Human Genome-Wide Association and Mouse Knockout Approaches Identify Platelet Supervillin as an Inhibitor of Thrombus Formation Under Shear Stress

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Background—High shear force critically regulates platelet adhesion and thrombus formation during ischemic vascular events. To identify genetic factors that influence platelet thrombus formation under high shear stress, we performed a genome-wide association study and confirmatory experiments in human and animal platelets.

Methods and Results—Closure times in the shear-dependent platelet function analyzer (PFA–100) were measured on healthy, nondiabetic European Americans (n=125) and blacks (n=116). A genome-wide association (P<5×10−8) was identified with 2 single-nucleotide polymorphisms within the SVIL gene (chromosome 10p11.23) in African Americans but not European Americans. Microarray analyses of human platelet RNA demonstrated the presence of SVIL isoform 1 (supervillin) but not muscle-specific isoforms 2 and 3 (archvillin, SmAV). SVIL mRNA levels were associated with SVIL genotypes (P≤0.02) and were inversely correlated with PFA-100 closure times (P<0.04) and platelet volume (P<0.02). Leukocyte-depleted platelets contained abundant levels of the ~205-kDa supervillin polypeptide. To assess functionality, mice lacking platelet supervillin were generated and back-crossed onto a C57BL/6 background. Compared with controls, murine platelets lacking supervillin were larger by flow cytometry and confocal microscopy and exhibited enhanced platelet thrombus formation under high-shear but not low-shear conditions.

Conclusions—We show for the first time that (1) platelets contain supervillin; (2) platelet thrombus formation in the PFA-100 is associated with human SVIL variants and low SVIL expression; and (3) murine platelets lacking supervillin exhibit enhanced platelet thrombus formation at high shear stress. These data are consistent with an inhibitory role for supervillin in platelet adhesion and arterial thrombosis. (Circulation. 2012;125:2762-2771.)

Key Words: genetics ■ genome-wide association study ■ murine model ■ platelets ■ thrombosis

Atherothrombosis is a major cause of myocardial infarction (MI) and stroke. Most clinical events occur when an atherosclerotic plaque ruptures to expose subendothelial collagen and von Willebrand factor (VWF). These proteins bind to platelets, triggering primary activation and granule secretion.1 Secretion of soluble agonists, including ADP, amplifies platelet reactivity, leading to integrin activation, platelet-platelet aggregation, and occlusive thrombus formation. Platelets play a more prominent role in arterial thrombus formation than in venous thrombosis because the highly specialized initial platelet-VWF interaction is enhanced by shear stress, such as occurs in coronary arteries.2-3

Clinical Perspective on p 2771

Platelet reactivity varies greatly among individuals. This variation exhibits strong heritability in both European Americans (EAs) and African Americans (AAs),4 which could explain some of the known genetic contribution to the risk of acute MI.5 Although genetic epidemiology screens have identified loci associated with MI risk,6 our understanding of...
causative genes is limited. The use of intermediate phenotypes to identify genes involved in the pathophysiology of arterial thrombosis can yield stronger genetic associations.\(^7\) Genome-wide association studies (GWAS) have led to the discovery of key molecules regulating human disease\(^8\) and have identified genetic variants and novel genes associated with platelet number, platelet volume, and in vitro platelet aggregation.\(^9\),\(^10\) However, no GWAS has identified genes associated with shear stress–dependent platelet function. The platelet function analyzer–100 (PFA-100) measures the time to platelet thrombus formation under a defined shear stress of 1500 s\(^{-1}\) in whole blood.\(^11\) The assay requires platelet tethering to VWF, firm adhesion to collagen, platelet activation and secretion, and platelet aggregation mediated by VWF and fibrinogen. Abnormal assay results correlate with platelet hyperfunction and hypofunction associated with acute coronary syndromes\(^12\)–\(^15\) and bleeding disorders, respectively.\(^16\),\(^17\) The aim of this study was to identify genetic factors that influence platelet reactivity and thrombus formation under high shear stress. We performed a genome-wide screen with the PFA-100 to identify novel gene variants in AAs and EAs. We identified a novel platelet gene, SVIL (encoding the cytoskeletal regulatory protein supervillin), whose expression negatively regulates platelet reactivity and thrombus formation in both humans and mice.

**Methods**

**Subjects and Platelet Phenotyping**

The Platelet Genes and Physiology Study was approved by the institutional review boards of Baylor College of Medicine and Thomas Jefferson University, and informed consent was obtained from all volunteers. Healthy donors were recruited between 2000 and 2006 in Houston, TX. Citrated whole blood was used to measure PFA-100 closure times in a collagen- and ADP-impregnated cartridge (hereafter referred to as PFA-100ColA) within 30 minutes of phlebotomy. A platelet aggregation response of <10% was considered as exposure to antiplatelet agents and reason for exclusion.

**Genotyping**

Genomic DNA was extracted from leukocyte buffy coats with the Qiagen DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer\'s instructions. DNA from AAs was genotyped on the Illumina HumM Beadarray. EA DNAs were genotyped on the Illumina Hum50K Beadarray. Because of the lower levels of linkage disequilibrium in African populations, a denser single-nucleotide polymorphism (SNP) microarray was selected to genotype the DNA samples from AA individuals to improve tagging of causal variants.

**Statistical Analysis**

Quality control was performed before statistical analysis. Subjects were excluded for relatedness to other participants and for failing stringent genotyping quality control. Individual genotypes that failed quality control were also removed (see Methods in the online-only Data Supplement for details). PFA-100ColA closure times were natural log transformed and tested for associations with the use of an additive model within a linear regression framework. All analyses of EA and AA samples were performed separately. The potential confounders age, sex, VWF activity, plasma fibrinogen level, and platelet CD41 level were tested for significance with the use of forward selection, and significant covariates were retained within the model (P<0.1). Principal components analysis was also performed on the data with a subset of the SNP markers that are in linkage equilibrium (r\(^2\)<0.3).\(^18\) To evaluate whether there is inflation of the test statistic due to population substructure/admixture, A was estimated both when no principal components analysis components and when 1 through 5 principal components analysis components were included in the linear regression model.

**Platelet Gene Expression Analysis**

RNA from leukocyte-depleted platelets (LDP) was prepared for gene expression profiling in 29 healthy subjects. LDP was prepared with the use of density centrifugation followed by CD45-positive cell depletion of platelet-rich plasma (PRP).\(^19\) As controls, RNA from PRP and from the CD45\(^+\) leukocyte fraction was extracted from 11 and 5 subjects, respectively, with the use of TRizol (Invitrogen, Carlsbad, CA). Gene expression analysis was performed with the Sentrix BeadChip and BeadStation system from Illumina, Inc (San Diego, CA).\(^20\)

**Platelet Supervillin Expression and Correlation With PFA-100ColA**

LDP RNA was used to validate the SVIL microarray data.\(^19\) Total LDP RNA was reverse transcribed and polymerase chain reaction (PCR) amplified with the use of a sense primer in coding exon 1 and an antisense primer in coding exon 5 of SVIL isoform 1. Immunoblotting of 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels was performed with 4 different anti-supervillin antibodies to verify platelet expression. Natural log–transformed mRNA expression data from microarrays were plotted versus PFA-100ColA closure times for the 23 individuals for whom both values were available. Pearson correlation r and P values were calculated with the use of GraphPad Prism software (La Jolla, CA).

**Mouse Platelet Phenotyping**

Mice lacking Svil were generated and back-crossed 10 times onto a C57BL/6 background. Svil\(^−/−\) and control C57BL/6 mice were maintained by homozygous breeding. Platelet thrombus formation under shear stress was measured with the use of microfluidic flow chambers with immobilized collagen.\(^21\) Blood from 3 wild-type and 3 Svil\(^−/−\) mice was studied. Experiments were performed over 4 different days, with 3 to 4 runs per day (a “run” was defined as data acquisition from platelet deposition in a single flow chamber) for a total of 15 runs per wild-type genotype and 15 runs per Svil\(^−/−\) genotype. P values were computed with a 2-way repeated-measures, linear mixed-effects ANOVA model that accounts for the main effect of genotype using GraphPad Prism.

**Flow Cytometry**

Whole blood was diluted into Tyrode’s buffer containing 1 mmol/L CaCl\(_2\) and stained with fluorescein isothiocyanate-α-CD41 (BD Pharmingen) or phycoerythrin-α–glycoprotein (GP)Iba (Emfret Analytics, Würzburg, Germany). After dilution with phosphate-buffered saline, the cells were analyzed on a FACScan.

**Immunofluorescence Confocal Microscopy**

Platelets adhered to glass slides statically or to collagen-coated coverslips statically or under high shear stress were stained with Alexa-568 phalloidin and Alexa-568 phalloidin and Alexa-488 anti-CD61 antibodies and imaged with confocal microscopy. Additional details are available in the online-only Data Supplement.

**Results**

**SNPs Within SVIL Are Associated With Platelet Function Under Shear Stress**

To assess the capacity for platelet thrombus formation under shear stress, PFA-100ColA was used to measure PFA-100 closure times in a cohort of healthy, nondiabetic subjects (Platelet Genes and Physiology Study). For this genetic study, only subjects self-identified as EA or AA were considered. Blood was collected for PFA-100ColA testing on 154 AAs.
and 157 EAs. The PFA-100CoA closure time data were normally distributed in both groups (not shown). The DNA from each subject was genotyped for 620,901 or 1,070,000 tag SNPs for EAs or AAs, respectively, with the use of the Infinium II platform. Exclusion criteria included nonsteroidal anti-inflammatory drug use, subject relatedness, and failing genotyping quality control (described in Methods and in the online-only Data Supplement). Table 1 summarizes the demographics of the 116 AA and 125 EA subjects who were analyzed.

The PFA-100CoA phenotype was tested for association with each genotype under an additive model. With the use of log-transformed values of the PFA-100CoA phenotype, analysis was performed with the use of linear regression, controlling for sex, age, CD41, and VWF activity in AAs and for sex and VWF activity in EAs. For AAs, age (P = 10^{-2}), sex (P = 10^{-2}), CD41 (P = 10^{-2}), and VWF activity (P = 10^{-6}) were significant and were retained in the model. For EAs, only sex (P = 10^{-2}) and VWF activity (P = 10^{-6}) were significant, and therefore only these covariates were included in the analysis. Principal components analysis components were not included in the analysis because λ = 1.0, indicating no inflation in the test statistic due to population substructure/admixture. Figure 1A shows Manhattan plots for genotype associations with PFA-100CoA closure times, with a prominent “peak” observed in chromosome 10 for AAs. Table 2 lists the SNPs with the 10 lowest P values for AAs. Notably, 4 of these SNPs were clustered within ~21.8 kb of 4 exons within the SVIL gene, which encodes differentially spliced isoforms of supervillin and archvillin. The most significant SNP, rs7070678 (P = 3.6 × 10^{-8}), which is a synonymous SNP in exon 14 of the SVIL gene, is in linkage disequilibrium with other SNPs within the SVIL gene but not with SNPs in neighboring genes (Figure 1B). A second SNP, rs10826650, within the SVIL gene (intron 13) also reached GWAS significance (P = 5 × 10^{-8}). Two additional SNPs (rs7910521, rs10826649) within introns 16 and 17 of the SVIL gene had P values in the order of 10^{-6}. There was no evidence for deviation from the additive model (analyses not shown). The closest other well-annotated genes in this region, LYZL1 (lysozyme-like 1) and KIAA1462, were >214 kb from the SVIL SNPs listed in Table 2, and there was no evidence of an association with either of these genes. The clustering of

### Table 1. Demographics of Subjects in Genetic Study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>African Americans</th>
<th>European Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>116</td>
<td>125</td>
</tr>
<tr>
<td>Women, %</td>
<td>69</td>
<td>50</td>
</tr>
<tr>
<td>Age, mean ± SD, y</td>
<td>35.0 ± 9.4</td>
<td>35.0 ± 11.1</td>
</tr>
<tr>
<td>Body mass index, mean ± SD, kg/m²</td>
<td>28.6 ± 5.3</td>
<td>25.5 ± 4.6</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>16.4</td>
<td>32</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>8.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Hematocrit, mean ± SD, %</td>
<td>36.1 ± 5.0</td>
<td>37.4 ± 4.4</td>
</tr>
<tr>
<td>Fibrinogen, mean ± SD, mg/dL</td>
<td>345 ± 99</td>
<td>300 ± 64</td>
</tr>
<tr>
<td>Von Willebrand factor activity, mean ± SD, %</td>
<td>88 ± 38</td>
<td>79.7 ± 35</td>
</tr>
<tr>
<td>Platelet-rich plasma platelet count (per μL), mean ± SD</td>
<td>406,060 ± 93,350</td>
<td>430,550 ± 111,600</td>
</tr>
</tbody>
</table>

**Figure 1.** Genome-wide association studies with platelet function analyzer–100 closure times in a collagen- and ADP-impregnated cartridge (PFA-100CoA). A, Manhattan plots for PFA-100CoA. The Manhattan plot is shown for all autosomes for European and African Americans. The y axis shows the −log 10 of the P values for the chromosomes numbered on the x axis. For the African Americans, the 2 most significant single-nucleotide polymorphisms (SNPs) are rs7070678 and rs10826650 in supervillin (SVIL); both meet genome-wide significance with P values of 3.6 × 10^{-8} and 4.4 × 10^{-8}, respectively. None of the SNPs within SVIL meet genome-wide significance for European Americans. B, SVIL region association plot on chromosome 10 for African Americans. The most significant SNP rs7070678 is displayed, and the amount of linkage disequilibrium between this SNP and nearby SNPs is shown as a heat plot with SNPs in strongest linkage disequilibrium with rs7070678 in darker colors. The significance level is displayed on the y axis as −log 10 of the P values. The rate of recombination within the region is shown as the light blue tracing. The regions containing the SVIL and LYZL1 genes are indicated by green arrows.
SNPs with $P$ values of $\approx 10^{-8}$ suggested that genetic variants in $SVIL$ (and not another gene) are associated with PFA-100ColA closure times in AAs. No associations with GWAS significance were detected with SNP marker loci within the $SVIL$ gene within EAs, although 3 additional $SVIL$ SNPs had weak associations in EAs ($P<10^{-2}$).

**Supervillin mRNA and Protein Are Present in Human and Mouse Platelets**

Figure 2 illustrates the $SVIL$ exon structure, the 2 major known mRNAs, and the location of rs7070678, the SNP with the strongest association with PFA-100ColA closure times. $SVIL$ encodes supervillin, which has a broad cell distribution, and 2 archvillin isoforms, which are enriched in muscle.22–24 Supervillin forms a high-affinity link between the actin cytoskeleton and the plasma membrane22 but has not been described in platelets. Because integrin function and rearrangements of the actin cytoskeleton are a crucial aspect of the platelet adhesive process,25 we therefore sought to characterize supervillin in platelets.

Platelet RNA expression analysis was performed with LDP RNA samples from 29 healthy subjects. Probes from the 2’-most and 3’-most exons (Figure 2A) were readily detectable. However, an RNA signal was not detected for probe ILMN_1659306, which is specific for archvillin and SmAV. Platelet $SVIL$ transcripts are expressed at moderately high levels and are higher than platelet/endothelial cell adhesion molecule-1 but lower than GPIbα mRNAs (Figure I in the online-only Data Supplement). Supervillin transcripts were easily detected in LDP RNA by reverse transcription PCR (Figure 2B), validating the microarray data. Figure 2C shows $SVIL$ mRNA levels for LDP, PRP, and CD45+ leukocytes (white blood cells) as box plots. Western immunoblotting identified supervillin at $\approx 205$ kDa in PRP (Figure 3A). This polypeptide migrated slightly higher than GPIbα.

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**Table 2. Most Significant $P$ Values Detected in Genotype Data From African Americans**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Chromosome:Position</th>
<th>Genes</th>
<th>$\beta$</th>
<th>SE</th>
<th>MAF</th>
<th>Type</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7070678</td>
<td>10:29786283–29963907</td>
<td>$SVIL$</td>
<td>-0.006807</td>
<td>0.001235</td>
<td>0.327</td>
<td>Exon 14 synonymous</td>
<td>$3.581 \times 10^{-8}$</td>
</tr>
<tr>
<td>rs10826650</td>
<td>10:29812602</td>
<td>$SVIL$</td>
<td>-0.006856</td>
<td>0.001253</td>
<td>0.340</td>
<td>Intron 13</td>
<td>$4.426 \times 10^{-8}$</td>
</tr>
<tr>
<td>rs1158159</td>
<td>15:24824188</td>
<td>PWRN1</td>
<td>0.005737</td>
<td>0.001169</td>
<td>0.366</td>
<td>Intron 7</td>
<td>$9.187 \times 10^{-7}$</td>
</tr>
<tr>
<td>rs3901472</td>
<td>15:24814582</td>
<td>PWRN1</td>
<td>0.005773</td>
<td>0.001182</td>
<td>0.379</td>
<td>Intron 3</td>
<td>$1.034 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs7656730</td>
<td>4:40159617</td>
<td>NABP2</td>
<td>0.025637</td>
<td>0.005250</td>
<td>0.675</td>
<td>3’ UTR</td>
<td>$1.044 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs7910521</td>
<td>10:29803661</td>
<td>$SVIL$</td>
<td>-0.006104</td>
<td>0.001279</td>
<td>0.326</td>
<td>Intron 16</td>
<td>$1.830 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs10826649</td>
<td>10:29792539</td>
<td>$SVIL$</td>
<td>-0.006142</td>
<td>0.001301</td>
<td>0.297</td>
<td>Intron 17</td>
<td>$2.382 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs17783459</td>
<td>18:42329076</td>
<td>$SETBP1$</td>
<td>0.032067</td>
<td>0.006898</td>
<td>0.042</td>
<td>Intron 3</td>
<td>$3.343 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs9890514</td>
<td>17:46738883</td>
<td>None</td>
<td>0.012391</td>
<td>0.002675</td>
<td>0.196</td>
<td>...</td>
<td>$3.604 \times 10^{-6}$</td>
</tr>
<tr>
<td>rs1507740</td>
<td>1:163075021</td>
<td>None</td>
<td>0.013215</td>
<td>0.002885</td>
<td>0.157</td>
<td>...</td>
<td>$4.640 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

SNP indicates single-nucleotide polymorphism; ID, identification; and MAF, minor allele frequency.
more quickly than that seen in control A549 cells, and therefore additional antisera were used to confirm platelet supervillin molecular weight and immunoreactivity. The locations of the epitopes recognized by these antibodies are shown in Figure 3B. In addition, because supervillin is present in leukocytes, we also analyzed LDP and leukocyte-enriched buffy coat. With the use of 2 other antibodies, 205-kDa supervillin was again detected in plate-
lets, as well as in the megakaryocyte cell line Meg-01, but was not detected in CD45-enriched leukocytes or K562 erythroleukemia cells (Figure 3C and 3D). Finally, supervillin was present in mouse platelets (Figure 3E). Sequencing of platelet SVIL mRNA reverse transcription PCR products revealed the presence of SVIL isoform 1, as in HeLa cells (not shown).

Supervillin mRNA Levels Correlate With Platelet Function Under Shear Stress, Platelet Size, and the rs7070678 Genotype

Because platelet protein was available from only a few of the 29 subjects used in the platelet RNA expression analysis, we used SVIL mRNA levels to test for a correlation with platelet function. Figure 4A shows that SVIL mRNA levels correlate with PFA-100ColA closure times (P=0.038). Because longer closure times indicate reduced platelet function, these data suggest that SVIL expression may inhibit human platelet thrombus formation under shear stress. A trend was observed for a similar relationship between SVIL mRNA expression and collagen-induced platelet aggregation (P=0.07) but not with ADP-induced platelet aggregation (P=0.23; data not shown). SVIL mRNA levels also correlated negatively with human mean platelet volume (P=0.016) (Figure 4B). Because both SVIL SNPs and SVIL transcripts were associated with PFA-100ColA closure times (Figures 1 and 4), we tested whether SVIL SNPs were associated with SVIL mRNA levels using a recessive model analysis. Figure 4C shows that SVIL expression differed significantly by rs7070678 genotype.

Platelets From Svil-Deficient Mice Form Thrombi Faster Under Shear Stress in Flow Chamber Studies

To further address the role of supervillin in platelet function, we generated mice from BayGenomics ES cells bearing an insertion in the supervillin (Svil) gene (Figure 5). In this mouse, a β-galactosidase/neomycin phosphotransferase (β-gal/neo) gene trap inserted into the large intron downstream of the 13th of the 34 coding exons (Figure 5A) disrupts expression of all characterized SVIL splice forms (not shown).23-24 The location of the insertion was verified by Southern blotting, which showed that a BglII restriction site from the pGT0lxf vector reduced the 11.5-kb BglII fragment including coding exon 13 to 7.2 kb (not shown), and by PCR (Figure 5B) with primers specific for either the wild-type or mutated locus (arrows). This insertion destabilizes the message or protein because supervillin is effectively absent from murine Svil−/− platelets (Figure 5C) and leukocytes (not shown).

To assess the role of supervillin in platelet thrombus formation under shear stress, we analyzed wild-type and Svil−/− platelets in microfluidic flow chamber assays on immobilized collagen.21 No difference was observed under the “venous” flow rate of 400 s⁻¹ (Figure 6A and 6B), but Svil−/− platelets showed greater deposition (P<0.05) under arterial flow rates of 1200 s⁻¹ (Figure 6C and 6E). Both percent coverage of the collagen-coated area, indicating platelet adhesion to collagen, and sum intensity, indicating thrombus formation, were affected.

Although there was no difference between wild-type and Svil−/− mice in platelet numbers, Svil−/− platelets appeared to be larger as measured by both forward scatter in flow cytometry and confocal microscopy (Table 3), consistent with the relationship in humans (Figure 4B). However, these larger platelets did not express correspondingly greater levels of major surface adhesion receptors integrin αIIb or GPIbα (Table 3).

Supervillin directly binds F-actin, myosin II heavy chain, filamin, and many other cytoskeletal proteins.28-31 To better elucidate the cytoskeletal and structural differences between wild-type and Svil−/− platelets, we analyzed F-actin and myosin IIA organization using immunofluorescence confocal microscopy. Although these platelet cytoskeletons are similar in appearance when statically adhered to glass (Figure 7A and 7B versus 7C and 7D), Svil−/− platelets are more highly spread after activation under high shear flow across collagen (Figure 7E and 7F versus 7G and 7H). The intensity of F-actin staining increases after activation of both types of platelets, as expected given the associated polymerization of actin.32,33

Discussion

Interindividual variation in platelet reactivity contributes to common arterial thrombotic disorders in humans, but there is only a limited understanding of the responsible molecular
mechanisms. We screened a cohort of healthy human subjects by genome-wide genotyping for associations with platelet function assessed under shear stress and identified a candidate gene in AAs that was validated using gene expression and platelet physiology approaches. Our major findings were that (1) genetic variants in \(\text{SVIL}\) were associated with closure times in the PFA-100CoA; (2) platelets contain supervillin and little or no archvillin or SmAV; and (3) low or absent platelet supervillin is associated with enhanced thrombus formation under high shear stress and increased platelet size in both humans and mice.

Growing evidence supports a role for supervillin as a regulator of cytoskeletal-membrane interactions. Among other functions, supervillin increases myosin II contractility, reduces integrin-mediated cell adhesion, and promotes rapid integrin recycling.\(^{34–36}\) Our data support an inhibitory role for supervillin in regulating the rapid activation and spreading of platelets during thrombus formation on collagen under shear stress, an important determinant of platelet responsiveness in arterial thrombosis. These results are consistent with supervillin inhibition of spreading and integrin function in other cell types.\(^{37,38}\)

**Table 3.** Platelet Parameters in Wild-Type and \(\text{SVIL}^{\text{WT}}\) Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type</th>
<th>(\text{SVIL}^{\text{WT}})</th>
<th>(P^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet No./μL(^\dagger)</td>
<td>1 083 000</td>
<td>969 000</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean forward scatter(^\ddagger)</td>
<td>19.4</td>
<td>23.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Surface area, (\mu\text{m}^2)(^\S)</td>
<td>4.7±0.1 (n=159)</td>
<td>5.3±0.1 (n=241)</td>
<td>0.0045</td>
</tr>
<tr>
<td>Integrin αIIb, MFI fold change(^\S)</td>
<td>1.02</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Glycoprotein Ibα, MFI fold change(^\S)</td>
<td>1.30</td>
<td>0.40</td>
<td></td>
</tr>
</tbody>
</table>

\(\S\)MFI indicates mean fluorescence intensity ratio of mutant/wild-type platelets.

\(\dagger\)By Hemavet 850FS (Drew Scientific).

\(\ddagger\)By FACScan (Becton Dickinson).

\(\S\)Surface area covered by platelets at indicated time points presented as percentage of coated collagen ±SEM measured at the indicated time points (n=15 for each genotype).

\(\S\)Sum of fluorescence intensity ±SEM by confocal microscopy of AlexaFluor568-phalloidin stained unactivated.

\(\S\)Flow cytometric comparisons between mice are shown as fold changes (WT/SVIL) to normalize for day-to-day variation in platelets, antibody fluorescence, binding, and acquisition.

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**Figure 6.** Enhanced adhesion and thrombus formation in platelets lacking supervillin (\(\text{SVIL}\)). Whole blood from wild-type (WT; black line) or \(\text{SVIL}^{\text{WT}}\) (\(\text{SVIL}\); red line) mice was perfused over immobilized collagen at venous (400 s\(^{-1}\)) or arterial (1200 s\(^{-1}\)) shear rates. Platelets in whole blood were labeled with AlexaFluor488-labeled antibodies to platelet GPIb before perfusion. A and C, Surface area covered by platelets at indicated time points presented as percentage of coated collagen ±SEM (n=15 for each genotype). B and D, Sum of fluorescence intensity ±SEM measured at the indicated time points (n=15 for each genotype). Experiments shown in A to D were conducted on 4 different days for a total of 15 runs for each genotype. E, Representative images were taken at the designated times during perfusion at arterial (1200 s\(^{-1}\)) shear rates. Bars shown in 1-minute images apply to all time points.

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**Genome-Wide Association Study**

The PFA-100 is dependent on shear stress and is relatively “high throughput.” We appreciate that some\(^{12–15}\) but not all\(^{39,40}\) clinical cardiovascular outcome studies have observed associations with PFA-100 results. The lack of an association between PFA-100 results and clinical outcomes may be due to some nonphysiological conditions, such as anticoagulated blood or an absence of vessel wall. It would be ideal to replicate our findings with other assays in humans; however, neither light transmission aggregometry nor impedance aggregometry is performed under conditions considered to apply shear stress to platelets. Our strategy was to use the GWAS as a screen for identifying novel genes associated with shear-dependent platelet reactivity. However, our study differs from prior platelet genomics studies in that our validation approach employed mRNA expression and physiology experiments. Our statistical analysis was strengthened by the ability to adjust for confounders known to affect platelet adhesion (VWF activity) and aggregation (CD41 levels), which were also shown to be significant in the analysis. Two SNPs in \(\text{SVIL}\) met the threshold for genome-wide significance in AAs, and 2 additional \(\text{SVIL}\) SNPs were associated with PFA-100CoA closure times, with \(P\) values of \(\sim 10^{-6}\).
The reason for our inability to detect a similarly strong association in EAs is unclear but probably relates to the limited power of a small sample size by typical GWAS standards and the fact that minor allele frequencies were ≈20% lower in EAs. Thus, much larger sample sizes might be necessary to detect an association in EAs. Indeed, supplemental data in the genomic analysis by Johnson et al\(^8\) show modest associations (≈10\(^{-5}\)) between SNPs in SVIL and in vitro platelet aggregation in both AAs and EAs.

None of the 5 SVIL SNPs associated with PFA-100ColA closure times were predicted to affect the protein coding sequence, and therefore we had no reason to suspect that any of these SNPs directly altered supervillin function. This is not unusual because GWAS uses indirect association mapping with tag SNPs that are in linkage disequilibrium with causal variants.\(^8\) However, despite a small sample size composed of both EAs and AAs in our RNA expression study, expression quantitative trait loci analysis supported an association between these SNPs and SVIL mRNA levels (Figure 4C). Thus, at least 1 genetic mechanism by which SVIL variants may regulate platelet reactivity is by altering SVIL expression, perhaps via effects on transcription or mRNA stability.

**SVIL Expression in Platelets**

SVIL mRNA was detected in platelets with the use of a 3’UTR probe that recognizes all SVIL transcripts and a 5’UTR probe specific for nonmuscle supervillin (Figure 2). Archvillin mRNAs were not detected in our microarray expression study, consistent with prior data indicating that archvillin and SmAV are mainly expressed in muscle.\(^9\) In addition to the RNA data, immunoblotting with multiple antibodies demonstrated the presence of abundant supervillin protein in platelets. Using microarray analysis, Watkins et al\(^7\) reported that SVIL mRNA is expressed in multiple hematopoietic cell types including CD4\(^+\) and CD8\(^+\) T cells, CD14\(^+\) monocytes, CD19\(^+\) B cells, CD56\(^+\) natural killer cells, and CD66b\(^+\) granulocytes. We also observed SVIL mRNA in CD45\(^+\) cells (Figure 2C); found supervillin protein in murine thymocytes, splenocytes, macrophages, and neutrophils by immunoblotting; and recovered Coomassie blue–staining amounts of supervillin from bovine neutrophils.\(^26\) Although high levels of proteases may have caused degradation in human leukocyte lysates (Figure 3), supervillin is clearly expressed at moderately high levels in platelets (Figures 2C and 3 and Figure I in the online-only Data Supplement). In addition, because PRP has some contamination with white blood cells, our gene expression data suggest that SVIL mRNA is expressed at higher levels in platelets than in white blood cells (Figure 2C). The basis for the reproducibly faster migration of platelet supervillin, compared with HeLa and A549 cell supervillin (Figure 3), is unclear because the predicted mRNAs are identical. A likely explanation is a cell type–specific difference in posttranslational modifications.

**Supervillin and Platelet Size**

Supervillin expression levels are inversely correlated with platelet size in both humans and mice. Although Svil\(^−/−\) platelets are larger, they do not have greater surface expression of several critical adhesive glycoproteins (GPIb\(α\) or integrin α\(IIb\)), suggesting a possible membrane cytoskeletal defect rather than premature release of larger platelets from megakaryocytes. There are several possible mechanisms by which supervillin might regulate platelet size. Filamin anchors the GPIb-IX-V complex to the platelet cytoskeleton and binds to supervillin\(^31,41\); an altered filamin-GPIb interaction could produce larger platelets, such as those characteristic of inherited mutations in the gene encoding GPIb\(α\).\(^42,43\) Other possible mechanisms include altered myosin II function similar to the MYH9-associated macrothrombocytopenias\(^44\) or a simple disruption of the actin cytoskeleton that maintains normal platelet size, as in the Wiskott-Aldrich syndrome.\(^45\) The increased platelet size may enhance thrombus formation because high platelet volumes have been associated with MI.\(^16\)

**Supervillin and Platelet Function**

Supervillin effects on platelet function are most prominent under shear stress. Healthy human subjects expressing higher levels of SVIL mRNA exhibit slower platelet thrombus formation in the PFA-100ColA. Platelets in blood from supervillin-deficient mice form thrombi faster under high shear rates in flow chamber studies than is observed for platelets with wild-type Svil (Figure 6). Although the effect of supervillin on platelet adhesion is not dramatic, it is difficult to demonstrate gain-of-function effects, and the increased adhesion observed in Svil-null platelets is well in agreement with other mouse mutants of signaling molecules that limit...
platelet activation.37,38 The mice used in these studies have Svil defects in all tissues, and we cannot exclude an indirect effect on platelets. However, the SVIL association studies used here and by Johnson et al9 utilized an in vitro platelet assay consisting only of platelets and plasma (ie, aggregation). Taken together, our results strongly suggest an inhibitory role for supervillin in platelet function.

An inhibitory effect during spreading is consistent with the larger surface profiles observed for supervillin-deficient platelets on collagen after 2 minutes of flow under high shear stress (Figure 7). The direction of this effect is consistent with a number of mechanisms. First, the larger initial volumes of unactivated Svil−/− platelets (Table 3) may contribute to their larger surface areas after activation. Second, the loss of supervillin could increase the rate of cell spreading by increasing integrin adhesion to the substrate or by decreasing myosin II–mediated slowing of the cell spreading rate, as observed in other cell types.34,35 Finally, decreased integrity of the membrane-cytoskeleton connection could increase the apparent cross-sectional surface area if Svil−/− platelets are more sensitive to cortical disruption by high shear forces. Further studies are needed to determine which supervillin interactions regulate the early phases of platelet activation and adhesion under high-shear forces.

There are potential clinical implications to our findings. If confirmed in additional studies, these data suggest that SVIL variants may contribute to predisposition to cardiovascular disease in AAs. Targeting supervillin in a manner that would enhance its activity might have antithrombotic benefit for arterial vascular diseases such as MI and stroke, a benefit that may be more pronounced in AAs. Conversely, drugs that interrupt supervillin function in platelets could have untoward effects of promoting thrombosis. Finally, SNPs in strong linkage disequilibrium with the causative SVIL variant may be useful as a biomarker for risk of thrombosis or hemorrhage.

Summary
Combined genome-wide technologies in a cohort with well-characterized platelet function have led to the identification of a novel protein in platelet biology. We have identified a candidate gene meeting GWAS significance, and both human and mouse studies support an inhibitory effect of supervillin in platelet thrombus formation under shear stress. Although the causative variant in SVIL has not been identified, these data indicate that genetic variations in SVIL expression contribute to variations in human platelet reactivity and support a role for supervillin in arterial thrombosis. Further analysis of SVIL variants may lead to a better understanding of the genetic basis for susceptibility to arterial thrombosis.

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Disclosures
None.

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CLINICAL PERSPECTIVE

Platelets play a central role in ischemic arterial vascular disease, and antiplatelet therapies are mainstays of treatment. The findings in this study identify a novel platelet protein, supervillin, which functions to dampen the early formation of platelet thrombi under high shear stress. Although these results will not alter current management of vascular disease, there are potential clinical implications. Supervillin is an interaction hub for many proteins that regulate cell adhesion and contractility. Drug targeting of supervillin or one of its binding partners in a manner that would decrease platelet adhesion under high shear forces may have antithrombotic benefit for arterial vascular diseases such as myocardial infarction and stroke. This benefit could be especially pronounced in African Americans, who suffer disproportionately from cardiovascular disease. Conversely, drugs that knowingly or unknowingly block supervillin function in platelets could have untoward effects of promoting thrombosis. The shear dependence of the supervillin effect presents an opportunity to develop therapies that differentially affect arterial and venous thrombosis by inhibiting platelet thrombus formation under high shear settings (eg, acute coronary syndromes or percutaneous coronary intervention) without altering the normal hemostatic function of platelets in low-shear veins or microcirculation. Finally, single-nucleotide polymorphisms strongly linked to the causative supervillin variant may be useful as a biomarker for risk of thrombosis or hemorrhage.
Human Genome-Wide Association and Mouse Knockout Approaches Identify Platelet Supervillin as an Inhibitor of Thrombus Formation Under Shear Stress
Leonard C. Edelstein, Elizabeth J. Luna, Ian B. Gibson, Molly Bray, Ying Jin, Altaf Kondkar, Srikanth Nagalla, Nacima Hadjout-Rabi, Tara C. Smith, Daniel Covarrubias, Stephen N. Jones, Firdos Ahmad, Moritz Stolla, Xianguo Kong, Zhiyou Fang, Wolfgang Bergmeier, Chad Shaw, Suzanne M. Leal and Paul F. Bray

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Platelet preparation and phenotyping. The details of subject recruitment, blood processing and platelet phenotyping have been previously reported.\textsuperscript{1,2} Citrated platelet-rich plasma (PRP) was used in light transmission platelet aggregometry (LTA) within 2 hours of phlebotomy. Unstimulated platelet CD41 (platelet fibrinogen receptor) levels were ascertained by flow cytometry, and platelet poor plasma was prepared and stored at \(-80°C\) for “batch” measurements of VWF activity and fibrinogen levels.\textsuperscript{2}

Data analysis. Statistical analysis was performed using the R libraries GenABEL,\textsuperscript{3} EIGENSTRAT\textsuperscript{4} and PLINK.\textsuperscript{5} Non-normalized BeadStudio gene expression summary data were processed using the variance stabilizing transformation (VST) method as implemented in the lumi R package.\textsuperscript{6}

Genotyping. An initial amplification of genomic DNA, followed by fragmentation and precipitation of the amplified DNA was performed. The fragmented, amplified genomic DNA was then hybridized to the BeadChip overnight. A single base pair extension reaction occurred directly on the BeadChip, in which dye-labeled nucleotides were incorporated onto the end of the oligonucleotide probes attached to each bead. The resulting products were imaged with the BeadArray Reader (Illumina, Inc., San Diego, CA, USA). Allele detection and genotype calling were performed by using the BeadStudio software (Illumina, Inc.). Quality standards for staining, single-base extension, hybridization, stringency, and nonspecific binding were verified.

Genotyping Quality control (QC). QC included removing 1) individuals who were outliers (>2 standard deviations [SD]) in the principal components analysis (PCA) when component 1 and 2 were plotted,\textsuperscript{4} 2) individuals missing >3% of their genotypes, and 3) samples with average
heterozygosity that differed by more than two SD from the mean. Additionally, any SNP locus that was missing > 5% of its genotypes was removed unless the minor allele frequency for the SNP was <5%, in which case a more stringent criterion was used in which SNPs were removed that were missing >1% of their genotype data. Identity by descent was examined to identify cryptic duplicates and related individuals. Those markers for which the test of Hardy Weinberg Equilibrium was highly significant (p-value<1 x 10^-8) were removed from the dataset. Related individuals were removed so that only a single family member remained in the sample.

*Statistical Analysis of human SVIL expression.* SVIL mRNA expression levels were determined by hybridization to the Illumina human Ref-8 BeadChips. Non-normalized BeadStudio summaries were processed using the variance stabilizing transformation (VST) method, as implemented in the lumi R package. Pearson correlation coefficients and p-values were calculated for the relationship between normalized SVIL mRNA level with PFA-100ColA closure time and mean platelet volume (MPV). The expression level, PFA-100ColA and MPV data all passed the Kolmogorov–Smirnov test for normal distribution. Differences between SVIL expression levels based on rs7070678 genotype were evaluated using a Student’s T-test. Both tests were performed using GraphPad Prism.

*Supervillin antibodies.* H340 (polyclonal, affinity purified IgG against supervillin residues 1-340), ab50856 (Abcam polyclonal against supervillin residues 474-492), HPA02013 (Atlas Antibodies, Stockholm, Sweden, polyclonal against residues 421-558) and S8695 (Sigma Aldrich, affinity purified polyclonal against supervillin residues 900-918).

*Tyrodes Buffer* (140 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.46 mM NaH₂PO₄, 5.5 mM Glucose, 10 mM HEPES, pH 7.4)
Platelet cDNA cloning. Human platelet SVIL cDNAs were reverse transcribed from LDP RNA using SuperScript II (Invitrogen). Overlapping N-terminal and C-terminal cDNA fragments were amplified using Platinum Taq DNA polymerase (Invitrogen) and SVIL-specific 5’-UTR and 3’-UTR primers, paired with gene-specific primers (GSP) in the coding region. Thirty cycles of amplification were carried out: 30 s at 98°C, 30 sec at 60°C, and 5 min at 68°C. Three clones of each product were sequenced, and the consensus was compared with reference SVIL isoforms.

Supervillin Gene Targeting. Mice bearing a homozygous insertion in the Svil gene were generated from the 129P2/OlaHsd embryonic stem cell clone RRF043 (UniTrapt UNI8247). This cell line was created by the NHBLI-funded consortium, BayGenomics (http://baygenomics.ucsf.edu/) in partnership with the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis. In this non-conditional allele, the trapping construct pGT0lxf was inserted downstream of the 13th of the 34 Svil coding exons. This construct contains a strong splice acceptor followed by the gene encoding the β-galactosidase/neomycin-resistance fusion protein and a strong SV40 polyadenylation signal. The location of the inserted gene trap sequence was confirmed by PCR and Southern blot analysis, and mice were generated by blastocyst injection. Genotyping was performed using touch-down PCR from genomic DNA recovered from mouse tail tips. Southern blots of genomic DNA digested with Bgl II were probed with an 806-bp PCR product corresponding to exon 13 (ENSMUSE00000390129) and surrounding sequence.

Mice were backcrossed onto the C57BL/6 background (Charles River Laboratories, Wilmington, MA) for ten generations. This allele is designated Svil<Gt(RRF043)Byg> (MGI:3513192). All studies were performed with the approval of Institutional Animal Care and Use Committees at the Thomas Jefferson University and the University of Massachusetts.
Medical School. The animals received food and water ad libitum and were subjected to a 12-hour light-dark cycle.

**Analysis of SVIL deficient mouse platelet parameters.** Platelet count was determined by diluting 50 μl of whole blood into 100 μl heparinized PBS and analyzed using the Drew Scientific Hemavet 850FS animal blood analyzer (Drew Scientific, Waterbury, CT). Forward scatter, Integrin αIIb, and GPIbα were determined using a FACScan cytometer (Becton Dickinson). Surface area was analyzed by confocal microscopy, described below. Normal distribution of data was tested with a Kolmogorov–Smirnov test. Statistical significance between the parameters of the different genotypes was determined by Student’s T-Test.

**In vitro flow chamber studies.** A microfluidic device fabricated in poly dimethylsiloxane (PDMS). Fabrication of microfluidic devices and microfluidic collagen patterning were performed as previously described. Briefly, a 100 μm-strip of fibrillar collagen type I (200 μg/ml) was deposited and immobilized by microfluidic patterning along the length of a glass slide. A PDMS device with 10 flow channels (width:250 μm, height: 60 μm, length: 6 mm) was oriented perpendicular to the patterned collagen. Blood was drawn from the retro-orbital plexus from mice anesthetized with isofluorane into heparinized tubes (30 U/ml Lovenox). Murine whole blood was incubated with 2.5 μg/ml of anti-GPIX-Alexa488 and infused at arterial (1200 S) or venous (400 S) wall shear rates for 5 minutes. Adhesion of platelets was monitored continuously with a Nikon Ti-U inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a Retiga EXL monochrome camera (QImaging, Surrey, Canada). Images were analyzed using Nikon NIS Elements software (NIS-Elements Advanced Research; Melville, NY, USA). Normality of the data was examined using the Shapiro-Wilk and Kolmogorov–Smirnov of the residuals, and we did not find evidence for departure from normality.
**Immunofluorescence confocal microscopy.** For statically adhered platelets, platelet rich plasma was deposited on either Superfrost Plus charged slides (Fisher Scientific, Pittsburgh, PA) or onto collagen coated coverslips for one hour at 37°C. For platelets adhered under flow, whole blood was allowed to flow across collagen-coated coverslips for two minutes in the microfluidic device, as described above. All preparations were quickly rinsed in PHEM buffer (100 mM PIPES, 5.25 mM HEPES pH 7.0, 10 mM EGTA, 20 mM MgCl₂) and then fixed and permeabilized with 2% paraformaldehyde and 0.04% IGEPAL in PHEM buffer for 10 minutes. The slides were stained using rabbit α-myosin IIA (Covance, Princeton, NJ), goat α-rabbit AlexaFluor488 F(ab')2, and AlexaFluor568 phalloidin (Life Technologies, Carlsbad, CA). Images were obtained with a 100 Plan Apo objective lens (NA 1.4) on a Nikon Eclipse TE-2000 microscope (Nikon Instruments, Melville, NY), using a Solamere CSU10 spinning disk confocal microscope (Solamere Technical Group, Salt Lake City, UT) and a Roper Scientific CoolSNAP HQ2 camera (Photometrics, Tucson, AZ).

Platelet surface areas were determined from the AlexaFluor568 phalloidin channel in basal sections of fixed unactivated platelets. Signals were inverted, converted to objects using the Threshold plug-in of ImageJ, and quantified with the Analyze Particles plug-in (rsbweb.nih.gov/ij/).
**Supplemental Figure 1.** Comparisons of SVIL mRNA levels with other well-known platelet-expressed genes
Supplemental References

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