Supporting Information

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SI Materials and Methods

Washed Platelet Isolation and Platelet Incubation on Polyacrylamide Gels. Whole blood was drawn from healthy donors into collection tubes with acid citrate dextrose (ACD). Platelet-rich plasma (PRP) was collected after centrifugation under 150 × *g* for 15 min. Additional ACD was added into PRP (10% of the PRP volume), which was then centrifuged at 900 × *g* for another 5 min. Platelet-poor plasma was then removed and the platelet pellet was resuspended in Tyrode's buffer containing 0.1% BSA. Platelets at 5.5 million per mL were then incubated on polyacrylamide (PA) gels with 1 mM Ca^{2+} and 2 mM Mg^{2+} for 2 h. After incubation, nonadherent platelets were removed and adherent platelets were fixed with 1% paraformaldehyde (PFA) for 10 min. The fixed platelets were then washed three times with PBS and then stored in PBS for further analysis. For inhibitor studies, platelets at 5.5 million per mL were preincubated with different inhibitors for 1 h before being incubated on PA gels. One millimolar Ca^{2+} and 2 mM Mg^{2+} were added into the suspension immediately before incubation on PA gels. The inhibitors used in this study included NSC23766 (Cayman; 5, 30, and 300 μM), latrunculin A (Sigma; 2 μM), ML-7 (Sigma; 10 μM), Y-27632 (Sigma; 10 and 30 μM), PP2 (Sigma; 10 μM), and focal adhesion kinase (FAK) inhibitor (Tocris Bioscience; 10 μM).

Analysis of Platelet Adhesion and Spreading on PA Gels. Fixed platelets were stained with CellMask Orange (Life Technologies; 1:1,000; cell membrane staining) for 30 min followed by washing with PBS for three times. Three gels were analyzed per stiffness group and three representative images were obtained via epifluorescence microscopy (Nikon TE2000-U) for each gel. The number of adherent platelets in each image was counted using ImageJ, and the numbers from three images were averaged for each gel. For platelet spreading, adherent platelets were imaged with *Z* stack using confocal microscopy (Zeiss LSM700). Similarly, three representative images were taken for each gel. The *Z*-stack images were then flattened, and the spreading area of each platelet was measured using ImageJ. Spreading area of platelets in all three images was averaged for each gel.

Analysis of Integrin $\alpha_{IIb}\beta_3$ Activation. One hundred microliters of PAC-1-FITC (BD Biosciences) was applied on each gel for 5 min before fixation. Stained platelets were then imaged with *Z* stack using confocal microscopy. The same laser light intensity and gain settings were used for all images. The *Z*-stack images were flattened, and both the average fluorescence intensity over the spreading area and total fluorescence intensity of each adherent platelet were measured with ImageJ.

Analysis of P-Selectin Expression. The fixed platelets were blocked with 5% (vol/vol) goat serum for 1 h. The primary antibody, mouse anti-human P-selectin (Abcam; AK4, SC-19996; 1:300) in 5% goat serum was then applied for 1 h. After washing with PBS three times, goat anti-mouse IgG–Alexa Fluor 488 (AF488) (Life Technologies; 1:300) was then added for 1 h. The platelets were double stained with CellMask Deep Red (Life Technologies; 1:1,000; cell membrane staining) for 30 min. Stained platelets were then imaged with *Z* stack using confocal microscopy. The imaging process and analysis with ImageJ are similar as that for activated integrin $\alpha_{IIb}\beta_3$. The spreading area was measured according to the fluorescence signal of P-selectin staining. Because some nonspread platelets did not express P-selectin, their spreading areas were measured with cell membrane fluorescence signal.

Analysis of PS Exposure. Adherent platelets were stained with Annexin V–AF488 (Life Technologies) for 5 min immediately before fixation. The fixed platelets were then double stained with CellMask Deep Red (Life Technologies; 1:1,000; cell membrane staining) for 30 min. Stained platelets were then imaged with *Z* stack using confocal microscopy. The platelets positively stained with Annexin V–AF488 were counted from flattened images with ImageJ.

Assessing Platelet Mechanosensing Under Flow Conditions. PA gels of different stiffnesses (0.5, 5, and 50 kPa) were polymerized on the bottom of microfluidic devices, and fibrinogen was conjugated on the top surface of the gels. Washed platelets were perfused through the microchannel at a shear rate of 100 s^{-1} for 2 h. The shear rate was calculated via Eq. **S1** (1):

$$\dot{\gamma} = \frac{6Q}{w * h^2}, \qquad \textbf{[S1]}$$

where $\dot{\gamma}$ is shear rate, Q is the flow rate, w is the width of the channel, and h is the height of the channel. Adherent platelets were then fixed by flowing 1% PFA for 10 min and washed by flowing 1× PBS for another 30 min.

Characterization and Measurement of Platelet PS Exposure and Bleb Formation in 3D Fibrin Networks. Two different concentrations of fibrinogen, 0.5 and 4 mg/mL, were used. $CaCl_2$ (10 mM), thrombin (1 U/mL), Annexin V–AF488 (1:20), and platelet (20 million per mL) concentrations were kept constant. To visualize the fibrin fibers, 37.5 μg/mL fibrinogen–AF543 was also added to the fibrinogen solution. Under these conditions, the bulk mechanical properties of fibrin gels are determined primarily by the fibrinogen concentration (2). The solutions were then mixed, allowing fibrin gels to form in the presence of thrombin-activated platelets. Time-lapse confocal microscopy images were then recorded for 30 min with an interval of 1 min for each fibrin gel. We observed that thrombin-activated platelets in stiffer fibrin networks exhibited much stronger PS staining than thrombin-activated platelets in softer fibrin networks (Fig. S9 *A* and *B*). Time-lapse confocal microscopy revealed that platelets in the stiffer fibrin networks exhibited increased PS-positive blebbing and at earlier time points than platelets in softer fibrin networks (Fig. S9*C*). Overall, nearly all platelets in the stiffer fibrin networks contained PS-positive blebs, whereas only a small minority of platelets in the softer fibrin networks contained PS-positive blebs (Fig. S9*D*).

Statistics. The significant difference in each group was determined with one way or two-way ANOVA using Minitab.

1. Son Y (2007) Determination of shear viscosity and shear rate from pressure drop and flow rate relationship in a rectangular channel. *Polymer (Guildf)* 48(2):632–637.

2. Ryan EA, Mockros LF, Weisel JW, Lorand L (1999) Structural origins of fibrin clot rheology. *Biophys J* 77(5):2813–2826.

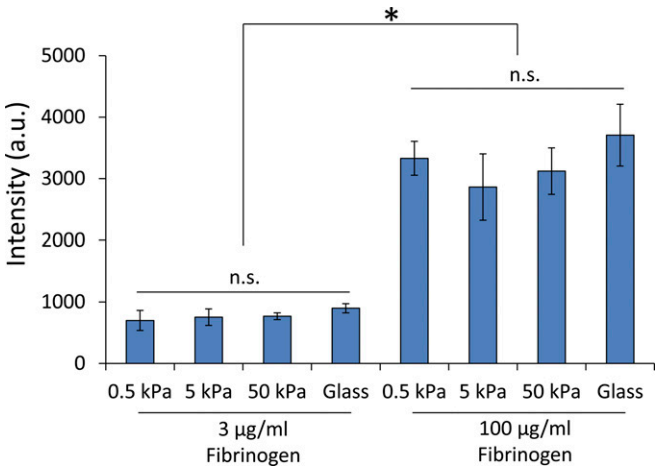

Fig. S1. No statistically significant differences in fibrinogen density, as measured via fluorescence intensity of conjugated fibrinogen–AF488, were observed among PA gels of different stiffnesses (0.5, 5.0, and 50 kPa) that were each conjugated with fibrinogen at 3 μg/mL. Similarly, no statistically significant differences in fibrinogen density were observed among PA gels of different stiffnesses (0.5, 5.0, and 50 kPa) that were each conjugated with fibrinogen at 100 μg/mL. Expectedly, statistically significant differences were observed between PA gels conjugated with fibrinogen densities of 3 μg/mL versus PA gels conjugated with fibrinogen densities of 100 μg/mL. n.s., not significant. *$P < 0.05$; $n = 3$ experiments.

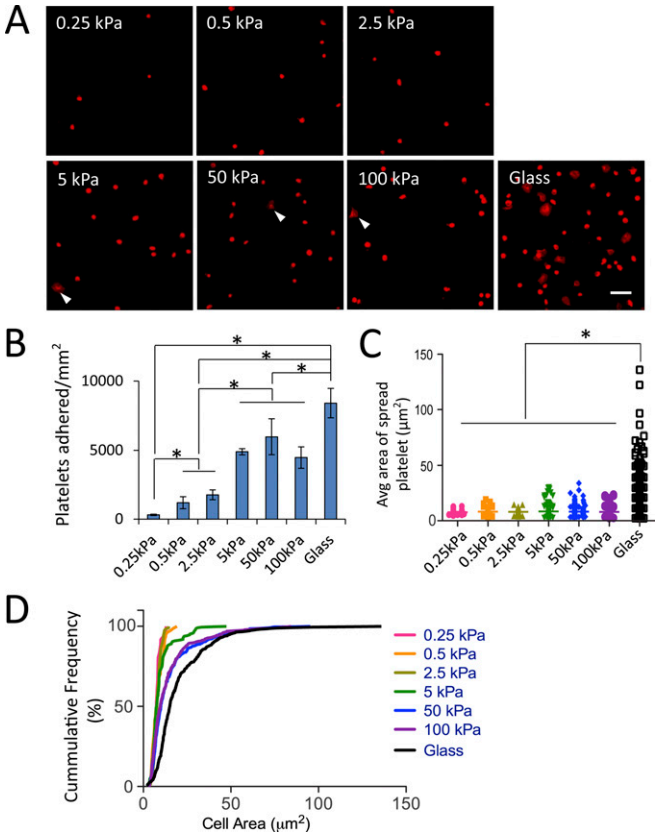

Fig. S2. Substrates of different stiffnesses conjugated at high fibrinogen concentrations (100 μg/mL) resulted in differential platelet adhesion and platelet spreading. (*A*) Representative confocal images of platelets adhesion and spreading on substrates of different stiffness conjugated with fibrinogen at a concentration of 100 μg/mL. The arrows indicate limited number of spread platelets on stiffer gel substrates. (Scale bar: 10 μm.) (*B*) Platelet adhesion reached a plateau when substrate stiffness reaches 5 kPa. (*C*) Higher fibrinogen and softer substrate stiffness synergistically decrease platelet spreading. (*D*) Cumulative frequency of spread platelet surface area on substrate of different stiffnesses. *$P < 0.05$; $n = 3$ experiments.

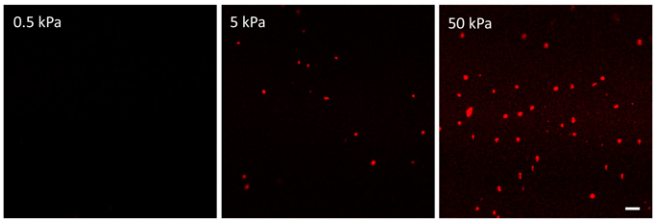

Fig. S3. Increases in substrate stiffness lead to increased platelet adhesion under flow conditions (shear rate, 100 s^{-1}). Almost no platelets were observed adhered on substrate of 0.5 kPa. However, platelets were found adhered on gels of 5 and 50 kPa under flow after 2 h. (Scale bar: 10 μm.)

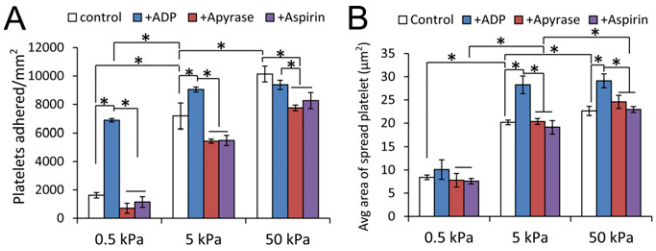

Fig. S4. Platelet mechanosensing during adhesion and spreading is not mediated by ADP or thromboxane A2. (*A*) The number of platelets adhered onto PA gels of different stiffness after 10 μM ADP, 0.05 U/mL apyrase, or 1 mM acetylsalicylic acid (aspirin) treatment. (*B*) Preactivation by exogenous ADP differentially affects substrate stiffness-mediated platelet adhesion on PA gels of different stiffnesses, whereas inhibition of ADP activation via 0.05 U/mL apyrase and inhibition of thromboxane A2 via 1 mM aspirin did not alter platelet mechanosensing during spreading on PA gels of different stiffnesses ($P < 0.05$; $n = 3$ experiments; error bars indicate SD).

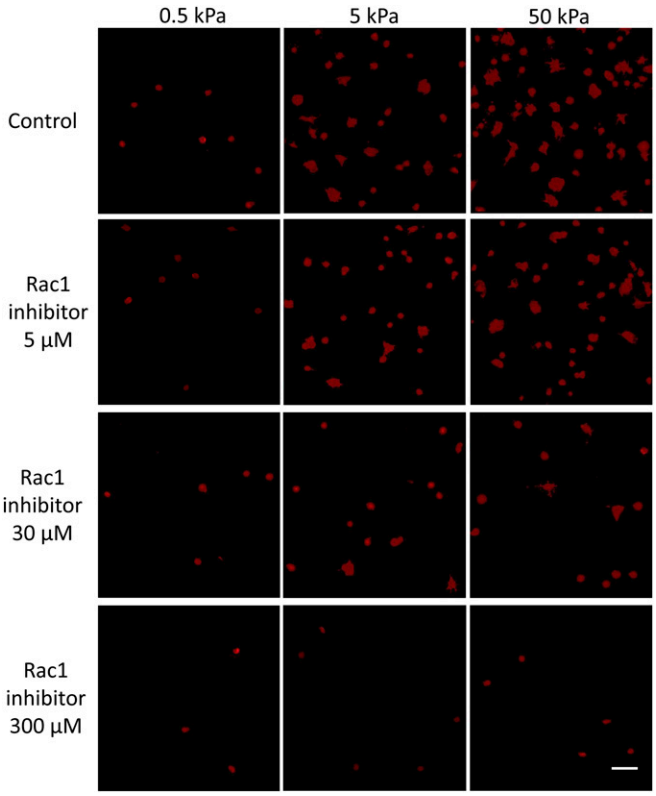

Fig. S5. Rac1 mediates platelet mechanosensing during adhesion and spreading. Representative epifluorescence images of adherent platelets (nontreated versus treated with Rac1 inhibitor) on 0.5-, 5.0-, and 50-kPa PA gels. (Scale bar: 10 μm.)

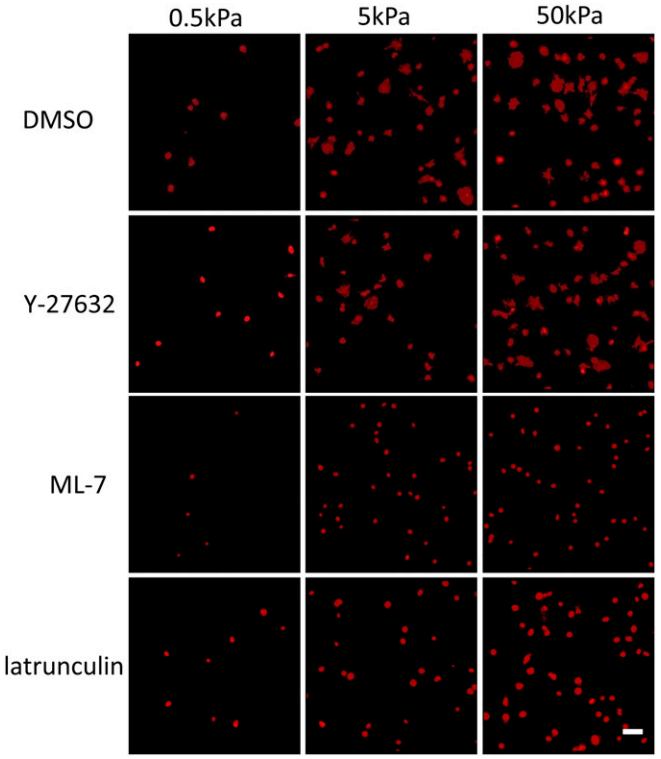

Fig. S6. Myosin activity and actin polymerization mediate platelet mechanosensing during spreading. Representative confocal images of platelets treated with DMSO only (control), Rho kinase (ROCK) inhibitor (Y-27632; 10 μM), myosin light chain kinase (MLCK) inhibitor (ML-7; 30 μM), or latrunculin (2 μM) after adhering onto PA gels with stiffnesses of 0.5, 5, and 50 kPa. (Scale bar: 10 μm.)

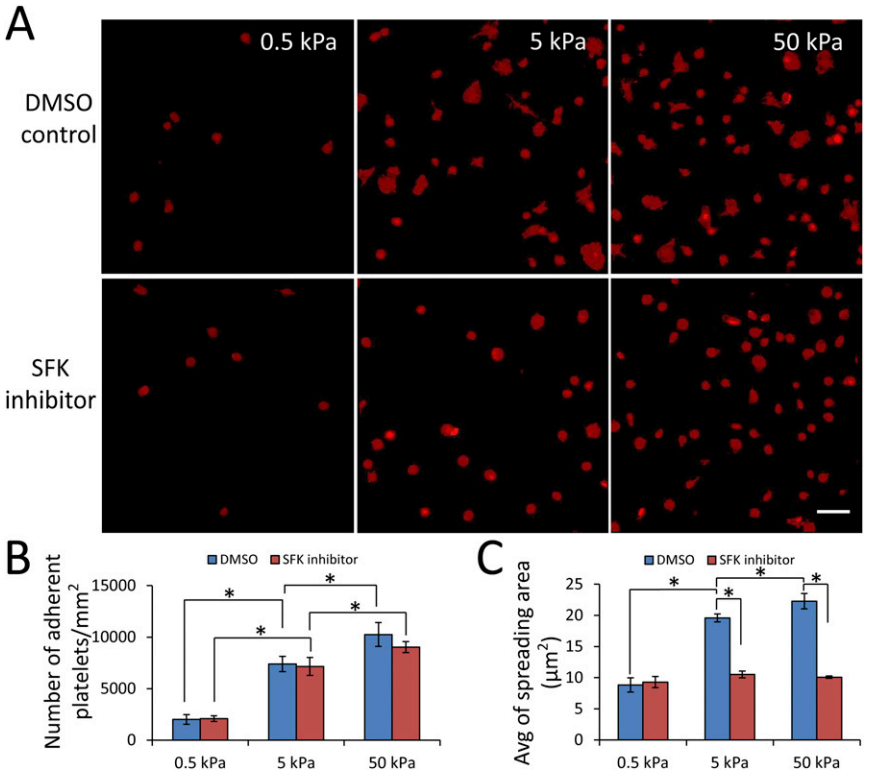

Fig. S7. Platelet mechanosensing during spreading is mediated by Src family kinase (SFK) activity. (*A*) Representative epifluorescence images of platelets treated with DMSO only (control), SFK inhibitor after adhering onto PA gels with stiffnesses of 0.5, 5, and 50 kPa. (*B*) Inhibition of SFK activity did not affect platelet mechanosensing during adhesion but (*C*) blocks platelet mechanosensing during spreading ($P < 0.05$; $n = 3$ experiments; error bars indicate SD). (Scale bar: 10 μm.)

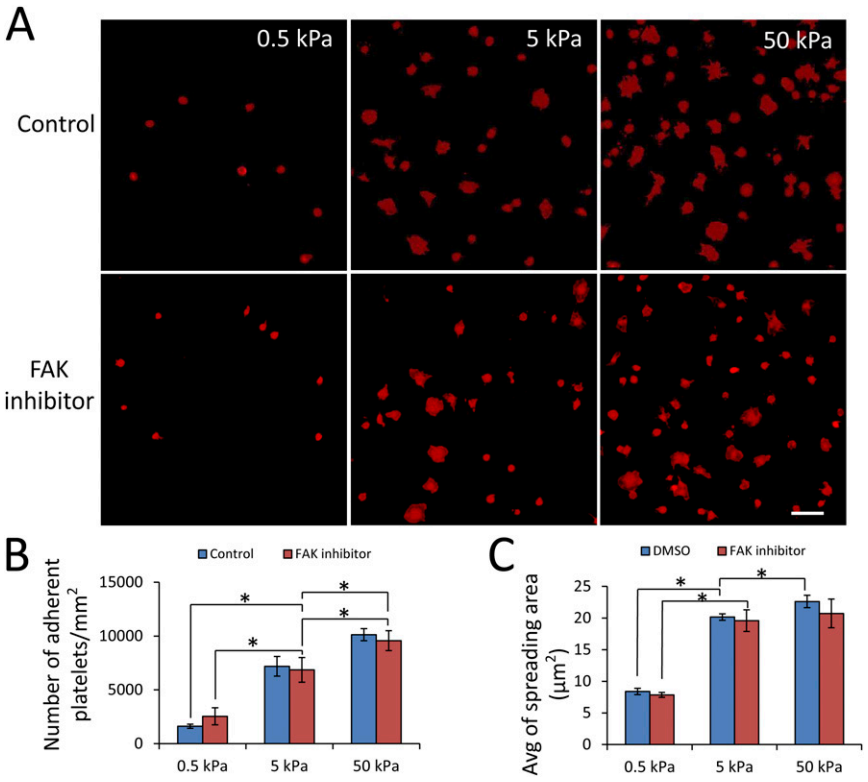

Fig. S8. Platelet mechanosensing is not mediated by FAK activity. (*A*) Representative epifluorescent images of platelets treated with DMSO only (control), FAK inhibitor after adhering onto PA gels with stiffnesses of 0.5, 5, and 50 kPa. (*B*) Inhibition of FAK activity did not affect either platelet mechanosensing during adhesion, or (*C*) platelet mechanosensing during spreading ($P < 0.05$; $n = 3$ experiments; error bars indicate SD). (Scale bar: 10 μm.)

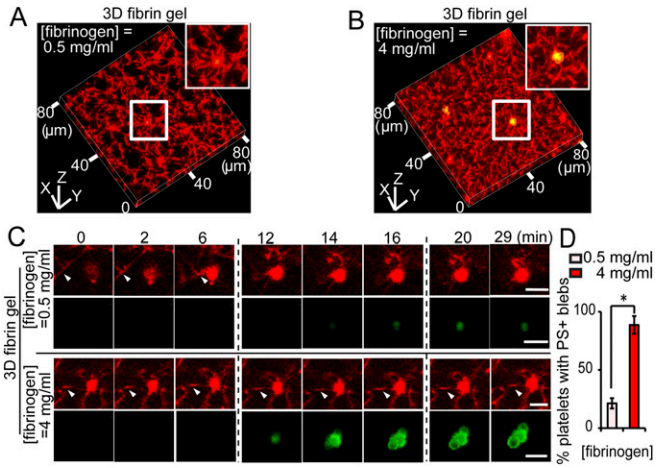

Fig. S9. Thombin-activated platelets in stiffer fibrin gels (4 mg/mL) form more phosphatidylserine (PS)-positive blebs than in softer fibrin gels (0.5 mg/mL). (*A*) A representative 3D rendering of confocal *z*-stack images of platelets in softer fibrin gels (0.5 mg/mL) after 30-min activation by thrombin. (*Inset*) A platelet attached to the fibrin scaffold exhibits weak PS staining via Annexin V conjugated with AF488. (*B*) A representative 3D rendering of confocal *z*-stack images of platelets in stiffer fibrin gels (4 mg/mL) after 30-min activation by thrombin. (*Inset*) A platelet attached to the fibrin scaffold exhibits blebbing with strong PS staining via Annexin V conjugated with AF488. (*C*) Time series of confocal images showing single thrombin-activated platelets interacting with and pulling on individual fibrin strands (arrows indicate the same fiber at different time points) and of PS staining via Annexin V conjugated with AF488 in softer (0.5 mg/mL) and stiffer (4 mg/mL) fibrin gels. (Scale bar: 5 μm.) (*D*) After 30 min, significantly more platelets exhibit PS-positive blebs in stiffer fibrin gels (4 mg/mL) than in softer fibrin gels (0.5 mg/mL). $P < 0.05$.