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REVIEW

Fibronectin maintains the balance between hemostasis and thrombosis

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Abstract Fibronectin is a dimeric protein widely distributed in solid tissues and blood. This major extracellular matrix protein is indispensable for embryogenesis and plays crucial roles in many physiological and pathological processes. Fibronectin pre-mRNA undergoes alternative splicing to generate over 20 splicing variants, which are categorized as either plasma fibronectin (pFn) or cellular fibronectin (cFn). All fibronectin variants contain integrin binding motifs, as well as N-terminus collagen and fibrin binding motifs. With motifs that can be recognized by platelet integrins and coagulation factors, fibronectin, especially pFn, has long been suspected to be involved in hemostasis and thrombosis, but the exact function of fibronectin in these processes is controversial. The advances made using intravital microscopy models and fibronectin deficient and mutant mice have greatly facilitated the direct investigation of fibronectin function in vivo. Recent studies revealed that pFn is a vital hemostatic factor that is especially crucial for hemostasis in both genetic and anticoagulant-induced deficiencies of fibrin formation. pFn may also be an important self-limiting regulator to prevent hemorrhage as well as excessive thrombus formation and vessel occlusion. In addition to pFn, cFn is found to be prothrombotic and may contribute to thrombotic complications in various diseases. Further investigations of the role of pFn and cFn in thrombotic and hemorrhagic diseases may provide insights into development of novel therapeutic strategies (e.g., pFn transfusion) for the maintenance of the fine balance between hemostasis and thrombosis.

Keywords Fibronectin · Platelet · Fibrinogen and fibrin · Collagen · Integrins · Coagulation factors

Introduction

Fibronectin is a key extracellular matrix protein that plays crucial roles in many biological processes. It is required for embryogenesis, and mice deficient in fibronectin die around embryonic day 10.5 due to severe mesoderm defects [1]. Fibronectin is secreted by various cell types into the surrounding intercellular space. This extracellular fibronectin niche plays essential roles in wound healing, malignant transformation, inflammation, infection, hemostasis, and thrombosis. The intensive investigations into the roles of fibronectin over the past few decades have catalyzed many groundbreaking advances in biology and medicine [2–6].

Fibronectin was first discovered in 1948 by Morrison et al. as "cold-insoluble globulin" during purification of fibrinogen from human plasma [7]. It was later realized that the "cold-insolubility" is not an intrinsic characteristic of

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the protein but an artifact of its interaction with fibrinogen and heparin [8, 9]. Cellular and cell-associated fibronectin was recognized by several laboratories as a protein on the fibroblast cell surface and in culture media in the 1970s using a radioactive labeling technique [10–17]. Historically, cellular and cell-associated fibronectin has had many names, such as large external transformation protein, surface fibroblast antigen, cell surface protein, and cell attachment protein, which reflect its widespread distribution and diverse functions [18]. The name "fibronectin" was first proposed in 1976 by Vaheri et al. to indicate its association with fibrinogen and fibrin [19, 20]. The name was finally cemented during a "fibroblast surface protein" conference organized by Vaheri et al. at the New York Academy of Sciences in 1977 [21].

Several decades of intensive investigation have revealed that fibronectin possesses the ability to bind cell surface integrins and a number of other extracellular matrix proteins, and plays major roles in cell adhesion, migration, differentiation, and transformation. Fibronectin was also one of the first genes discovered to undergo alternative splicing and has become a model for the study of transcription and splicing mechanism [22, 23]. In the last two decades, many investigators of fibronectin have turned their focus to the functions of different fibronectin splicing variants in animal models in vivo. The availability of murine models expressing various fibronectin splicing variants has greatly facilitated research in this field [1, 4, 24–29]. In particular, many controversial findings made in vitro regarding the role of fibronectin in hemostasis and thrombosis can now be tested in vivo.

Major functional domains on fibronectin

Fibronectin is a dimer made of two 250-kDa subunits linked by two C-terminal disulfide bonds. Each monomer contains three types of homologous modules, including 12 type I modules, 2 type II modules, and 15–17 type III modules (Fig. 1). Two alternative splicing sites exist within type III modules, called extra domain (ED) A and EDB [30]. A third alternative splicing site, called the variable region, exists close to the N-terminus. The alternative splicing of EDA, EDB, and the variable region results in approximately 20 monomeric isoforms in humans and 12 in mice, with even higher numbers of dimeric isoforms [31–33]. These splicing variants can be categorized into two groups, namely plasma fibronectin (pFn) and cellular fibronectin (cFn). pFn is secreted specifically by the liver with exclusion of both EDA and EDB, while cFn can be secreted by various cell types and contains at least one of the EDs.

The organization of fibronectin into its fibrillary form is mediated through its interaction with cell surface integrins. All fibronectin variants contain the RGD (arginine–glycine–aspartic acid) motif in the tenth type III module, which is a common binding site for several integrins [34–36] including α5β1, αvβ3, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α8β1, and αIIbβ3 integrins [37]. In solid tissue, α5β1 integrin is the major receptor on cells to mediate fibronectin fibrillogenesis. Although fibronectin fibers form in the absence of α5 integrin, likely through the interaction of fibronectin with other integrins, α5-deficient mice exhibit significant mesoderm defects and die at approximately embryonic day 11–13 [38, 39]. Upon binding of fibronectin, α5β1 integrin begins to translocate along the plasma membrane through the interaction of its cytoplasmic tail with actin [40, 41]. In this way, the intracellular cytoskeleton tension is transmitted to the extracellular fibronectin molecules, which leads to a conformational change of fibronectin and exposure of its cryptic binding sites. These newly exposed binding sites then bridge the adjacent fibronectin molecules and form a stable insoluble fibrillar matrix [42]. To reach optimal affinity with α5β1 integrin, a synergistic site adjacent to the RGD motif in the fibronectin ninth type III module is required [43, 44]. Interestingly, although the fibronectin RGD motif is required for survival of the fetus beyond embryonic day 10, fibrillogenesis still occurs in the absence of RGD [25]. It was found that a non-enzymatic rearrangement of NGR into iso-DGR (iso-aspartic acid-glycine-arginine) in fifth type I module enabled the matrix formation through binding of αvβ3 integrin [25].

In the circulation, although different types of integrins can bind to the RGD motif of fibronectin under static conditions, it has been demonstrated that the interaction of pFn with platelets under flow conditions mainly depends on the presence of activated platelet surface αIIbβ3 integrin, with a smaller but significant contribution from other less abundant platelet integrins, such as platelet α5β1, and αvβ3 [45]. Platelets from Glanzmann's thrombasthenia, a bleeding disorder characterized by αIIbβ3 integrin deficiency, exhibit markedly reduced surface binding of fibronectin compared to wild-type platelets after thrombin activation [46, 47]. Interestingly, thrombin-induced fibronectin expression on the platelets was also reported to be dependent on the presence of fibrin, suggesting the complex of fibronectin–fibrin–αIIbβ3 integrins was formed on platelet surface after thrombin stimulation [47, 48]. The αIIbβ3 integrin can also mediate the uptake of pFn into platelet α-granules, and this process is competitively inhibited by fibrinogen [49].

Apart from the RGD motif, the N-terminus is also crucial for fibronectin function and matrix formation. The first five type I modules (first to fifth type I modules, also

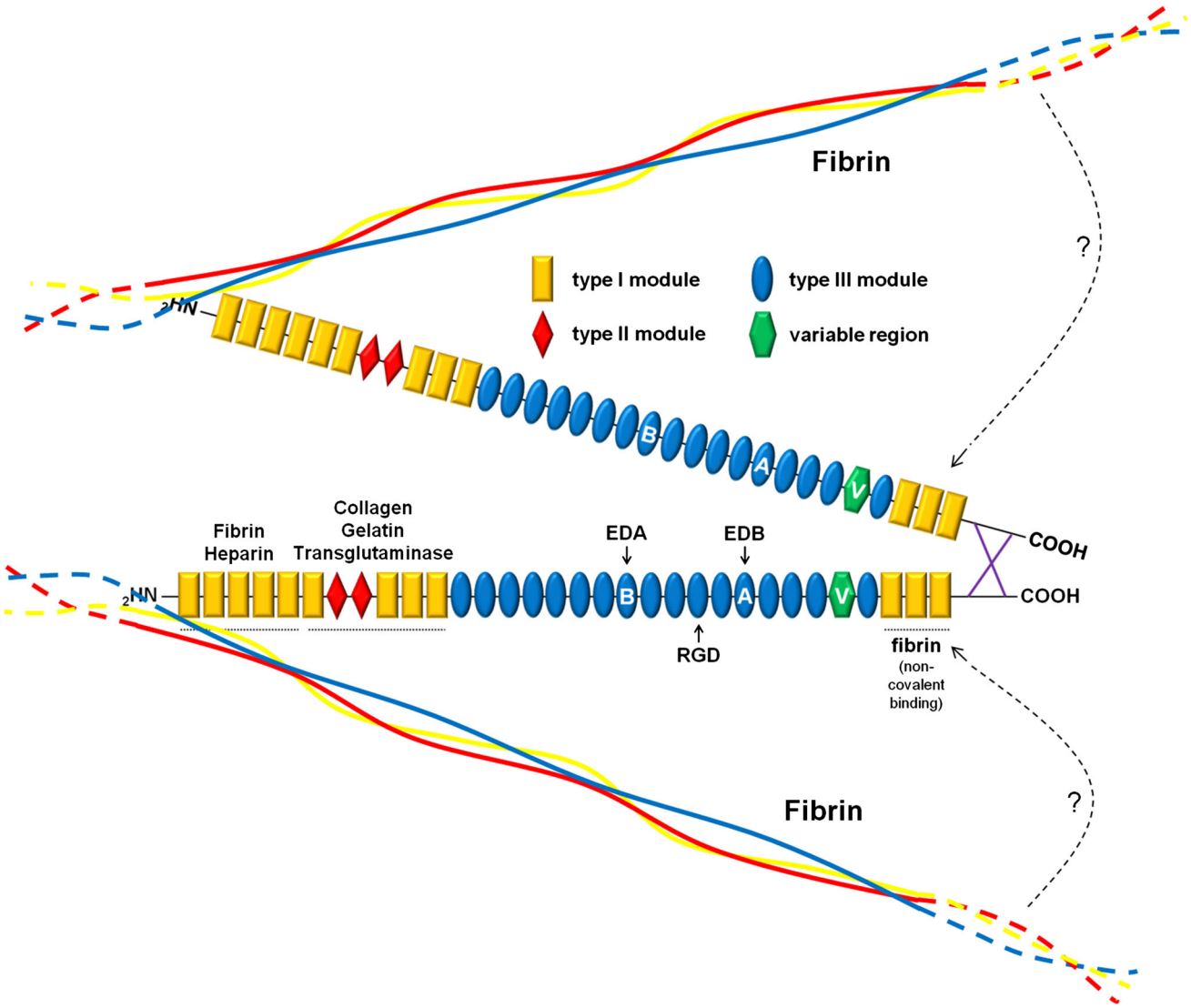

Fig. 1 Structure of fibronectin. Fibronectin is a dimer made of two 250-kDa subunits. Each monomer contains three types of homologous modules, including 12 type I modules, 2 type II modules, and 15–17 type III modules. Two alternative splicing sites exist within type III modules, called EDA and EDB. *Dotted lines* represent the binding sites of various ligands. While cFn contains EDA, EDB, or both, pFn lacks EDA and EDB. The N-terminal 27 kDa region of fibronectin can be covalently linked with fibrin. This interaction may enhance lateral aggregation of fibrin fibers. The fibronectin–fibrin complex formed at the site of vascular injury also provides multiple binding sites for platelets and other cells in the circulation

commonly referred to as N-terminal 27 kDa fragment) at the N-terminus are required for fibronectin fibril formation [50]. This region may also directly interact with cell surface receptors [51], although the specific receptor(s) has yet to be identified. Importantly, the 27 kDa region can also be covalently linked with the C-terminus of the fibrin α chain by formation of ε-(γ-glutamyl)-lysyl bonds under the catalyzation of factor XIIIa, thus enabling pFn incorporation into fibrin clots and enhancing the mechanical strength of the clot [52, 53]. A small 49 amino acid peptide derived from the fibronectin binding protein F1 of *S. pyogenes*, called functional upstream domain (FUD) can specifically inhibit the pFn–fibrin cross-linking and pFn self-assembly [54, 55]. Although the C-terminus fibrin non-covalent binding site has also been identified [56], the N-terminus fibrin binding site appears to play a dominant role in pFn incorporation into the blood clot, since an N-terminus mutant of fibronectin was undetectable in the fibrin network formed [57]. In addition, each subunit of the fibronectin dimer harbors an N-terminus collagen-binding site, which can be covalently linked to collagen by factor XIIIa [58]. As collagen is a major sub-endothelial matrix protein, this interaction may play a role in mediating the rapid incorporation of pFn into the vessel wall under flow

conditions [53]. Interestingly, it has been reported that the majority of sub-endothelial and tissue fibronectin in adults is pFn derived from the circulation [59].

Fibronectin splicing variants

The fibronectin splicing variants are the result of alternative splicing of a single pre-mRNA. EDA and EDB are each encoded by a single exon, which can be excluded or included in the mature mRNA. The inclusion of EDA is promoted by SR (serine-arginine) proteins, most notably SF2/ASF, while exclusion of the EDA exon is favored by hnRNPA1 [60–63]. The inclusion of the EDB exon is mediated by the binding of SRp40 to the intronic splicing enhancers downstream of EDB or to the purine-rich sequence within EDB [23, 64–66]. The alternative splicing of the variable region is more complicated; the variable region can be included or skipped entirely or partially, depending on the presence of various splicing factors [67]. The inclusion of the variable region in pFn was found to be required for its secretion from hepatocytes [68].

The functional importance of EDA and EDB has been studied using various animal models with constitutive inclusion or exclusion of these modules. EDA has been reported to harbor the binding sites for α4β1 and α9β1, but the in vivo significance of these interactions is still unclear [69]. While both $EDA^{-/-}$ and $EDB^{-/-}$ mice are viable, approximately 80 % of EDA and EDB double deficient mouse embryos die at embryonic day 10.5 due to severe cardiovascular defects, including vascular hemorrhage, failure of remodeling embryonic and yolk sac vasculature, defective placental angiogenesis, and heart defects [28, 29, 70]. Although EDA deficiency alone is not lethal, the $EDA^{-/-}$ mice present with a shortened life span, abnormal wound healing, and impaired motor coordination [27, 71]. On the other hand, $EDA^{-/-}$ fibronectin is less prothrombotic, and EDA deficiency protects the mice from atherosclerosis [70, 72]. The EDB deficient mice develop normally, but the fibroblasts obtained from these mice exhibit a decreased ability to form a fibronectin matrix, suggesting a role for EDB in extracellular matrix modeling [28].

The variable region is especially important for the secretion of pFn from hepatocytes [68]. Although pFn contains neither EDA nor EDB, at least one variable region is found in pFn in the circulation [68]. The variable region also contains binding sites for α4β1 and α4β7 integrins [73, 74]. However, the in vivo significance of these interactions for fibronectin matrix assembly and cell migration is still largely unknown.

Plasma fibronectin in thrombosis and hemostasis: in vitro and ex vivo evidence

pFn circulates in the blood at a relatively high concentration (230–650 μg/mL) [75, 76]. Since pFn can bind to platelet surface integrins and various sub-endothelial matrix proteins, it has long been suspected that pFn plays a role in hemostasis and thrombosis [3, 5, 77].

Hemostasis is the physiological process to stop bleeding after an injury. In contrast, thrombosis is the pathological process of inappropriate activation of platelets and the coagulation system that causes vessel occlusion and downstream ischemia, inducing heart attack and stroke, the leading causes of mortality and morbidity worldwide [78, 79]. According to classical theory, as two sides of the same coin, arterial hemostasis and thrombosis both involve the following chain of events [80, 81]: (1) a breach of vessel integrity and exposure of the sub-endothelial matrix due to physiological (e.g., traumatic injuries) or pathological (e.g., rupture of atherosclerotic plaques) events; (2) platelet adhesion to the sub-endothelial matrix through von Willebrand factor (VWF) [82–84]; (3) platelet activation through interaction of platelet surface GPVI and α2β1 with sub-endothelial collagen [85, 86]; (4) additional platelet aggregation onto the initial adhered platelets through platelet surface αIIbβ3 integrin binding to fibrinogen or other integrin ligands [26, 87–90]; and (5) activation of the coagulation cascade and clot formation [78, 91]. Notably, thrombin generated from blood coagulation process and soluble agonists released from activated platelets (e.g., thromboxane A2, adenosine diphosphate) can further enhance the platelet activation and thrombus formation [78, 83, 92]. Based on extensive in vitro and ex vivo studies over the last few decades, it is apparent that pFn is involved in multiple steps of hemostasis and thrombosis.

Studies have consistently shown that pFn can support platelet adhesion [93–99]. Platelet adhesion to purified pFn is a relatively weak interaction and unstable compared to platelet adhesion to the natural endothelial cell matrix or collagen [93, 94]. However, when pFn coating a flow chamber was cross-linked to fibrin by factor XIIIa, robust platelet adhesion and thrombosis was observed [54]. Furthermore, while platelets adhered to a pFn alone coated surface cannot induce pFn matrix formation on platelets under shear stress, platelets adhered to pFn cross-linked to fibrin promoted robust pFn matrix formation [45]. Since both factor XIIIa and fibrin generation require thrombin, this observation suggests a pivotal role of thrombin and fibrin in the regulation of pFn function.

The role of pFn in platelet aggregation is controversial and has only recently been elucidated [6, 53, 100]. pFn has been shown to inhibit ionophore A23187- and collagen-

induced platelet aggregation [101, 102]. However, monoclonal antibodies targeting fibronectin inhibited platelet aggregation mediated by these agonists [103, 104], suggesting that pFn supports platelet aggregation. Another report showed that pFn corrected the platelet aggregation defect in a patient with Ehlers–Danlos syndrome (a connective tissue disorder), although the mechanism remains unclear [105]. In a recent study, we found that fibrin is required for pFn to enhance platelet aggregation. When agonists that do not induce fibrin formation were used, such as adenosine diphosphate (ADP), collagen, and thrombin receptor activating peptide (TRAP), plasma pFn and platelet-contained pFn both inhibited platelet aggregation. A likely explanation for this phenomenon is that the two RGD motifs on the pFn dimer are so close to each other that it is difficult for the two RGD motifs to bind to αIIbβ3 integrins on two adjacent platelets simultaneously. The binding of pFn RGD motifs to αIIbβ3 integrin on one platelet, however, prevents αIIbβ3 integrin from being approached by fibrinogen, thereby inhibiting platelet aggregation. In contrast, when thrombin was used as agonist, pFn supported platelet aggregation [53], likely through formation of a pFn–fibrin complex, since thrombin can convert both plasma- and platelet α granule-released fibrinogen to fibrin [47]. The multiple RGD motifs in pFn and fibrin molecules allow the pFn–fibrin complex to serve as a bridge between platelets, enhancing platelet aggregation. Consistent with this, our studies in patients indicate that fibrin is required for retention of pFn on the platelet surface [47].

The involvement of pFn in blood coagulation has been recognized for several decades. pFn may be covalently linked to fibrin by factor XIIIa, thereby incorporated into the fibrin network of the blood clot [52]. However, the mechanical and structural changes in the clot induced by pFn incorporation are controversial. Some studies found that pFn increased clot shear modulus and turbidity, while others found that pFn prolonged clotting time and decreased turbidity, possibly through blocking fibrin polymerization [106–109]. The key challenge for these studies was that the pFn used was purified from wild-type animal or human plasma, which is inevitably contaminated by fibrin or fibrinogen (hence the name "fibr*in*-ectin") [53]. Variation in the extent of contamination may have subsequently determined the results of these studies. In a recent study, we used pFn purified from fibrinogen-deficient mice and demonstrated that this "non-fibrin-associated" pFn enhanced the mechanical strength of the clot by increasing the diameter of the fibrin fibers formed [53]. The increased fibrin fiber diameter is likely due to an increased fibrin fiber lateral aggregation caused by the pFn dimer. With both N-termini of the monomer cross-linked to fibrin, the dimeric pFn is able to shorten the lateral distance between fibrin protofibrils and promote lateral growth, which has been demonstrated to be associated with a stronger clot network [53, 110].

As described above, the roles of pFn in platelet adhesion, activation, aggregation, and blood coagulation are dictated by the presence of fibrin and the formation of a pFn–fibrin complex through ε-(γ-glutamyl)-lysyl bonds. The thrombin and fibrin concentration and generation in the local microenvironment around the thrombus could determine the function of pFn. Furthermore, pFn may be similar to fibrinogen and can induce platelet P-selectin synthesis, although the roles of these de novo synthesized P-selectin in thrombosis and hemostasis remain to be elucidated [111, 112].

Plasma fibronectin in thrombosis and hemostasis: in vivo evidence

In the last decade, the application of intravital microscopy models has significantly advanced our knowledge of hemostasis and thrombosis. A series of groundbreaking discoveries have been made in platelet physiology, which paved the way for a new understanding of pFn function in vivo. One such discovery is the recognition of VWF- and fibrinogen-independent thrombus formation [87–89]. For decades, VWF and fibrinogen were considered required for platelet thrombus formation. However, using fibrinogen/VWF double deficient mice, Ni et al. revealed that platelet aggregation and thrombosis persist in the absence of fibrinogen and VWF, suggesting that other ligand(s) could mediate thrombosis independent of fibrinogen and VWF [87]. It was later confirmed that platelet aggregation does occur in the absence of fibrinogen, when no anticoagulant is used during the bleeding process [26, 87, 88].

In vivo study of pFn began after Sakai et al. developed the first pFn conditional deficient mouse model using the Cre-loxP system, in which the hepatic specific deletion of pFn production was induced through polyinosinic-polycytidylic acid injection in the pups and >98 % of pFn in the blood circulation was depleted [24]. Using a $FeCl_3$ arterial injury intravital microscopy model, Ni et al. found that pFn plays a significant role in thrombus initiation, growth, and stability at arterial shear rates [4]. Although pFn deficiency did not affect initial platelet adhesion, the time needed to form the first thrombi larger than 20 μm was significantly prolonged. In addition, the thrombi formed in pFn-deficient mice constantly shed platelets or small platelet clumps, which significantly prolonged the vessel occlusion time [4]. Similar impairment of thrombus formation was observed in a fibronectin$^{+/-}$ mouse model, in which pFn concentration in plasma is about half that of wild-type mice [113].

Since platelet fibronectin content increased in both fibrinogen$^{-/-}$ and fibrinogen$^{-/-}$/VWF$^{-/-}$ mouse models and in afibrinogenemic patients as a result of increased pFn internalization, it was hypothesized that pFn may be the factor mediating the fibrinogen/VWF independent thrombus formation [4, 47, 49, 87, 114]. Surprisingly, further depletion of pFn in fibrinogen$^{-/-}$/VWF$^{-/-}$ mice did not abolish or decrease thrombus formation, but rather increased platelet aggregation and thrombosis in vitro and in vivo [26]. Fibrin was later identified to be the factor that mediates this switch in pFn function. pFn supports platelet aggregation and thrombosis in the presence of fibrin, while pFn inhibits platelet aggregation and thrombosis in fibrinogen-deficient mice [53]. This in vivo observation is consistent with the in vitro aggregation finding that pFn inhibits platelet aggregation in the absence of fibrin, but promotes platelet aggregation in the presence of fibrin, as described in the previous section. These observations have important implications: it has been shown that fibrin mainly formed in the "inner core" of the thrombi, while at the peripheral or "outer shell" of the thrombi, minimal fibrin formation is observed [53, 115, 116]. Therefore, fibronectin in the "inner core" of the thrombi could support platelet aggregation by covalently linking with fibrin, while the non-fibrin-linked pFn at the periphery of the thrombi could inhibit further thrombus growth and prevent vessel occlusion, maintaining blood supply to downstream tissues and organs.

Another important discovery made using an in vivo mouse model was the recognition of the vital hemostatic function of pFn [53]. Depletion of pFn in fibrinogen-deficient mice markedly prolonged the tail bleeding time and resulted in a 2.5- to 4-fold increase in mortality caused by excessive bleeding. In wild-type mice treated with anticoagulants, such as heparin and hirudin, pFn depletion also significantly impaired the ability to achieve hemostasis. Infusion of pFn into either fibrinogen-deficient or anticoagulant-treated mice significantly shortened bleeding time. These data provided the first in vivo evidence that pFn supports hemostasis and is key for prevention of life-threatening hemorrhage in genetic and anticoagulant-induced coagulation deficiencies.

The hemostatic benefit of pFn is attributed, at least partly, to the fast response of pFn in the blood to vessel injury. Unlike fibrin, which is observed at the site of vessel injury only after considerable platelet accumulation has already occurred, pFn deposits at the injury site immediately after laser-induced vessel injury, even before platelet accumulation. Moreover, the pFn deposition is independent of fibrinogen, VWF, αIIbβ3 integrin, or platelets, as demonstrated in these individual gene deficient mice [53]. Traditionally, platelets are considered the initial responders to the site of vessel injury, so platelet accumulation is called "primary hemostasis" or the "first wave of hemostasis". The identification of pFn deposition (possibly together with other pFn-associated proteins) before platelet accumulation suggests that a "fibronectin wave" or "protein wave" of hemostasis is actually an even earlier response to the disruption of vessel integrity. The high quantity of deposited fibronectin may then self-assemble or incorporate into the sub-endothelial matrix to prevent leakage of blood cells and plasma into the extravascular space. Future studies on the detailed network structure and organization of the integrin-mediated assembly of insoluble or fibrillar pFn with electron microscopy and other imaging techniques in these tissues will further elucidate the mechanism of the hemostatic function of pFn.

It is worth noting that platelets could internalize pFn from plasma through αIIbβ3 integrin, and this process is markedly enhanced in afibrinogenemic patients and fibrinogen-deficient mice, leading to 3- to 5-fold increase of pFn in platelets [47, 49, 87, 114]. Platelets could deliver their pFn content specifically to the injury site through granule release, and the local pFn concentration could be significantly boosted by the platelet-derived pFn. The exact hemostatic function of platelet pFn still requires further investigation, but it is conceivable that the platelet-released pFn has the potential to bind to the platelet surface immediately after its secretion and therefore exert an immediate effect on platelet function. At the site of vessel injury, the locally released pFn can deposit onto the injured vessel wall and be efficiently cross-linked with fibrin at the "inner core" of the hemostatic plaque to promote platelet aggregation and to control bleeding.

Plasma fibronectin maintains the balance of hemostasis and thrombosis: a novel model

There are major differences between physiological hemostasis and pathological thrombosis. Hemostasis is a regulated, self-limiting response to vessel injury, which usually abates when vessel integrity is re-established. Thrombosis, on the other hand, is a self-propagating reaction, during which the intensity of platelet thrombus formation and coagulation surpasses the level required to seal the endothelium or vessel wall breakage, leading to vessel occlusion and cessation of downstream blood flow. To develop either anti-thrombotic therapies that will not cause bleeding, or hemostatic agents that will not cause thrombosis, it is pivotal to identify and study the factors that maintain the balance of hemostasis and thrombosis. Here we propose a novel model for pFn as the self-limiting regulator of hemostasis and thrombosis [6, 53, 117] (Fig. 2). After vessel injury, pFn deposits on the exposed sub-endothelial matrix as the initial hemostatic response.

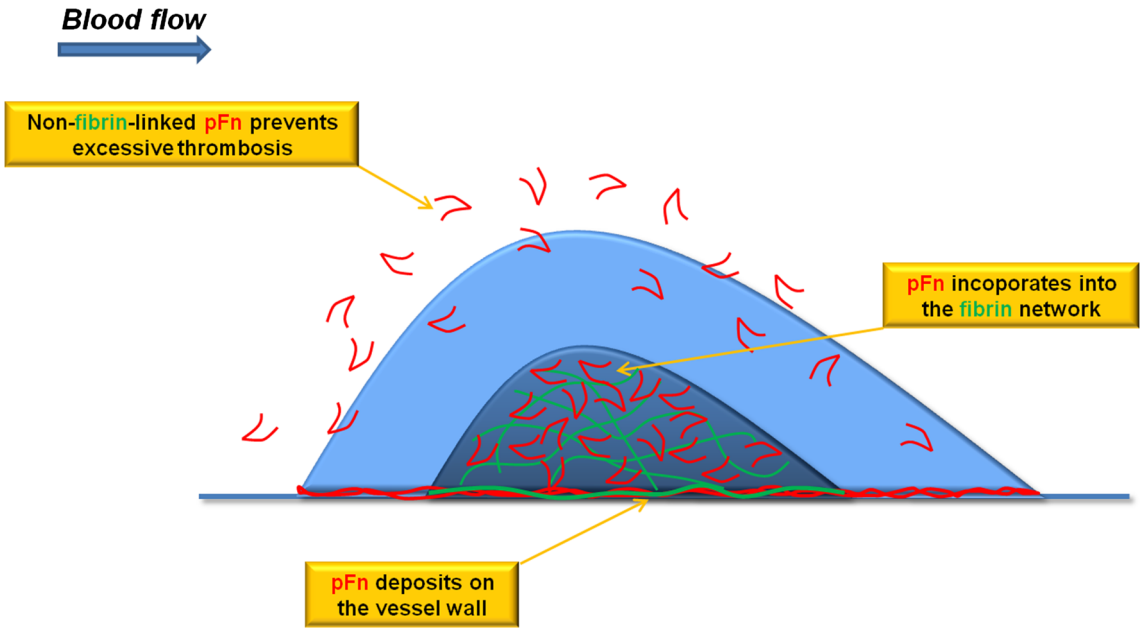

Fig. 2 Plasma fibronectin maintains the fine balance of thrombosis and hemostasis. After vessel injury, pFn (*red*) quickly deposits on the injured vessel wall as an acute hemostatic response. Platelets then accumulate on the deposited pFn and release their intracellular pFn content. At the "inner core" of the hemostatic plug (*dark blue*) close to the injury site, pFn is cross-linked to fibrin (*green*) to promote platelet aggregation and strengthen the fibrin network. As the hemostatic plug extends into the vessel lumen, fibrin is almost undetectable at the "outer shell" of the hemostatic plug (*light blue*). Here, non-fibrin-linked pFn prevents excessive thrombus growth and vessel occlusion

Following platelet accumulation at the injury site, pFn is released from the activated platelets to further boost the local pFn concentration. As the thrombi grow, fibrin begins to form in the center of the thrombi adjacent to the injured vessel wall, due to a high local thrombin level. The subsequent pFn–fibrin complex formed promotes the early platelet aggregation at the injury site to prevent hemorrhage. pFn is also incorporated into the fibrin fibers by FXIIIa to strengthen the clot fibrin network and to stabilize the "inner core" of the platelet thrombi. As the thrombi extend into the vessel lumen, the newly incorporated platelets at the periphery of the thrombi are further away from the injured vessel wall, where the thrombin level gradually reduces to the point that no fibrin generation is observed. In this "outer shell" region, the non-fibrin-linked pFn plays a predominantly inhibitory role for platelet aggregation, thereby limiting excessive thrombus growth and vessel occlusion.

Emerging role of cellular fibronectin in thrombosis and hemostasis

Only trace amounts of cFn have been detected in the circulation of healthy individuals. However, the level of circulating cFn, especially the EDA-containing (EDA^+) cFn, is markedly increased in patients with atherosclerosis, ischemic stroke, vascular trauma, and diabetes [118–121]. Using a mouse model with constitutive EDA expression, it was identified that EDA^+ cFn has stronger prothrombotic activity compared to pFn, due to the interaction of EDA^+ cFn with platelet TLR4 [72, 100, 122]. EDA^+ cFn promoted atherosclerosis and hypercholesterolemic stroke [123, 124]. Aside from circulating cFn, fibrillar cFn also promoted platelet thrombus formation and fibrin generation [125]. Together, these studies suggest a potent prothrombotic effect of cFn, which may contribute to increased risk of thrombosis in certain disease conditions.

In addition, platelets contain a small amount of cFn from megakaryocytes [24, 45]. These cFn may also be secreted upon activation of platelets, thereby contributing to platelet aggregation and thrombosis. Further studies with megakaryocyte-specific cFn-deficient animal models are required to elucidate the significance of platelet-associated cFn in hemostasis and thrombosis.

Potential roles of fibronectin in deep vein thrombosis and fetal hemostasis

Deep vein thrombosis (DVT) and its complication, pulmonary embolism (PE), are major health care challenges affecting 900,000 people in the US alone [126]. In contrast to arterial thrombosis, deep vein thrombosis is initiated by

an inflammatory response that involves neutrophils, platelets, the coagulation system, macrophages, and neutrophil extracellular traps (NETs) [127–130]. Since fibronectin regulates platelet thrombus formation and strengthens the fibrin clot, it is conceivable that fibronectin may contribute to the pathogenesis of deep vein thrombosis [53]. In addition, fibronectin contains DNA binding sites and was found to be associated with NETs [130–132]. Thus, fibronectin could potentially contribute to NETs formation as well as platelet adhesion/clot propagation on NETs. Indeed, increased circulating fibronectin was observed in patients with DVT, although further clinical investigation is required to demonstrate a causative relationship between elevated fibronectin levels and DVT [133, 134]. Interestingly and paradoxically, DVT has been reported in a significant number of immune thrombocytopenia (ITP) patients, even though these patients have a low platelet count [135]. A recent large cohort study demonstrated that patients with chronic ITP have a twofold increase of DVT risk compared to the general population [136]. Whether fibronectin contributes to DVT in these patients and whether the involvement of fibronectin is different depending on which antibody is causing ITP (e.g. anti-αIIbβ3 integrin and the pFn binding sites versus anti-GPIb, which may lead platelet activation and aggregation) are interesting questions for future investigation [137–142].

Fibronectin may be a key hemostatic factor in fetuses. In a model of fetal neonatal alloimmune thrombocytopenia (FNAIT), platelet depletion alone did not cause severe bleeding, such as intracranial hemorrhage [143–145]. It seems that fibrinogen deficiency, even combined deficiencies of both platelet and fibrinogen in murine fetuses, does not lead to bleeding disorders and the fetuses were morphologically indistinguishable from their wild-type controls at day 18.5 of gestation [146]. Given that the fetal coagulation system is relatively immature and that multiple coagulation factors are expressed at significantly lower levels compared to adults [147], it is intriguing how the majority of FNAIT mice and patients maintain hemostasis in their major organs and survive. It is suspected in the fetal/neonatal medicine field that there may be other plasma proteins beside the traditional coagulation factors that contribute to fetal hemostasis independent of platelet. As pFn deposition has been identified as a crucial early phase of hemostasis, independent of platelets and the coagulation cascade, it is possible that pFn could be an important contributor to fetal hemostasis. On the other hand, as placental fibrin-rich thrombosis formation has been identified as a cause of fetal miscarriage [137], and the presence of a high level of fetal fibronectin in the cervicovaginal fluid is a major risk factor for pre-term labor with fetal loss [148], it is possible that a high level of fibronectin in fetal circulation may affect fetal thrombosis, which is deserved for the future investigation.

Conclusions and perspectives

Since its discovery half a century ago, fibronectin has been the subject of intensive investigation. Aspects of fibronectin transcription, alternative splicing, translation, secretion, and structure have been elucidated in great detail. However, the in vivo function of circulating pFn in hemostasis and thrombosis was largely unidentified for decades. The recent advances in intravital microscopy and various pFn mutation mouse models have greatly facilitated the study of the in vivo function of pFn and other fibronectin splice variants. It is recognized that pFn could support hemostasis by depositing on the injured vessel wall, strengthening the fibrin clot, and promoting platelet aggregation though pFn–fibrin complex formation. The non-fibrin-linked pFn may play a crucial role in limiting excessive thrombus growth. Under certain disease conditions, increased circulating cFn may also contribute to the development of thrombotic complications.

Clinically, it is vital to determine whether there is a causative relationship between the levels of plasma fibronectin (both pFn and cFn) and thrombotic diseases, and whether a certain level of circulating pFn is required to limit excessive thrombus growth. This is an especially pressing question, given that high concentrations of pFn are already routinely used in patients through transfusion of various blood products. Commonly used blood products, such as fresh frozen plasma and cryoprecipitate, contain considerable amounts of pFn [149, 150]. For example, up to 25 % of protein contained in cryoprecipitate is pFn [151]. However, the effect of pFn transfusion on hemostasis and thrombosis has never been studied in patients. More importantly, the anti-thrombotic effect of the non-fibrin-linked pFn may be crucial in preventing vessel occlusion in heart attack and ischemic stroke. Future studies in this field will not only address the benefits and risks of pFn transfusion, but will also suggest novel therapeutic strategies for hemorrhagic and thrombotic disorders.

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