction while the inhibition of its activity partially restores physiological EC homeostasis.[7]

Consequently, extensive research has been carried out to probe into the angiogenic role of thrombin and its impact on tumor development. Notably, thrombin elevates the expression of various angiogenic factors like angiopoietin-2, growth-regulated oncogene (GRO-α) and VEGF through c-FOS transcriptional regulation in *in vitro* EC culture.[8] It has also been described as increasing cancer invasion enhancing the expression of matrix metalloproteinase-9 and integrin βeta1 (β1) on the cell surface[9] and promoting vasculogenic mimicry, a process which transforms tumor cells into EC, through PAR1 and nuclear factor kappaB (NF-κB) signaling.[10] Its angiogenic potential is also evident in rat brains, where it enhances new vessel formation after intracranial hemorrhage, and its inhibition preserves blood-brain barrier integrity.[11] In agreement with this, a role in murine embryonic vascular development has been shown for PAR1, and its inhibition results in reduced angiogenesis and attenuated permeability in *in vivo* models.[12,13] Interestingly, thrombin has been observed to control EC functions not only through PAR, but also directly binding integrin alpha-v-beta3 (αvβ3) and promoting EC attachment, migration and survival.[14] The binding of thrombin with integrins should not be surprising, considering the pivotal role of these receptors in finely regulating EC functions.

However, the role of thrombin on the *in vitro* formation of vascular tubule networks remains a topic of debate, contingent upon the quantity of thrombin employed for EC stimulation: high concentrations enhance EC permeability through great PAR1 activation, while lower doses exhibit a protective effect, mainly cleaving PAR3.[15] Additionally, thrombin has been associated with both venoconstriction and arterial vasodilation, highlighting the complexity of its role in endothelium biology.[16]

Current data suggest that thrombin induces unique but conflicting responses within the human vasculature (Table 1), emphasizing the need for new studies to confirm its action on EC.

Factor VIII and von Willebrand factor

Factor VIII (FVIII) and its partner, von Willebrand factor (vWF), engage in a tightly co-ordinated interplay within the coagulation cascade, forming a complex that is pivotal for hemostasis. vWF acts as a carrier protein for FVIII, safeguarding it from premature degradation in the bloodstream. Upon vascular injury, vWF adheres to exposed collagen at the injury site, facilitating platelet adhesion and aggregation. This localization of vWF-bound FVIII primes the coagulation process, enabling FVIII to interact with activated platelets and initiate the coagulation cascade, increasing the catalytic activity of factor IX (FIX) and, thus, activating factor X (FX).[17]

Given the primary production of FVIII by EC,[18] recent studies have been investigating the link between FVIII and EC functionality. Specifically, FVIII has been shown to induce transcriptional and functional changes in EC leading to a decrease in *in vitro* adherence and increased permeability, with paxillin as main mediator of these changes.[19] An altered and uncontrolled joint vascular remodeling has also been shown in FVIII-deficient mice after induction of hemarthrosis, suggesting a non-physiological angiogenic mechanism which could be a contributing cause of prolonged and repetitive bleedings.[20] Indeed, hemophilia A (HA) patients show reduced flow-mediated dilation (FMD) and hyperemic velocity time integral (VTI) compared to healthy controls,[21] pointing to alterations of both macrovascular and microvascular endothelial functions for

Table 1. Summary of endothelial cell functions regulated by selected pro-coagulation factors.

Pro-CF	Study	*In vitro* or *in vivo* models	Regulated EC function	Identified receptor
Thrombin	Rabiet *et al.*[4]	HUVEC and EA.hy926	Permeability	-
	Hirano *et al.*[5]	Porcine aortic EC	Permeability	-
	Lin *et al.*[6]	EA.hy926	Permeability	PAR1
	Koller *et al.*[7]	HUVEC and mice	Capillary tube regression	-
	Catar *et al.*[8]	HMEC-1	Angiogenesis	PAR1
	Radjabi *et al.*[9]	U2-OS osteosarcoma cells	Invasion	PAR1
	Zhao *et al.*[10]	A549 cells and Lewis cells	Vasculogenic mimicry	PAR1
	Zhou *et al.*[11]	Rat	Angiogenesis	-
	Griffin *et al.*[12]	PAR1-deficient mice	High embryonic lethality	PAR1
	Li *et al.*[13]	BMX-KO mice	Permeability	PAR1
	Tsopanoglou *et al.*[14]	HUVEC	Attachment, migration and survival	Integrin αvβ3
	Zolotoff *et al.*[15]	HBEC-5i EC	Alteration of BBB depending on the dose	PAR1 PAR3
	Guðmundsdóttir *et al.*[16]	Human subjects	Vasoconstriction and vasodilation	PAR1
Factor VIII	Cadé *et al.*[19]	HUVEC	Permeability	-
	Bhat *et al.*[20]	FVIII-deficient mice	Angiogenesis	-
	Sun *et al.*[21]	HA patients	FMD and VTI	-
	Manon-Jensen *et al.*[22]	HA patients	ECM production	-
Von Willebrand factor	Starke *et al.*[24]	HUVEC and healthy and vWD BOEC	Proliferation, migration and angiogenesis	Integrin αvβ3
	Randi *et al.*[25]	vWF-deficient mice	Angiogenesis	-
	Xu *et al.*[26]	vWF-deficient and anti-vWF antibody-treated mice	Angiogenesis	-
	de Vries *et al.*[27]	vWF-deficient mice	Angiogenesis	-
	Ishihara *et al.*[28]	vWF-deficient mice and HUVEC	Angiogenesis	-
Tissue factor	Giannarelli *et al.*[33]	HUVEC and human aortic EC	Angiogenesis	Integrins
	Van Den Berg *et al.*[34]	ECRF cell line and HUVEC	Angiogenesis and migration	Integrin α6β1 Integrin αVβ3
	Kocatürk *et al.*[35]	MCF-7	Proliferation	Integrin β1
	Sluka *et al.*[36]	asTF knock-in mice	Embryonic lethality due to vascular instability	-
	Carmeliet *et al.*[37]	flTF-deficient mice	Embryonic lethality due to vascular instability	-
	Zhu *et al.*[38]	pCMVEC	Angiogenesis	PAR2

Pro-CF: pro-coagulation factor; HUVEC: human umbilical vein endothelial cells; EA.hy926: human umbilical vein cell line; EC: endothelial cells; HMEC-1: human microvascular endothelial cells; U2-OS: osteosarcoma cell line; A549: adenocarcinomic human alveolar basal epithelial cells; Lewis cells: non-small-cell lung cancer (NSCLC) of mice; BMX: bone marrow kinase on the X chromosome; HBEC-5i: cerebral microvascular endothelial cell line; ECRF cell line: immortalized vascular endothelial cells (EC); MCF-7: human breast cancer cell line; pCMVEC: porcine cerebral microvascular endothelial cells; asTF: alternative splicing tissue factor; flTF: full length TF; PAR: protease-activated receptor; BBB: blood barrier brain; HA: hemophilia A; FMD: flow-mediated dilation; VTI: velocity time integral; ECM: extracellular matrix; BOEC: blood out-growth endothelial cell; vWD: von Willebrand disease.

which no clear culprit has yet been identified; we could speculate that the absence of FVIII itself might induce these impairments. This hypothesis is further sustained by the fact that treatment of HA patients with FVIII results in a reduced extracellular matrix dysfunction,[22] likely responsible for the increased vessel permeability in these individuals. The potential role of FVIII in EC hemostasis needs to be further characterized and the mechanism(s) triggered by FVIII should be elucidated. Many receptors control the bioavailability of FVIII, but none of them have been described as transducing a signaling related to EC functionality after FVIII binding. There is some evidence to show that these receptors are involved in the regulation of EC; in particular, low-density lipoprotein receptor-related protein 1 (LRP-1) has been demonstrated to have a role in vascular permeability and angiogenesis, but its expression in EC is still controversial.[23]

Despite playing a significant role in blood coagulation, vWF is typically not classified as a CF in the traditional sense. However, it not only has a pivotal function during coagulation and hemostasis, but it has also been described to have several direct effects on EC stability. Indeed, vWF has been shown to negatively control vascular formation, decreasing EC migration, proliferation, and angiogenesis.[24] Moreover, mice lacking vWF display an elevated vessel formation and a large vascular network in the ear.[25] These data suggest

that vWF could be an anti-angiogenic factor and this is supported by the demonstration that hypoxia induced an increased vessel formation in the brain microvasculature of both vWF$^{-/-}$ and anti-vWF antibody-treated mice.[26] In contrast, another study showed that vWF$^{-/-}$ mice have reduced angiogenesis after ischemia[27] and, in line with these results, vWF was found to play a pro-angiogenic function, binding and recruiting several growth factors thanks to its heparin-binding domain.[28] Although it is well-known that vWF binds integrin αvβ3 on the EC surface, little is understood about how it regulates vascular stability through this receptor,[24] and the downstream mediators activated by vWF in EC have not yet been clarified, with the exception of MAP kinase p38, found activated in EC after vWF treatment, and resulting in an increase in mesenchymal cell adhesion to EC.[29]

The available data on FVIII controlling EC functions are still not complete, and vWF effects on EC appear, in some cases, controversial, suggesting that it may display both pro- and anti-angiogenic roles (Table 1). Thus, further investigations are needed to elucidate their role in EC homeostasis.

Tissue factor

Following damage to the blood vessel, tissue factor (TF) joins with activated factor VII (FVII) in inducing the activation of FX, leading to fibrin deposition. TF is mainly expressed by platelets, neutrophils, eosinophils, fibroblasts, pericytes, keratinocytes, while monocytes increase its expression after stimulation with lipopolysaccharide (LPS) through activator protein 1 (AP-1) and NF-κB activation.[30] This protein has two isoforms: one is full-length (fl-TF), bound to the cell membrane, and the other one is the soluble alternatively spliced (as-TF) isoform. Both isoforms have been demonstrated to play major roles beyond coagulation; specifically, they are involved in the regulation of angiogenesis, tumor growth, metastasis, and inflammation.[31] Indeed, most of the present data suggest that TF is a pro-metastatic and angiogenic factor,[32] and that the main isoform involved in the angiogenic process is the as-TF, which can exert its action through the hypoxia-inducible factor-1 (HIF1)/VEGF pathway.[33] Moreover, as-TF has been shown to increase tubulogenesis, binding integrin α6β1 and activating MAP kinase p42/p44, while it enhances EC migration through integrin αvβ3 and MAP kinase p38.[34] Congruently, as-TF promotes cancer proliferation through integrin β1 signaling, further highlighting the pivotal interconnection of CF with integrins.[35]

However, as-TF alone is not enough to promote the correct formation of the vasculature during embryogenesis,[36] suggesting that both isoforms are necessary to regulate blood vessel formation and maintenance. Indeed, already in 1996, it was demonstrated that fl-TF$^{-/-}$ mice die during embryonic development due to vascular failure.[37] Moreover, it was described that fl-TF can induce EC migration through PAR2.[38]

Overall, these data show that both TF isoforms can directly bind integrins and GPCR, regulating EC functionality (Table 1).

Anti-coagulation factors

The intricate system of coagulation within the bloodstream is a finely tuned process and a delicate equilibrium is maintained by the interplay of pro- and anti-CF. These proteins with anti-coagulant activity act as essential regulators, counteracting the clot-forming cascade to prevent excessive blood clotting.

One of the major anti-CF is antithrombin (AT), which inhibits most pro-coagulant proteases of the coagulation cascade, with thrombin and activated FX and factor IX (FIX) as main targets of its action.[39] It also displays an anti-inflammatory role, binding heparan sulfate proteoglycans (HSPG) on the EC surface (especially syndecan-4) and increasing prostacyclin.[40] Notably, cleaved and latent forms of AT have been demonstrated to be potent anti-angiogenic factors down-regulating the expression of genes related to extracellular matrix assembly in EC, such as perlecan, byglycan and syndecans.[41] Its anti-proliferative function has been demonstrated in EC as well as in tumorigenic cell lines[42] and, specifically, its heparin-binding site was shown to inhibit proliferation, migration, capillary-like tube formation, and perlecan expression.[41] HSPG have been described as interacting with integrins to regulate many EC functions; thus, it has been hypothesized that the interplay between the AT-HSPG complex and the extracellular matrix (ECM) organization could be orchestrated through integrin signaling.[40]

Interestingly, also thrombomodulin (TM) seems to be implicated in angiogenesis regulation. Indeed, TM has been shown to be a pro-angiogenic factor, activating focal adhesion kinase and binding fibronectin.[43] This observation was strengthened by the demonstration of the *in vitro* and *in vivo* activity of recombinant EGF-like domain plus the O-glycosylation site-rich domain of TM.[44] In contrast, recombinant lectin-like domain of TM has been shown to interact with Lewis-Y carbohydrate antigen, inhibiting angiogenesis.[45] Recently, its role has also been assessed in TM-deficient EC and, several assays have shown it to tightly regulate EC quiescence.[46] Taken together, these findings reveal that TM exhibits a dual role as both a pro- and an anti-angiogenic factor.

Importantly, another well-known anti-CF, activated protein C (APC), has been described to protect the endothelial barrier binding angiopoietin-1 receptor (Tie2)[47] or PAR1, inducing a signaling in which β-arrestin-2 and dishevelled-2 are involved.[48] Further investigations have revealed that APC induces anti-apoptotic signaling in EC in a caveolin-1-dependent manner,[49] and increases phosphorylation of proteins mainly related to gene expression and actin binding, corroborating the role of APC in stabilizing the EC barrier.[6]

Table 2. Summary of endothelial cell functions regulated by selected anti-coagulation factors.

Anti-CF	Study	*In vitro* or *in vivo* models	Regulated function	Identified receptor
Antithrombin	Panicker *et al.*[40]	EA.hy926 and HDMEC	Apoptosis	HSPG
	Zhang *et al.*[41]	HUVEC	Angiogenesis	-
	O'Reilly *et al.*[42]	EC (unspecified)	Angiogenesis	-
Thrombomodulin	Hsu *et al.*[43]	HUVEC	EC adhesion, migration and angiogenesis	-
	Shi *et al.*[44]	HUVEC	Angiogenesis	-
	Kuo *et al.*[45]	HUVEC	Angiogenesis	Lewis Y Ag
	Giri *et al.*[46]	TM-deficient EA.hy926	Quiescence	-
Activated protein C	Minhas *et al.*[47]	HUVEC and mice	Permeability	Tie2
	Soh *et al.*[48]	EA.hy926	Permeability	PAR1
	Molinar-Inglis *et al.*[49]	EA.hy926	Apoptosis	PAR1
	Lin *et al*[6]	EA.hy926	Permeability	PAR1

Anti-CF: anti-coagulation factor; EA.hy926: human umbilical vein cell line; EC: endothelial cells; HDMEC: human dermal microvascular EC; HUVEC: human umbilical vein EC; HSPG: heparan sulfate proteoglycans; Lewis Y Ag: Lewis-Y carbohydrate antigen; TM: thrombomodulin; Tie2: angiopoietin 1 receptor; PAR: protease-activated receptor.

Overall, as described above for the pro-CF, even the anti-CF exert pivotal roles in EC maintenance (Table 2), but the way they do this requires further studies in order for these mechanisms to be fully defined.

Conclusions

New evidence has recently emerged on the complex interplay between coagulation and vascular homeostasis; CF possess a different role beyond their conventional involvement in coagulation cascade. Indeed, these factors intricately regulate the stability and the functionality of EC, suggesting a profound relationship between hemostasis and endothelial functions. Some observations also highlight the important effect of these CF, not only in mature EC, but also in vascular development during embryogenesis. Indeed, the absence of some CF (e.g., TF) or their receptors (e.g., PAR1) results in embryo lethality.[12,37]

Our review has explored specific pro-CF, including thrombin, FVIII, TF, and a few anti-CF, as the primary focus was to elucidate their roles in orchestrating endothelial functionality. While spotlighting these critical CF, it is important to acknowledge the relevance of other factors, such as factor V (FV) and factor XIII (FXIII), which have been recognized as enhancing EC migration and maintaining the vascular homeostasis,[50,51] and FIX, described as controling EC permeability.[52] Additionally, factor I (FI), FVII, and factor XII (FXII), have been shown to play a pro-angiogenic role.[53] On the other hand, FX angiogenic potential activity is still a subject of debate and it is not fully understood if it plays a pro- or an anti-angiogenic role.[53] This evidence further corroborates the hypothesis that CF can be main players both in coagulative and angiogenic processes.

The intricate pathways through which CF influence EC stability mainly involve direct receptor binding, triggering signaling pathways regulating angiogenesis, proliferation, migration, and permeability (Table 1, 2). Notably, CF demonstrate versatility by binding to diverse receptors such as GPCR, receptor tyrosine kinases (RTK), and especially integrins (Figure 1). The CF-receptor interactions underscore the significance of these factors in modulating various physiological endothelial functions. Yet, for certain CF, such as FVIII, whose specific receptors remain elusive, there is speculation as to how they act: is it through known EC surface receptors? Or is it by affecting thrombin generation, which in turn impacts EC functionality? Interestingly, integrins consistently emerge as being crucial membrane transmembrane receptors in mediating CF activity on EC. The persistent prominence of integrins as the principal receptors for CF among various studies reinforces their critical role in regulating endothelial responses to these factors, establishing them as the possible, but not the only, key players in the intricate landscape of EC functionality.

Despite their diverse effects on EC stability, those pathways triggered by CF that have been studied mainly converge on the regulation of EC angiogenesis through the expression of angiogenic factors (e.g., thrombin and TF), and in controlling EC permeability through ECM remodeling (e.g., FVIII and AT). As the ECM profoundly impacts tissue health, further investigation into how CF guide ECM organization remains critical to validate the initial findings. Additionally, elucidating whether CF act individually or collaborate in finely tuned regulatory networks to regulate EC, as in the coagulation cascade, remains an intriguing path for future exploration. Importantly, these findings would help define a more holistic treatment strategy, primarily for coagulation disorders (e.g., coagulopathies), but also

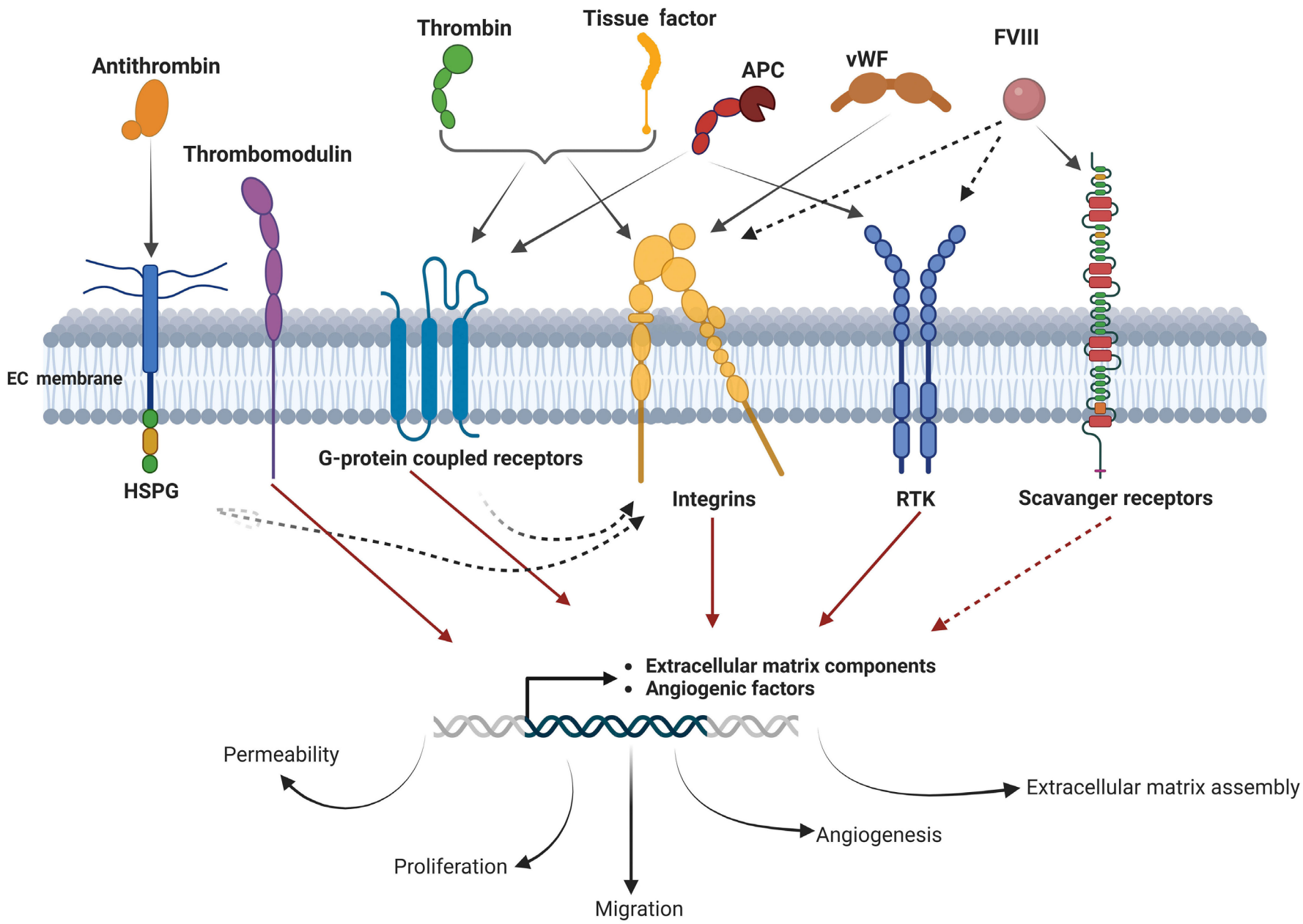

Figure 1. Coagulation factors binding to receptors involved in endothelial cell functions. Antithrombin binds to heparan sulfate proteoglycans (HSPG) to exert its function in endothelial cells (EC). Thrombomodulin can induce the expression of genes involved in extracellular matrix and angiogenesis. Thrombin and tissue factor bind G-protein coupled receptors and integrins. von Willebrand factor (vWF) binds integrin on EC and activated protein C (APC) binds G-protein coupled receptors and receptor tyrosine kinases (RTK). Factor VIII (FVIII) binds to known scavenger receptors, but its involvement with integrins or RTK in the regulation of EC functionality has never been fully explored. The activation of these receptors induces the expression of genes involved in extracellular matrix organization and angiogenesis modulating EC functions.

for conditions where both coagulation and angiogenesis are impaired, such as vascular diseases and cancer. For example, FVIII is expressed by several tumors independently of vWF expression, suggesting a possible unique role of FVIII in cancer physiopathology.[54]

Revealing the multifaceted roles of CF uncovers novel physiological mechanisms, shedding light on their functions beyond conventional hemostasis. While selected CF have already been the subject of initial studies, others await deeper exploration, suggesting an exciting trajectory in understanding their involvement in endothelial biology.

Disclosures

No conflicts of interest to disclose.

Contributions

CO, SA, AC and AF wrote and revised the manuscript. CO generated the figure using BioRender tool (Agreement N: HV26FVNYFH). AF conceived, wrote, and revised the manuscript.

Acknowledgments

The authors thank Dr. Chiara Borsotti and Saicharan Akula for their help in critically reading and revising the manuscript.

Funding

This work was partially supported by a Telethon grant (N. GGP19201 to AF) and Bando per la Ricerca Roche to CO.

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