Blood Reviews 29 (2015) 377–386

Contents lists available at ScienceDirect

Blood Reviews

journal homepage: www.elsevier.com/locate/blre

REVIEW

# Platelets and physics: How platelets "feel" and respond to their mechanical microenvironment

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ARTICLE INFO

*Keywords:*
Hydrodynamic forces
Mechanosensing
Binding kinetics
Stiffness
vWF
Fibrin(ogen)
GPIb
Integrin

ABSTRACT

During clot formation, platelets are subjected to various different signals and cues as they dynamically interact with extracellular matrix proteins such as von Willebrand factor (vWF), fibrin(ogen) and collagen. While the downstream signaling of platelet–ligand interactions is well-characterized, biophysical cues, such as hydrodynamic forces and mechanical stiffness of the underlying substrate, also mediate these interactions and affect the binding kinetics of platelets to these proteins. Recent studies have observed that, similar to nucleated cells, platelets mechanosense their microenvironment and exhibit dynamic physiologic responses to biophysical cues. This review discusses how platelet mechanosensing is affected by the hydrodynamic forces that dictate vWF–platelet interactions and fibrin polymerization and network formation. The similarities and differences in mechanosensing between platelets and nucleated cells and integrin-mediated platelet mechanosensing on both fibrin(ogen) and collagen are then reviewed. Further studies investigating how platelets interact with the mechanical microenvironment will improve our overall understanding of the hemostatic process.



## 1. Introduction

Platelets are anucleate megakaryocyte fragments that circulate in the bloodstream and rapidly detect and respond to vascular injury [1–4]. Upon vascular injury, the subendothelium is exposed and von Willebrand factor (vWF) deposits on the exposed collagen. Platelets are arrested at the site of injury *via* interactions with these adhesive extracellular molecules, and once adhered, activated platelets aggregate and interact with the polymerizing fibrin network to form a hemostatic plug that stops bleeding. The biochemical cues that promote clot formation such as ADP, thromboxane A2, and thrombin have been extensively studied, less is known about how the physical environment affects platelet function. Indeed, biophysical cues, such as hydrodynamic forces and mechanical stiffness of the underlying substrate, also mediate those specific platelet–ligand interactions and affect the binding kinetics of platelets to those specific ligands (Fig. 1). For example, recent studies have observed that platelets also physiologically respond to the mechanical cues of their microenvironment, such as the stiffness of the extracellular matrix or the nascent fibrin network [5].

Numerous studies in the last several years have documented that nucleated cells interact dynamically with their mechanical microenvironment [6–9]. Specialized surface membrane receptors connect cells with the extracellular domain and allow them to detect the mechanical properties of the microenvironment. Mechanotransduction of these signals results in various cell responses, including morphological changes, altered proliferation rates, migration, and differentiation [8–10]. The group of proteins that mediates sensing of the mechanical microenvironment in nucleated cells is also expressed in platelets. Indeed, it was recently found that platelets detect mechanical changes in their microenvironment *via* similar mechanisms as nucleated cells [5]. As there are physiological differences between platelets and nucleated cells, additional comprehensive studies on platelet mechanosensing are necessary to fully understand how mechanical stimuli affect platelets. In this review, we will first discuss how hydrodynamic forces regulate vWF conformational changes and cleavage *via* enzymatic proteolysis to alter the platelet microenvironment, and thus ultimately regulate vWF–platelet interactions. We will then focus on the microenvironment's influence on the formation of a fibrin network that can exhibit a wide range of mechanical properties that will, in turn, affect platelet mechanotransduction. Finally, we will provide a framework for integrin-based mechanosensing by platelets, adhered to both fibrinogen and collagen that is based on the well-documented responses of nucleated cells. By comparing the

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http://dx.doi.org/10.1016/j.blre.2015.05.002
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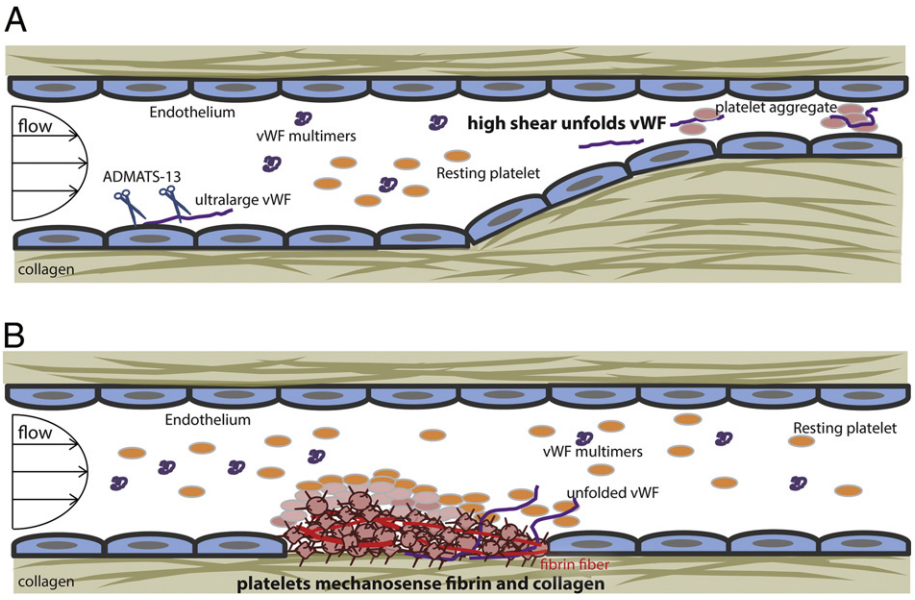


**Fig. 1.** Platelets interact with ECM proteins during clot formation. Hydrodynamic forces and the mechanical microenvironment affect each of these platelet–ligand interactions. A) vWF regulation and vWF-dependent platelet aggregate formation at high shear rates. Ultralarge vWF molecules (purple) are released from the Weibel–Palade bodies of activated endothelial cells and attach to the endothelium. A2 domain exposure on adhered ultralarge vWF leads to cleavage of ultralarge vWF by ADAMTS-13 into vWF multimers. vWF multimers adopt a globular conformation, which prevents both platelet binding and cleavage by ADAMTS-13. However, under pathological high shear stress, for example at the site of stenosis, vWF multimers are unfolded by hydrodynamic forces to switch to an extended conformation, thus causing platelets to bind and form aggregate. B) Clot formation at the vessel injury site. In vascular injury, the endothelium is damaged and underlying collagen (brown lines) is exposed to the bloodstream. vWF multimers adhere to exposed collagen and adapt an unfolded conformation due to wall shear stress. Platelets are arrested at the injury site *via* interactions with both collagen and vWF, both of which initiate platelet activation and recruit additional platelets. At the injury site, platelets interact with fibrin fibers (red), which display a wide range of mechanical properties, to form a clot to stop bleeding. The mechanical properties of the underlying collagen and fibrin, in and of themselves, affect platelet physiology and function.

similarities and differences in mechanosensing and mechanotransduction between platelets and nucleated cells, we hope to provide insight into platelet mechanosensing that promotes better understanding of platelet physiology during clot formation.

## 2. von Willebrand factor (vWF)

vWF is a large plasma glycoprotein that plays an integral role in platelet adhesion and activation (Fig. 1A). It consists of 275 kDa disulfide-linked subunits, and each subunit contains domains that specifically interact with platelets, the extracellular matrix, and plasma proteins [11]. A1, the major functional domain, contains the binding site for platelet membrane receptor glycoprotein (GP) Ib [12]. In addition, A2 domains contain an enzyme cleavage site to ADAMTS13, a plasma metalloprotease, which regulates the size of vWF multimers *via* proteolysis. Further, A3 domains bind collagen and C1 domains contain an RGD sequence that interacts with platelet receptors integrin αIIbβ3 and αvβ3 [12]. Hydrodynamic forces regulate the exposure of these domains by changing the conformation of vWF multimers, thereby modulating both the affinity and avidity of vWF for platelet binding and vWF-mediated platelet aggregation. In this section, we will first discuss how hydrodynamic forces, enzyme proteolysis, and collagen collectively regulate vWF size and conformation, which in turn regulate its interaction with platelets. We will then focus on the role of GPIb-A1 binding kinetics and GPIb-mediated mechanosensing in regulating platelet recruitment, adhesion, and signal transmission.

### *2.1. vWF production, storage and ultralarge vWF*

vWF is synthesized in megakaryocytes and stored in their α-granules (which are later partitioned amongst platelets); vWF is also synthesized in endothelial cells where it is stored in Weibel–Palade bodies and released upon endothelial cell stimulation. Stored and newly released vWF primarily exists as ultra-large multimers, which have a total molecular weight of greater than 50, 000 kDa [13]. Ultra-large vWF multimers are highly prothrombotic and bind to platelets with high avidity even at low shear rates [14]. Upon release into the bloodstream, ultra-large vWF attaches to the cell surface. ADAMTS13 then cleaves vWF at the Tyr 1605–Met1606 scissile bond in the central vWF A2 domains to form smaller, less adhesive vWF multimers. Cleavage reduces the high avidity of ultra-large vWF, thereby preventing platelet clumping and regulating vWF-mediated clot formation [13,15,16]. Importantly, cleavage of ultra-large vWF into smaller multimers functionally shifts the shear regime in which vWF-mediated platelet activation occurs, as higher forces are required to expose platelet binding domains in the smaller multimers.

### *2.2. Shear-induced conformation change and unfolding of vWF*

In circulation at low shear rates, cleaved vWF multimers adopt a globular conformation, in which the A1, A2, and A3 binding domains (sequentially located near the center the monomer) are covered [11,15]. vWF unfolds above a critical shear rate, both in free flow and when one end is surface bound. Mechanistically, unfolding is thought to proceed from hydrodynamic forces acting on small extensions projecting from the tumbling globular vWF [17]. This process is rapid and reversible, and simulations suggest that the critical shear rate for unfolding is more dependent on the multimer radius than on its total length [17]. Additional simulations suggest that elongation flow, such as that occurring in stenosis, more readily unfolds vWF at physiological flow rates [18].

Experiments on individual A2 domains showed that cleavage by ADAMTS13 only occurs when locally applied tension is above a threshold value [19]. As the force exerted on a vWF multimer is dependent on shear rate and multimer size, ADAMTS13 should not be able to cleave vWF multimers that remain below a critical length [14]. In addition, physiological levels of calcium play an important role in stabilizing the native conformation of vWF, and thereby increase the force needed for unfolding [20]. Careful kinetic measurements predict that at physiologic ADAMTS13 concentrations, cleavage occurs on time scales of 200 s,

suggesting that its effect on the initial vWF recruitment of platelets is minimal [14]. These findings together suggest that vWF is a complex force- and shear-modulated protein with monomer cleavage and platelet adhesion based on locally applied forces and local molecule concentrations.

### 2.3. Adhesion-induced conformation change of vWF

Upon blood vessel injury, shear induced conformation change enables vWF to bind to exposed collagen *via* the A3 domain. This binding enhances vWF's conformational change to expose its A1 and C1 domains (Fig. 1B) [17,21]. The interactions of GPIb with A1 and integrin αIIbβ3 with C1 facilitate platelet recruitment to the growing clot and stabilize platelet aggregates. As integrin αIIbβ3-mediated interactions are most stable at low physiological shear rates (less than 600–900 $s^{-1}$), platelet arrest on the exposed surface is increasingly dependent on GPIb–A1 interactions at high shear rates (1000–10,000 $s^{-1}$) [22]. In pathological conditions where shear rates are higher than 10,000 $s^{-1}$, such as in arterial stenosis, GPIb–A1 interactions play a critical role in platelet recruitment: they support the slow transit and rolling of platelets that promotes subsequent stable platelet adhesion. The versatility of vWF is thus highlighted by its active conformation changes that allow it to facilitate arrest of platelets at injury sites under various flow conditions.

### 2.4. The binding kinetics of GPIb–A1, force-induced conformation change of GPIb, and signal transmission

The platelet integrin complex GPIb–IX–V consists of four transmembrane proteins: GPIbα, GPIbβ, GPIX and GPV [23]. The A1 domain of vWF directly interacts with the extracellular region of GPIbα through the leucine-rich repeat of one of its N-terminus domains. This interaction regulates platelet rolling and arrest on the vessel wall. Studies at the single molecule level indicate that the significant role of vWF at high shear rates is dictated by the unique kinetics of GPIbα–A1 binding. Two theories currently exist to explain the mechanism of GPIbα binding to the vWF A1 domain under hydrodynamic forces. One theory proposes that at hydrodynamic forces <25 pN, GPIbα–A1 forms a catch bond that displays prolonged bond lifetimes and a decreased off-rate [24,25]; while the other theory suggests that forces cause the GPIbα–A1 bond to switch between two different slip bonds, forming a so-called 'flex-bond', with one slip bond having a much higher on-rate than the other [26]. In either case, the increased lifetime of the GPIbα–A1 bond is necessary for platelet rolling, slowing down, and eventual arrest on the vessel wall.

While it is known that the leucine-rich repeat domain of the GPIbα N-terminus governs binding to the A1 domain, it is largely unknown how the remaining domains affect A1 binding and force transmission. It is also unknown how interactions between GPIbα and the other components, such as GPIbβ and GPIX, affect force and signal transmission into platelets. Following the leucine-rich repeat domain of the N-terminus, GPIbα contains a highly glycosylated macroglycopeptide region; a stalk region; a pair of cysteine residues that connect to GPIbβ *via* disulfide bonds; a transmembrane helix; and a relatively short cytoplasmic domain at the C-terminus [27,28]. It was recently discovered that the stalk region acts as a mechanosensor that unfolds under pulling forces [29]. This unfolding occurs at forces ranging from 5 to 20 pN, which is a range similar to that reported for catch and flex bonds [29]. It is likely that the interplay between the unfolding of the stalk region and the binding kinetics of vWF–A1 under hydrodynamic forces governs platelet recruitment to the vessel wall. It is also proposed that unfolding of this stalk region induces conformational changes in GPIbβ and GPIX, which triggers further signaling [29].

### 2.5. The downstream signaling of the GPIb–A1 interaction

vWF is considered to be a weak agonist for platelet activation; however, binding of vWF to GPIbα triggers intracellular signaling events such as increased calcium concentration, integrin activation, thromboxane A2 production, and phosphoinositide 3-kinase (PI3K) activation. In resting platelets, phosphorylation of GPIbβ leads to the binding of 14-3-3-ζ, an intracellular adaptor/signaling molecule, to both GPIbα and GPIbβ, which maintains GPIb in a low-affinity state [23]. Upon vWF binding, 14-3-3-ζ dissociates from GPIbβ and associates with GPIbα, leading to downstream activation of signaling molecules such as Src and Lyn, two members of the Src family kinases (SFK). Src and Lyn then associate with GPIb–IX and become activated in what appears to be a PI3K p85 subunit-dependent process [23]. Shear-regulated GPIb–A1 interactions thus transmit signals into platelets through these cytoplasmic proteins.

### 2.6. The interaction of GPIb with the cytoskeleton

Early research has suggested that a link between the cytoskeleton and the GP1b receptor is important in platelet binding to the extracellular matrices at high shear. The cytoplasmic tail of GPIbα binds to actin filaments *via* filamin A, a dimeric scaffold protein that crosslinks GPIbα to actin [30]. The filamin A-mediated connection between GPIbα and actin is constitutively maintained in both resting and activated platelets. It appears that the link between GPIb and the membrane cytoskeleton does not affect the binding function of GPIbα or the downstream signaling of GPIbα–A1 binding [31]. However, anchoring of GPIbα to the membrane cytoskeleton *via* filamin A influences the integrity of platelet membranes at high shear rates (5000 to 40,000 $s^{-1}$) [31]. Under these conditions, mouse platelets expressing human GPIbα that are defective in filamin A binding develop unstable membrane tethers and lose membrane integrity, which in turn results in defective platelet adhesion at high shear.

### 2.7. GPIb–A1-regulated reversible platelet aggregation

During clot formation, vWF mediated platelet rolling is followed by platelet aggregation. Although it has long been assumed that soluble agonists generated at the injury site initiate platelet aggregation and thrombus growth, recent studies discovered that these processes are primarily driven by changes in blood rheology, and that the GPIbα–A1 interaction plays a significant role in initiating platelet aggregation. The GPIbα–A1 interaction induces integrin αIIbβ3 activation and αIIbβ3–vWF binding, leading to the formation of discoid platelet aggregates. Platelets are thus able to mechanosense the shear microgradient and form aggregates in response [32]. Further, the elimination of soluble agonists, such as ADP, thromboxane A2, and thrombin do not affect platelet aggregation dynamics induced by local shear microgradients [32]. The soluble agonists do, however, cause global activation of platelets, stabilize the aggregates, and lead to consolidation of platelet aggregation. Thus, discoid platelet aggregates form in the absence of soluble agonists in a shear-dependent manner but are completely reversible as they dissociate with cessation of flow.

## 3. The clot backbone: fibrinogen and fibrin networks

Although vWF initiates reversible platelet aggregation, clots stiffen with global platelet activation and consolidation of platelet aggregates. The mechanical properties of clots are essential to their function, as the clot must not only be elastic enough to sustain the shear forces of blood flow, but also stiff enough to resist rupture before bleeding is stemmed [33,34].

Typically, clots are composed of platelets surrounded by fibrin meshwork. The fibrin network is considered to be the backbone of blood clots, and is essential for stemming blood flow at sites of vascular

injury. The fibrin network forms as a consequence of biochemical cascades, during which fibrinogen is activated by thrombin and polymerized to form fibrin; the fibrin network is further stabilized *via* transglutaminase crosslinking [35,36]. The structure of the fibrin network is dependent on the kinetics of fibrin polymerization, and this structure ultimately determines clot mechanics [37]. A clot rheology study demonstrated that fibrin fiber thickness and branch point density are the two key determiners of network stiffness [38]. Larger fibrin fiber diameter and higher branch density result in a stronger or stiffer clot, and these properties can be obtained by increasing the concentration of fibrinogen, thrombin and/or $Ca^{2+}$ [39]. Unlike many commonplace polymers, such as rubber, the fibrin network exhibits non-linear strain stiffening due to the properties of fibrin and the overall structure of the fibrin clot [40]. Although it is composed of long, straight fibers, the fibrin network is both permeable and extensible: [41] the fibrin network can be stretched to three times its original length. When exposed to stretching forces, randomly oriented fibrin fibers align with the direction of the force [41]. This reorganization of fibers reduces intra-fiber spacing and excludes water from the fibrin meshwork, thus increasing the stiffness of fibrin gels dramatically. In fact, the stiffness of fibrin gels can be increased up to 20 fold under application of tensile forces [39]. It was also recently found that the stepwise unfolding of fibrin(ogen) molecules at the γC nodule and the reversible extension–contraction at the αC coiled-coil region further contribute to the extensibility and strain stiffening properties of fibrin gels [42–45]. The unfolding of these regions exposes hydrophobic domains that cause a further loss of water in fibrin clots. Therefore, the micromechanics of fibrin gels are not only controlled by the concentration of biochemical molecules, such as fibrinogen and thrombin, but are also governed by the local mechanical stretch or force applied. For example, the local twist of fibrin gels leads to the formation of a stiffness gradient proceeding from the twisting site [46].

The complex hydrodynamic flow environment in which clots form greatly affects the dynamics of fibrin polymerization as well as the ultimate fibrin structure, thus playing a crucial role in clot mechanics [47]. Biochemical signals can also be regulated by hydrodynamics; for example, during clot formation, the local concentration of thrombin, a soluble enzyme released from the surface of platelets or tissue-factor-bearing cells, is largely affected by blood flow [48]. Experiments using microfluidic devices have shown that fibrin clots of different morphologies, ranging from thin films to 3D gels, are controlled both by thrombin wall flux and wall shear rate [49]. Polymerization of fibrin fibers and the resulting fibrin network also influences thrombin transport in the 3D network: limited thrombin transport to the core of the clot results in dense fibrin at this location [50–52]. In addition, blood flow also affects the orientation of fibrin fibers. Compared to *in vitro* fibrin clots, *in vivo* thrombi show increased organization in the orientation of fibrin fibers. As modeled in a parallel plate flow chamber, newly formed fibrin fibers align with the direction of flow, and alignment increases with increasing flow rates [53]. Taken together these studies suggest that during clot formation, platelets are exposed to complex microenvironments with a wide range of mechanical stiffnesses that are dynamically affected by hydrodynamic forces.

## 4. Platelet–fibrin(ogen) interaction *via* integrin αIIbβ3

Although the structure of the fibrin network is governed by biochemical cascades and hydrodynamic forces, clot mechanics are also strongly affected by platelet–fibrin(ogen) interactions themselves. The interaction between platelets and fibrin(ogen) is facilitated by integrin αIIbβ3, the most abundant transmembrane receptor expressed on platelets [54,55]. A resting platelet's plasma membrane contains approximately 80,000 integrin αIIbβ3 receptors, and an additional pool of intracellular integrin αIIbβ3 can be recruited to the membrane upon platelet activation [56]. These membrane receptors serve as physical connections between fibrin(ogen) and the platelet cytoskeleton. Cytoskeletal contraction forces are transmitted *via* αIIbβ3 to fibrin(ogen), where the forces propagate through the clot and result in the clot contraction that stops bleeding. The binding of αIIbβ3 to fibrin is regulated by both the fibrin network and platelets themselves, as the integrin αIIbβ3 signals bidirectionally [57]: intracellular signaling triggers the activation of integrin αIIbβ3 and the sequential extracellular binding of fibrin(ogen) (inside-out signaling); while binding to fibrin(ogen) triggers the downstream signaling pathway of integrin αIIbβ3 to further amplify platelet activation, platelet contraction, and clot formation (outside-in signaling) [58]. Although integrin αIIbβ3 is the most abundant integrin receptor on platelets, they also express other integrins, such as αvβ3 and α2β1 [59]. As activation of all integrins occurs by conformational change and bidirectional signaling is similar amongst integrins [60,61], we will first discuss the structure of integrins, activation-related global integrin conformation changes, and force-enhanced integrin activation. We then specifically discuss the kinetics of the binding of αIIbβ3 to fibrin(ogen). Additionally, because most integrin-based mechanosensing is studied in nucleated cells, we will discuss platelet mechanosensing as it relates to that of nucleated cells, and further discuss the similarities and differences between nucleated cells and platelets. Lastly, we will discuss potential intracellular platelet mechanosensors and the role of the cytoskeleton in regulating platelet mechanosensing.

### 4.1. Integrin activation induces global integrin conformation changes

The integrin protein family in vertebrates includes 24 different members, derived from the 18α and 8β subunits. All integrins are heterodimers composed of non-covalently associated α and β subunits; the α subunit determines binding specificity and the β subunit attaches to actin [57,61,62]. The conformation of all integrins is similar: both α and β subunits have a large ectodomain, a single transmembrane domain, and a short cytoplasmic tail. The two subunits interact with one another to adopt a conformation of a large "head" on two "legs". The ectodomain of inactivate integrins has a bent or 'closed' conformation in which the head-piece is less than 5 nm from the plasma membrane. In this bent conformation, integrins have low affinities for their ligands. Activated integrins, on the other hand, exhibit an extended or 'open' conformation with the head-piece ~19 nm away from the plasma membrane, thus allowing for higher affinity ligand binding [63,64]. Integrins can also exist in an intermediated extended conformation with a closed head-piece. Long-range conformational change occurs when integrins switch from a bent to an extended conformation [63,65], which is triggered either by the binding of intracellular proteins, such as talin [66] or kindling [67], to the β tail, or by binding of extracellular molecules, such as ECM, $Mn^{2+}$, or antibodies, to the integrin ectodomain [58]. Steered molecular dynamic modeling and experimental studies also suggest that external forces can extend integrins and trigger their activation [68]. External forces can also accelerate the allosteric activation pathway of integrins [69]. In the absence of force, the time scale of integrin activation is on the order of seconds; however, external forces may decrease activation time to sub-seconds, thereby stabilizing bonds and preventing their dissociation [69]. External forces are transmitted from the ligand to the integrin, thus if the ligand is associated with the surrounding extracellular matrix, the integrins will respond to the mechanical properties (stiffness) of the extracellular matrix. More specifically, the mechanical properties of fibrin(ogen) and collagen matrices could potentially affect the activation and functions of platelet integrins.

### 4.2. Immobilized fibrinogen might be mechanically different from soluble fibrinogen in regulating platelet–fibrinogen interaction

In the blood stream, resting platelets co-exist with a high concentration of soluble fibrinogen (~2–4 g/L) [70], but it is well known that soluble fibrinogen molecules do not bind resting platelets due to the low binding affinity of the inactive integrin αIIbβ3 (low on-rate and high off-rate) [61]. In other words, while soluble fibrinogen may transiently

associate with bent integrin αIIbβ3, the molecules will dissociate before inducing downstream signaling or platelet activation. Therefore, the binding between inactive αIIbβ3 and soluble fibrinogen can be neglected. However, platelets readily adhere to immobilized fibrinogen molecules, and this association results in platelet spreading and activation. It has been suggested that fibrinogen immobilization causes a conformation change in the fibrinogen molecule that exposes binding sites that facilitate platelet adhesion [71,72]. This hypothesis is not fully supported as soluble fibrinogen binds activated platelets and fibrinogen molecules are dumbbell shaped with specific platelet-binding sequence in the γC domains on each end [73]. Furthermore, upon clot initiation, the HHLGGAKQAGDV sequences at each end of soluble fibrinogen are readily available for rapid platelet–fibrin(ogen) interactions. It is therefore possible that soluble fibrinogen is mechanically different from immobilized fibrinogen. Indeed, when fibrinogen is immobilized on substrates of different stiffnesses, the adhesion of platelets increases as the stiffness increases [5] (Fig. 2). Substrate stiffness-mediated platelet adhesion is regulated by Rac1, as the inhibition of Rac1 by treatment with NSC23766 decreases platelet adhesion on stiff substrates in a dose-dependent manner. It has been reported that the β3 tail is sufficient to regulate Rac1 activation, and that Rac1 activation is related to integrin clustering [74], where integrin clustering is the initial step in the formation of integrin-based adhesions. In both nucleated cells and platelets, integrin clustering increases binding avidity, stabilizes ligand engagement, and initiates interactions between integrins and the cytoskeleton [61,75,76]. Although recent studies on nucleated cells indicate that integrin clustering is force-independent [77], it is possible that the resistant forces of substrates facilitate integrin clustering by accelerating integrin activation and stabilizing their extended conformations. Therefore, it is likely that resistant forces from stiff substrates facilitate initial integrin activation and stabilize the integrin clustering that induces Rac1 activation. In addition, activation of Rac1 can activate Rap1b, a small GTPase, which then associates with talin and results in further integrin activation [78]. Therefore, substrate stiffness-mediated platelet adhesion may be regulated by an integrin–Rac1–Rap1b positive feedback loop [79] (Fig. 2). Although more mechanistic studies are needed to explore the underlying mechanism, this study suggests that platelets could sense and respond to the mechanical properties of fibrin(ogen).

### 4.3. The binding kinetics of αIIbβ3 and fibrinogen

The binding kinetics of integrin αIIbβ3 and fibrinogen at the single molecule level have been studied using both optical tweezers [80] and atomic force microscopy [81]. It was found that αIIbβ3–fibrinogen complexes exist in at least two mechanically stable states which correspond to low ligand affinity and high ligand affinity. Pulling force is able to induce a slow reversible conformational transition from lower to higher affinity. The applied tensile force increases the off-rate of the lower affinity form of αIIbβ3, while the forced off-rate of the higher affinity form decreases. The pulling forces may extend αIIbβ3, lead to a tighter binding pocket, and cause more side chain contacts [80,82,83]. However, the pulling force must be slow enough to form stable αIIbβ3–fibrinogen bonds as the bond's stability depends on contact duration [84]. αIIbβ3–fibrinogen complexes are shown to correlate to classic slip bonds when exposed to forces in the 5–50 pN range [84]. This is consistent with platelet adhesion in flow conditions: flowing platelets are arrested on immobilized fibrinogen only at wall shear rates below 600–900 $s^{-1}$ due to the slow rate of αIIbβ3–fibrinogen bond formation and their low resistance to tensile stress [22,85]. Further, it is possible that αIIbβ3–fibrinogen bonds are more stable when the force is less than 5 pN, or at lower loading rates with longer contact times. The sensitivity of the αIIbβ3–fibrinogen bond to small forces may implicate it in the fine-tuning of the platelet response to the underlying clot structure and formation.

### 4.4. General differences between the integrin-based mechanosensing of nucleated cells and platelets

Integrin-based adhesions between cells and their environments are the best-studied aspects of the transmission of mechanical stimuli [6–8]. Nucleated cells are subject to external mechanical forces, as

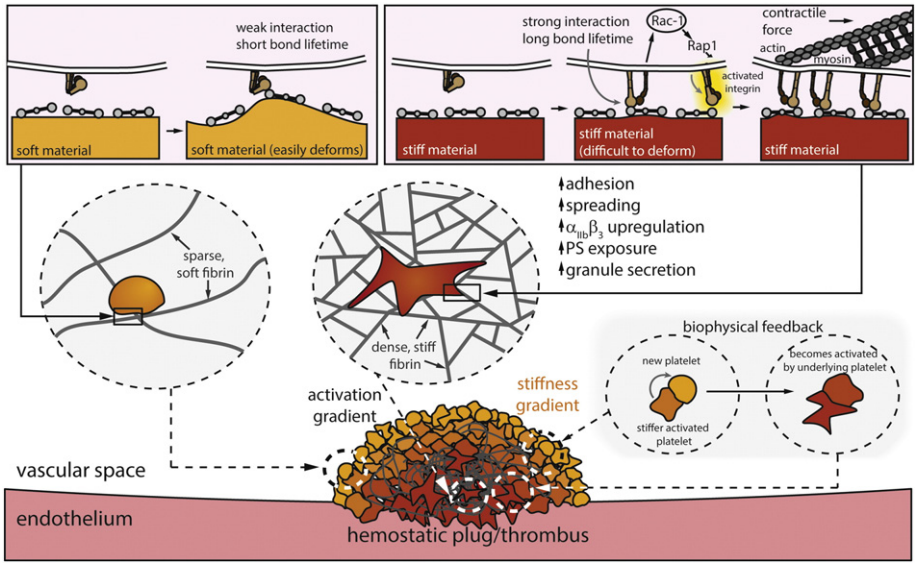


**Fig. 2.** Proposed multi-scale effects of platelet substrate mechanosensing on hemostasis and thrombosis. Platelet mechanosensing of the microenvironment (which includes heterogeneous fibrin polymer networks of differing mechanical properties) likely starts with the initial adhesive event, during which the resistant substrate forces balance the inertial motion of the platelet. A platelet engaging with a relatively soft substrate results in a weak $\alpha_{IIb}\beta_3$–fibrinogen interaction and short bond lifetime. A stiffer substrate, however, provides more resistant force, leading to more platelet adhesion and outside-in $\alpha_{IIb}\beta_3$ signaling. This in turn, generates high acto-myosin-mediated internal balancing force, resulting in additional platelet spreading on stiffer substrates. Downstream signals will then further trigger platelet activation, thus upregulating $\alpha_{IIb}\beta_3$ activation, granule secretion, and PS exposure. In addition, as activating platelets stiffen, interacting platelets bridged by fibrinogen will act as physical substrates for one another, and mechanosense the increased stiffness of adjacent platelets. This leads to a biophysical positive feedback loop that further drives platelet aggregation and could possibly interact with biochemical signals that inhibit platelet aggregation such that the process is controlled. Taken together, platelet mechanotransduction of the microenvironment likely synergizes with biochemical signals to fine-tune platelet activity and function during clot formation.

well as intracellular forces generated by actin polymerization and myosin contraction [86]. The magnitude of these forces affects the dynamic formation of integrin-based adhesions and the interactions between adhesions and the cytoskeleton [87]. In integrin-based adhesions, a set of proteins acts as a mechanical sensor by exhibiting force-driven conformation changes that regulates enzyme activity and exposes phosphorylation sites or binding sites on downstream proteins [86]. Using this protein machinery, nucleated cells sense the mechanical properties of their microenvironments and transduce mechanical signals into biochemical signals that result in cytoskeletal reorganization and morphological change, as well as regulate cell migratory behavior. On a longer time scale, force-driven signaling also mediates cell proliferation and differentiation [88].

Although platelets are anucleate cell fragments, they have many of the same mechanosensing proteins as do nucleated cells, including Src, talin, FAK and vinculin. It therefore raises the question as to what role mechanosensing plays in clot formation. Recent findings suggest that platelets mechanosense their microenvironments and respond with different levels of adhesion, spreading and activation [5]. However, the lack of a nucleus as well as their rapid, relatively short-lived response to endothelial cell injury, means that the long-term effects of mechanosensing in nucleated cells, such as gene expression regulation and cell migration, do not exist in platelets. Furthermore, maturation of integrin-based adhesions from adhesion complexes to focal adhesions is unnecessary for platelet outside-in signaling and spreading. Platelet mechanosensing then likely works in concert with biochemical signals to regulate platelet activation behaviors such as spreading, granule release, and granule membrane fusion with the plasma membrane. In the following sections, we will discuss potential platelet mechanosensors, the most important steps in platelet integrin outside-in signaling, and the potential mechanosensing process in platelets.

### 4.5. Potential mechanosensors in platelet adhesion

In nucleated cells, mechanosensor proteins respond to force with conformation change and activation [86], and this force-driven signal initiation is important for a cell's interactions with its microenvironment. However, the roles of mechanosensing proteins in platelet function and physiology are largely unknown. Talin has been identified in nucleated cells as one of the most important mechanosensors for focal adhesion assembly [89,90]. Talin binds directly to the integrin cytoplasmic tail through its head FERM (four point one protein/Ezrin/Radixin/Moesin) domain [91], and its rod domain contains 5 potential vinculin binding sites [92]. Applied forces can extend the length of talin by 140 nm, leading to exposure of the vinculin binding sites [93]. The force-driven recruitment of vinculin matures focal adhesions and stabilizes integrin-based adhesions. However, the role of vinculin does not seem to be as essential to platelet activity [94]. Mice deficient in megakaryocyte/platelet vinculin have normal platelet counts, and a majority exhibit only mild platelet function defects as evidenced by prolonged tail bleeding times. Furthermore, these mice form normal occlusive thrombi in response to carotid artery injury. Vinculin deficiency thus does not appear to impact agonist-induced αIIbβ3 binding to fibrinogen; platelet aggregation and spreading; actin polymerization/organization; or clot retraction. Mice platelets deficient in vinculin are also able to adhere to immobilized fibrinogen or collagen normally, regardless of flow conditions. Taken together, this either suggests that mature focal adhesions are not essential to platelet function (as they are in migratory nucleated cells), or that vinculin deficiency in mice is compensated for by other proteins. It also suggests that for most platelet functions, proteins such as talin and myosin readily facilitate the necessary connections between integrin and actin.

Src family kinases (SFKs), including Src, Fyn, Lyn and Yes, have been identified as critical in initiating and propagating activation signals in platelets [95–97]. While Src is involved in the downstream signaling of the GPIb–A1 interaction [23], it also constitutively associates with the cytoplasmic tails of β3 and is essential to αIIbβ3 outside-in signaling in platelets. Upon fibrinogen ligation, integrin clustering causes protein tyrosine phosphatase (PTP) 1B-dependent dissociation of c-terminal Src kinase (Csk) from cytoplasmic tails of β3 [98,99], which in turn activates Src. Activated Src is found to localize in filopodia and at the edges of spreading platelets. This localization triggers downstream signaling pathways, cytoskeleton reorganization, and platelet spreading. In nucleated cells, Src is also activated by mechanical stimuli [100]: using optical tweezer traction of fibronectin-coated beads adhered to cells, it was found that force triggers directional propagation of Src activation, as well as distal Src activation. This begs the question as to whether mechanical forces can trigger the global activation of Src in platelets in order to accelerate platelet activation and clot formation.

Src activation in platelets is regulated by additional proteins, such as the receptor-like protein tyrosine phosphatase (RPTP) CD148 [101]. CD148 is identified as the only RPTP expressed on human platelets, and a deficiency in CD148 reduces Src activity and decreases platelet spreading on fibrinogen. As compared to normal platelets, CD148-deficient platelets form filopodia at a slowed rate and form fewer filopodia. The filopodia also tend to retract over time. Interestingly, in nucleated cells, a member of the transmembrane RPTP family, RPTP-α, colocalizes with $\alpha_v$-integrins at the leading edge of the cell during early spreading and acts as a mechanical transducer that controls the activation of SFKs, particularly Fyn [102]. This force-dependent pathway regulates the formation of focal complexes, thereby enhancing integrin–cytoskeleton connections during the initial phases of ECM contact. In platelets, Fyn also associates with the cytoplasmic tails of β3 independently of Src–integrin binding [103]; and deficiency of Fyn in mice has a modest effect on αIIbβ3-dependent platelet function, for example, Fyn-deficient platelets exhibit delayed spreading on immobilized fibrinogen. The similarities between protein machinery in platelets and nucleated cells pose interesting questions about the possible interplay of CD148 and SFKs, and whether this could contribute to platelet mechanosensing during initial spreading.

### 4.6. The role of actomyosin activity in platelet mechanosensing

Signaling downstream of SFKs leads to cytoskeletal reorganization, including actin polymerization and myosin activity [104]. Actomyosin activity dynamically mediates ECM–integrin–cytoskeleton connections, and impacts their mechanical stability. While initial integrin clustering and cell spreading is force-independent in nucleated cells, later cell spreading is regulated by forces generated within the cells as well as by resistive forces from the substrates underlying the spreading cells. The forces generated within cells include those from actin polymerization [105], contractile forces by myosin [106] and membrane tension [107,108]. At the leading edge of the lamellipodia, the force from actin polymerization pushes the outward movement of integrin clusters and furthers lamellipodia extension. Contractile forces from myosin pull on lamellipodia actin, causing cell edge retraction and initiating nascent adhesion complexes on the ECM. Further, forces from myosin release lamellipodia actin from the tip of the extension to create new edge protrusions by actin polymerization. Thus, periodic contraction and actin polymerization allow cells to spread with stable adhesions [109,110].

The forces associated with myosin contraction are chiefly controlled by phosphorylation of its light chain (MLC), which is regulated by two distinct signaling pathways: myosin light chain kinase (MLCK) and Rho kinase (ROCK) [111,112]. MLCK directly phosphorylates MLC in a $Ca^{2+}$/calmodulin-dependent manner, while ROCK promotes phosphorylation of MLC by inhibiting myosin phosphatase. It seems that these distinct signaling pathways differentially regulate myosin activity, and impact different platelet functions. It has been found that cell spreading is mediated by MLCK, in which MLCK binds to actin at the cell's leading edge and is carried rearward with actin flow during cell spreading. As MLCK moves rearward towards myosin, it is stretched to release MLCK auto-inhibition which further activates myosin [109,110]. Rigid

substrates favor contraction-mediated cell spreading as they provide increased resistant forces, and thus increased cell contraction, as compared to soft substrates. It was recently found that platelets employ a similar mechanism for mechanosensing during spreading. Platelets spread more on stiff substrates than on soft substrates, and the spreading of platelets reaches a plateau when the stiffness of the substrates is more than 5 kPa. Moreover, treatment with the MLCK inhibitor ML-7, but not with the ROCK inhibitor Y-27632, eliminates substrate stiffness-mediated spreading, confirming that MLCK and downstream myosin activity govern mechanosensing in platelet spreading [5]. More importantly, this substrate-mediated platelet adhesion and spreading further affect the major platelet activation pathways including inside-out integrin signaling, granule secretion, and phosphatidylserine (PS) exposure. On the substrates stiffer than 5 kPa, adherent platelets exhibit significantly more integrin αIIbβ3 activation, higher P-selectin expression and PS exposure than those on softer substrates.

ROCK is co-expressed with MLCK in platelets to regulate myosin activity *via* the RhoA–ROCK axis [113], though it differently impacts platelet functions than does MLCK. During platelet activation, the (Thr)18 and (Ser)19 residues of myosin light chain are phosphorylated; and it is found that Ser(19) phosphorylation is sufficient for shape change, whereas (Thr)18 phosphorylation acts in concert with Ser(19) phosphorylation for full platelet contraction and dense granule release. MLCK and ROCK exhibit differential regulation of these two residues: MLCK leads to phosphorylation of both (Thr)18 and (Ser)19 residues, while inhibition of ROCK does not affect (Ser)19 phosphorylation, but results in a substantial inhibition of (Thr)18 phosphorylation [113]. A study with RhoA-deficient mice further indicates that RhoA/ROCK is essential for integrin-mediated clot retraction, though it does not affect actomyosin rearrangements or platelet spreading on fibrinogen [114].

Myosin activity is also essential in forming a clot that is stiff enough to stop bleeding. Clots stiffen over time and their mechanical properties are largely determined by the fibrin network, the interaction between fibrin and platelets, and platelet contraction (myosin activity). In addition, the mechanical microenvironment of a clot is nonhomogeneous, so interactions between platelets and the heterogeneous microenvironment are critical to fine-tuning the clot's mechanical properties. Measurement of mechanics and dynamics of single platelets indicate that individual platelets generate an average maximum contractile force of 29 nN and form adhesions stronger than 70 nN. Moreover, platelets can mechanosense the stiffness of their fibrin(ogen) substrate and generate higher contraction forces in response to stiffer substrates, thus allowing for dynamic alterations in the clot's mechanical properties of clot and more uniform contraction of the heterogeneous clot [115]. An *in vitro* model of platelets embedded in 3D fibrin networks of different stiffnesses also indicates that thrombin-activated platelets mechanosense the microenvironment. During platelet contraction, the fibrin fibers of mechanically stiffer gels provide increased resistant forces that lead to faster and increased platelet bleb formation and PS exposure [5]. Taken together, these studies suggest that myosin activity is important in all stages of platelet activation and clot formation, while the myosin activity might be regulated through different pathways at different stages.

### 4.7. Direct αIIbβ3–cytoskeleton interactions

Integrin clusters recruit adaptor proteins, such as talin, α-actinin, vinculin and focal adhesion kinase (FAK), to connect integrins to the cytoskeleton in nucleated cells [116]. However, in platelets, myosin directly binds the tail of integrin β3 [117], thereby acting as a direct bridge between integrin and the actin cytoskeleton. Indeed, the selective binding of platelet myosin heavy chain to the integrin cytoplasmic tyrosine domain after tyrosine phosphorylation is important for outside-in αIIbβ3 signaling in platelet aggregation and clot retraction [118]. This suggests that forces generated by myosin can directly regulate αIIbβ3-mediated platelet–fibrin(ogen) interactions, thereby acting as an alternative pathway for platelet mechanosensing through αIIbβ3.

## 5. Collagen — the stiffness of the vessel wall may affect platelet physiology

### 5.1. Collagen in the vessel wall and mechanics of collagen matrices

Blood vessels must be strong enough to sustain cyclic loading and pressure, and this characteristic is largely provided by extracellular matrix components including collagen and elastin [119]. *In vivo*, collagen molecules self-assemble into fiber networks that form the structural basis for the integrity and strength of blood vessels, and due to its structure, collagen is the ubiquitous load-bearing and reinforcing element in arterial walls [120]. *In vitro*, collagen molecules self-assemble into a fiber network through a process similar to that which occurs *in vivo* in which monomers assemble into fibrils that further bundle into fibers and form networks [121]. *In vitro* studies of collagen gels provide insight into how the structure of the collagen fiber network affects the bulk mechanical properties of collagen gels. Similar to the fibrin network, the bulk mechanical properties of collagen gels are chiefly determined by the diameter of individual collagen fibers, the fiber density, and branch point density of the network [122]. Collagen matrices also exhibit a nonlinear stress–strain relationship in response to tensile forces, and force leads to the alignment of collagen fibers [123]. The mechanics of blood vessel walls mirror that of collagen gels, for example, arteries exhibit highly non-linear stress–strain responses in that they stiffen at higher pressure. *In vivo*, collagen fibers are also circumferentially aligned with cyclic loading, thereby preventing over-distension and damage to the vessels at high luminal pressures [124,125]. In addition, the stiffness of the vessel wall increases with aging due to changes in the microscale structure of the vessel wall's extracellular matrix: collagen deposition and matrix protein crosslinking increase during aging resulting in increased stiffness [126]. This stiffening process is accelerated by hypertension. An increase in vessel stiffness in other disease states, such as atherosclerosis, has also been linked to changes in composition and structure of the extracellular matrix [127].

### 5.2. Platelet mechanosensing on collagen matrices

Upon blood vessel injury, collagen matrices underlying the injured endothelium are exposed causing platelet adhesion and activation, and initiating (irreversible) clot formation. The transmembrane receptors glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$, are both responsible for platelet adhesion and activation on collagen [128–132]. Human GPVI is composed of 2 IgG-C2-like extracellular domains, a mucin-like stalk, a transmembrane region, and a short 51-amino acid cytoplasmic tail. It acts as a signal generator for integrin activation and other downstream signaling cascades, rather than as an adhesion receptor [131]. The binding of GPVI to collagen leads to activation of both Fyn and Lyn, followed by activation of Syk, which generates downstream signaling cascades [133]. It also leads to activation of both integrin αIIbβ3 and α2β1 from inside-out signaling [130]. The stable adhesion of platelets to collagen is governed by integrin α2β1, which binds to collagen in an $Mg^{2+}$-dependent manner and mediates outside-in regulation of platelet spreading. Similar to the αIIbβ3–fibrinogen interaction, engagement of α2β1 and collagen induces activation of Src, Syk, SLP-76 and PLC-γ2, leading to cytoskeleton reorganization and platelet spreading [129,134].

Since integrin α2β1 is implicated in outside-in signaling, it raises questions as to whether platelets can mechanosense the stiffness of collagen matrices, and whether the stiffness mediates platelet spreading on collagen. A recent study showed that, similar to platelets in a fibrin gel, platelets spread more on stiff collagen-coated substrates than on soft substrates, and that the substrate stiffness-mediated spreading on collagen is also regulated by myosin activity *via* the MLCK signaling pathway [135]. Interestingly, this substrate stiffness-mediated spreading is also affected by extracellular $Ca^{2+}$ levels. It is suggested that collagen be considered as a strong agonist that causes $Ca^{2+}$ release and influx, and results in a sustained high cytosolic $Ca^{2+}$ level [136]. The

sustained increased levels of $Ca^{2+}$ promotes the activation of the $Ca^{2+}$-dependent protease, calpain, which cleaves the connection between cytoskeletal proteins and integrins, and regulates bleb formation and shedding of microvesicles from activated platelets [137,138]. Upon activation of platelets *via* elevated $Ca^{2+}$ signaling, substrate stiffness-mediated spreading is reduced on collagen. Consistent with this, inhibition of ADP and thromboxane A2 signaling increases substrate stiffness-mediated platelet spreading on collagen. It was also found that the mechanical properties of collagen matrices can affect PS exposure of adherent platelets in the absence of exogenous agonists [135]. Taken together, since the stiffness of blood vessels increases with age and in diseased states, such as atherosclerosis, these findings suggest that biomechanical factors should be considered in these circumstances.

## 6. Summary and future directions

The key mechanism of cellular mechanosensing and mechanotransduction is that the binding and conformation of proteins are regulated by forces. This principle is also applied to molecular processes of clot formation. Complex hydrodynamics regulate the conformation of vWF for its enzymatic degradability, avidity for platelet binding, and exposure of the A1 domain for effectively arresting platelets on the vessel wall at high shear rates (Fig. 1A). Hydrodynamic forces also direct the structure of the fibrin network, such as fibrin fiber thickness, branch points, and fiber alignment. Although platelets are considered to be the simplest of human cells as they even lack mechanisms for motility, they are nevertheless able to mechanosense their microenvironment and physiologically respond in a graduated manner (Fig. 1B). In a manner similar to nucleated cells, platelets employ integrins and adaptor proteins to link the cytoskeleton, actin, and the motor protein myosin with extracellular proteins. The balance of hydrodynamic forces stemming from the flow environment, resistant forces resulting from extracellular matrix stiffness, and actomyosin driven intracellular forces can also affect integrin activation and the binding between integrins and the extracellular matrix. The dynamic force environment is thus inherently important to regulating platelet activity. Although the role of forces has been extensively studied in nucleated cells and many mechanosensors have been identified, platelet mechanosensing is a relatively untapped area of study. It will be interesting to further explore the differences between platelets and nucleated cells in mechanosensing and mechanotransduction to provide further insight into both platelet physiology and mechanobiology.

Clot formation is a complex process that requires the dynamic interplay between platelets and extracellular matrix proteins. Moreover, this interplay is regulated both by complex hydrodynamics and by biochemical molecules, such as ADP, thromboxane A2 and thrombin. Furthering the complexity of the microenvironment is that hydrodynamics also determine the local concentration and diffusion of these biochemical agonists. It is plausible that each of these effects dominates the microenvironment at different stages of clot formation. For example, a local shear microgradient can initiate reversible discoid platelet aggregation without any agonists, but agonists are necessary to further consolidate the aggregation. As a dynamic component of clotting, platelets may mechanosense their microenvironment, and dynamically change their own mechanical properties during activation. In addition, in a thrombus, or platelet aggregate, platelets are physical substrates for one another, and thus likely mechanosense the stiffening of adjacent platelets. This would then result in further activation and release of granule contents, forming a positive feedback loop of platelet activation within the microenvironment (Fig. 2). Further exploration of these processes will promote a better understanding of clot mechanics and dynamics of clot formation.

## Conflict of interest statement

None.

## Acknowledgments

Financial support for this work was provided by a National Science Foundation CAREER Award 1150235 (to W.A.L.); an American Heart Association Innovative Research Grant (to W.A.L.); National Institutes of Health Grants U54HL112309 (to W.A.L.), and R01HL121264 (to W.A.L.); and American Heart Association Postdoctoral Fellowships 12POST12080069 (to D.R.M.).

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