Genetic Diminution of Circulating Prothrombin Ameliorates Multi-Organ Pathologies in Sickle Cell Disease Mice

Paritha I. Arumugam,1^ Eric S. Mullins,2^ Shiva Kumar Shanmukhappa,3 Brett P. Monia,4 Anastacia Loberg,1 Maureen A. Shaw,2 Tilat Rizvi,1 Janaka Wansapura,5 Jay L. Degen1* and Punam Malik1,2*

1Divisions of Experimental Hematology and Cancer Biology; 2Hematology, Cancer and Blood Diseases Institute; 3Department of Pathology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio; 4Isis Pharmaceuticals, Carlsbad, CA; and 5Imaging Research Center, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio, USA

^PIA and ESM Contributed Equally

*Co-corresponding authors
* Punam Malik, MD
Professor of Pediatrics
Cincinnati Children’s Hospital Medical Center (CCHMC)
Mail Location 7013
3333 Burnet Avenue
Cincinnati, OH 45229
Phone: 513-636-8588
Email: Punam.Malik@cchmc.org

*Jay Degen, PhD
Professor of Pediatrics
CCHMC
Mail Location 7013
3333 Burnet Avenue
Cincinnati, OH 45229
Phone: 513-636-4679
Email: Jay.Degen@cchmc.org

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Reduced prothrombin improves survival, ameliorates inflammation and end-organ damage without spontaneous bleeding in sickle cell mice.

An individual procoagulant, prothrombin, represents a novel therapeutic target that can improve sickle cell disease outcome.
Abstract
Sickle cell disease (SCD) results in vascular occlusions, chronic hemolytic anemia, and cumulative organ damage. A conspicuous feature of SCD is chronic inflammation and coagulation system activation. Thrombin (FIIa) is both a central protease in hemostasis and a key modifier of inflammatory processes. To explore the hypothesis that reduced prothrombin (FII) levels in SCD will limit vaso-occlusion, vasculopathy, and inflammation, we employed two strategies to suppress FII in mice with SCD. Weekly administration of a FII antisense oligonucleotide “gapmer” to Berkeley SCD mice to selectively reduce circulating FII levels to ~10% of normal for 15 weeks significantly diminished early mortality. More comprehensive, long-term comparative studies were done employing mice with genetic diminution of circulating FII. Here, cohorts of FIIlox-/- mice (constitutively carrying ~10% normal FII) and FIIWT mice were tracked in parallel for a year following the imposition of SCD via hematopoietic stem cell transplantation from Berkeley SCD donors. This genetically-imposed chronic suppression of FII levels resulted in an impressive reduction in inflammation (reduction in leukocytosis, thrombocytosis and circulating IL-6 levels), reduced endothelial cell dysfunction (reduced endothelial activation and circulating sVCAM), and a significant improvement in SCD-associated end-organ damage (nephropathy, pulmonary hypertension, pulmonary inflammation, liver function, inflammatory infiltration and micro-infarctions). Notably, all of these benefits were achieved with relatively modest 1.25-fold increase in prothrombin times, and in the absence of hemorrhagic complications. Taken together, these data establish that prothrombin is a powerful modifier of SCD-induced end-organ damage, and presents a novel therapeutic target to ameliorate SCD pathologies.

(Abstract word count: 247)
Introduction

Sickle cell disease (SCD) is a common monogenic disorder that affects millions worldwide and is caused by a mutant β-globin gene. It is characterized by erythrocyte sickling, chronic hemolytic anemia, episodic acute vaso-occlusions, chronic systemic inflammation at baseline, and acute and chronic cumulative organ damage.\textsuperscript{1-7} Therapeutic options to prevent organ pathologies are limited to chronic transfusions, hydroxyurea or an allogeneic hematopoietic stem cell transplant (HCT).\textsuperscript{8-12}

A conspicuous feature of SCD is chronic activation of the coagulation system, often characterized by high levels of circulating D-dimer, thrombin-antithrombin (TAT) complexes, prothrombin fragment 1.2, increased tissue factor (TF) expression and high TF-bearing microparticles.\textsuperscript{13,14} Sickle cell-induced tissue damage is likely one driver of procoagulant activation, but sickle RBC membrane alterations and phosphatidylserine (PS) exposure may further augment procoagulant function.\textsuperscript{15,16} Thrombocytosis and platelet activation are also well-recognized features of SCD.\textsuperscript{4,13,14,16-35} Thrombotic events including pulmonary embolism, deep vein thrombosis and SCD-related stroke are common.\textsuperscript{17,36-40} SCD-associated pulmonary hypertension (PHT) is associated with endothelial cell activation (as measured by soluble vascular cell adhesion molecule-1 [sVCAM-1]), which would also support procoagulant activity.\textsuperscript{14,20,23,41} However, the precise contribution of hemostatic factors to SCD-induced pathobiologies and particularly progressive end-organ damage has not been thoroughly explored.

A linkage between hemostatic system activation and inflammation is firmly established and this linkage was underscored in SCD by two recent studies in sickle mice.\textsuperscript{42,43} TF activity on endothelial cells was reported to support increased levels of IL-6 in SCD mice, and antibody blockade of TF activity suppressed circulating IL-6, sVCAM and pulmonary neutrophil infiltration.\textsuperscript{42} Interestingly, rivaroxaban, a small molecule inhibitor of the prothrombin-activating protease, factor Xa (FXa) decreased IL-6, but not sVCAM; and dabigatran, a small molecule inhibitor of thrombin (FIIa), did not suppress either of these parameters. Thus, TF and FXa, but not FIIa, were linked to endothelial activation and/or inflammatory changes in SCD, and it was proposed that FIIa-independent signaling mechanism(s) mediated inflammatory effects in mice with SCD.\textsuperscript{43} However, these fascinating studies were short-term in design, and did not explore the influence of hemostatic factors on the wide spectrum of multi-organ pathologies that manifest over long time frames in SCD. Furthermore, human studies exploring the role of the
coagulation system activation employing anti-thrombotics in SCD patients have primarily focused either on reduction in acute sickle episodes and/or circulating markers of thrombin generation/inflammation as study endpoints [reviewed in\textsuperscript{44}]. The overall results from human studies show that while low-intensity anticoagulation diminished circulating markers of thrombin generation, effects on acute painful episodes were mixed, with either absent, modest, or significant reduction depending on the study.\textsuperscript{44,45}

The central role of FIIa as a hemostatic protease is underscored by the fact that it controls fibrin deposition and stability (through fibrinogen, FXIII, and TAFI), regulates platelet and other cell signaling events (through PAR-1,-3 and -4) as well as positively and negatively mediating its own generation (through factors XI, VIII and protein C). FIIa also constitutes a key regulatory bridge between hemostasis/thrombosis, tissue repair, and inflammatory processes through multiple substrates, including fibrin, PARs, and other effectors.\textsuperscript{46-49} However, a causative role for FIIa in SCD pathophysiology has remained elusive. We hypothesized that thrombin contributes significantly to the inflammatory changes and vascular occlusions in SCD and, ultimately worsens the cumulative end-organ damage. To test this concept, we employed both pharmacological and genetic strategies to reduce circulating FII in mice with SCD, such that it only caused mild changes in standard clinical coagulation parameters and no apparent hemorrhagic events. Consequently, a remarkable improvement in inflammation, endothelial dysfunction, multiple SCD-associated organ pathologies and overall survival was observed.
Methods

Experimental Mice. Berkeley SCD mice [Tg(Hu-miniLCRα1γδβ5) Hba0/Hba0 Hbb0/Hbb0] that exclusively express human sickle hemoglobin (HbS), and C57Bl/6-inbred FlIlox/- mice have been previously described.50,51 Berkeley mice expressing normal human hemoglobin (HbA) were generously provided by Dr. Cheryl Hillery (Medical College Wisconsin, Milwaukee, Wisconsin). All animals enrolled in these studies were of mixed gender and with generally similar proportions of males and females in each experimental arm. In accordance with standard IACUC practices, Kaplan-Meier studies did not assume death as a primary end-point. However, in Berkeley sickle mice, death was rarely preceded by morbidity based on overt health status and behavior, and, therefore, death in individual mice was generally unanticipated and occurred without animals appearing distressed or moribund. Mouse breeding and experiments were conducted in the Cincinnati Children’s Research Foundation Vivarium with appropriate IACUC approvals.

FII antisense oligonucleotide (ASO) gapmers. FII-ASO gapmer (ISIS 401025; Isis Pharmaceuticals, Inc., Carlsbad, California) known to selectively reduce circulating FII or control gapmer (ISIS 141923) were injected weekly into Berkeley SCD mice at 50mg/kg beginning at weaning. For further details see supplemental material.

Generation of FII-deficient Chimeric Sickle Mice. Bone marrow cells from Berkeley mice expressing HbS or HbA was transplanted intravenously into lethally irradiated FIIlox/- or FII WT recipients in a 1 donor: 7 recipient ratio to generate HbS/FIIlox/-, HbS/FII WT, HbA/FIIlox/-, HbA/FII WT chimeras. Donor chimerism was assessed at 4 months via HPLC analysis of hemoglobin (Alliance 2960; Waters, Milford, Massachusetts) using a Poly-CAT column, as previously described.53

Hematological assessment. Complete blood count was determined on the Hemavet analyzer and reticulocyte count by flow cytometry using Retic-Count reagent (BD Biosciences).

Plasma analysis. Platelet poor plasma from citrated blood after inferior vena cava phlebotomy was utilized for determining prothrombin time (PT), D-dimer, thrombin-antithrombin (TAT) complexes, sVCAM and IL-6.
**Urine albumin and osmolality.** Twenty four-hour urine collections were used for determining albumin and creatinine, using ELISA kits from Bethyl Laboratories (Montgomery, TX), and R&D Systems (Minneapolis, MD) and osmolality, using a vapor pressure osmometer Vapro5600 (Wescor Biomedical Systems, South Logan, Utah).

**Histopathology analysis.** Mice were weighed before sacrifice. The heart was dissected to separate RV free wall, and LV+septum was weighed separately. All organs were fixed in 10% buffered formalin and embedded in paraffin. Sections (3-5 µm) were prepared for microscopic evaluation by a pathologist blinded to animal genotype following hematoxylin and eosin (H&E) and periodic acid Schiff’s (PAS) staining. Lungs were inflation fixed with 4% paraformaldehyde solution in PBS.

**Immunohistochemistry.** Formalin-fixed, paraffin embedded sections of lung stained with anti α-SMA antibody (Sigma-Aldrich, St. Louis, MO) and secondary goat anti-mouse Alexa-Fluor-488 antibody were used for immunohistochemistry; 4’,6’-diamino-2-phenylindole.2HCL solution was added for nuclear staining, described in detail in supplemental material. Liver sections were stained for endothelial cells with rabbit anti-CD31 antibody (Abcam, Cambridge, MA) (1:50 dilution) followed by incubation with species-specific secondary antibodies and detected with UltraMax DAB kit (Ventana Laboratories, Tucson, AZ).

**Cardiac Magnetic Resonance imaging (CMR).** CMR was performed as previously described.54

**Statistical Analysis** Data were analyzed using Graphpad Prism v6 (La Jolla, CA). Values are expressed as mean ± S.E.M. The primary comparison of interest for sickle and normal genotypes, separately, was between normal or low prothrombin mice; hence pair-wise comparisons were employed. Non-parametric Mann-Whitney U-tests were used unless otherwise indicated.
Results

FII Specific Antisense Oligonucleotide Decreases Early Mortality in Berkeley Sickle Mice.

As an initial approach, to explore the impact of prothrombin on SCD outcome, we tested the hypothesis that reducing FII levels improves SCD survival. Our Berkeley sickle mouse colony has a relatively high early mortality, with 40% SCD mice dying within 4-5 months (Supplemental Figure 1). Cohorts of Berkeley sickle mice were injected with either a murine FII-specific or a control ASO-gapmer starting at 3 weeks of age for up to 15 weeks of age (Supplemental Figure 3A). The FII-specific ASO-gapmer is complementary to the 3' noncoding portion of the F2 mRNA (a sequence found to be unique in the mouse genome), and specifically reduces hepatic F2 mRNA (thereby reducing circulating FII levels) without any impact on other coagulation system components (Supplemental Figure 2). FII-specific ASO dose employed reduced circulating FII levels to approximately ~10% of normal (Figure 1A), a level of FII found here (Figure 1B) and previously to prolong prothrombin times mildly without causing spontaneous bleeding. More importantly, sickle mice treated with FII ASO showed significantly improved survival compared to sickle mice administered control ASO (Figure 1C). Indeed, the survival of Berkeley SCD mice treated with control ASO did not differ from the untreated Berkeley sickle mouse colony (Supplemental Figure 1). These data show that low levels of FII exert a major survival advantage to sickle mice, conceivably reducing the general severity of SCD complications.

While this study was primarily designed to be a survival analysis, we attempted to analyze differences in the surviving mice, with the caveat that we were comparing “surviving fit” control-ASO mice with FII-ASO mice. The body weights were not significantly different between control-ASO or FII-ASO treated sickle mice (Supplemental Figure 3B). Prothrombin times were modestly, but significantly (1.2-fold) higher in FII ASO mice compared to controls (Figure 1B). D-dimer levels were reduced to 371 ± 48 ng/mL in FII ASO group as compared to 468 ± 69 in control ASO mice, although these differences were not statistically significant. TAT complexes, which are short-lived, and only reflect any proximal/short-term thrombin generated, as opposed to the sum of episodic thrombin generation over long-time frames (as likely in SCD) were not different in either group of sickle mice surviving at 15 weeks of age.

In addition, we attempted complementary studies using separate cohorts of sickle mice randomized at 3 weeks of age to FII-specific or control ASO, that were sacrificed at 9 weeks of
age, the mid time-point where significant early mortalities occur in sickle mice to assess differences in organ pathology (the experimental schema is shown in Supplementary Figure 4A). All sickle mice exhibited some organ pathology. However, at this early age, no major pathological differences in kidney, lung, liver, heart and other tissues other than vascular congestion were seen between control and FII ASO mice (Supplemental Figure 4B), suggesting that the survival benefits conferred by FII ASO gapmer in young SCD mice may be due to the evasion of an acute sickle event rather than cumulative end-organ damage. While the precise cause(s) of death in very young sickle cell mice was not obvious based on histological tissue surveys, and remains to be formally established, reduced prothrombin presented a clear benefit in life expectancy.

**Genetically Imposed Reduction in FII levels in SCD Mice Reduces Thrombin Generation.**
To gain better functional insight into the long-term advantages of diminished FII levels in sickle mice, and to do so without potential animal stress or challenges associated with weekly ASO gapmer administration, we employed a FII germline mutation approach. Here, using HCT, we generated sickle hematopoietic chimeras on FIIlox/- background to derive SCD mice carrying approximately 10% of the normal level of FII (HbS/FIIlox/−). This approach allowed for a fixed diminution of FII from the time FIIlox/- mice develop SCD through the entire study period (~1 year following HCT). Control mice were sickle chimeras with normal FII levels (WT mice transplanted with Berkeley sickle bone marrow; HbS/FIIWT). Two additional controls were FIIWT or FIILox/- mice that received bone marrow transplant from Berkeley donors that express normal human hemoglobin (HbA), to generate HbA/FIIWT, HbA/FIIlox/- chimeras. The experimental schema for these experiments with the different analysis performed and timelines is shown in Supplemental Figure 5. Only mice that were fully chimeric for donor HbS or HbA and had negligible mouse hemoglobin (<0.3%, as shown in Supplemental Figure 6) were studied.

HbS/FIIlox/- and HbA/FIIlox/- chimeric mice had a mild (1.25-fold) increase in prothrombin time (PT) (Figure 2A) in comparison to HbS/FIIWT or HbA/FIIWT chimeric animals. This is a similar difference in PT as was described in the original description of the FIIlox/- mice50 and to the PT achieved in the FII ASO treated mice (Figure 1B). Predictably, in the HbA/FIIlox/- chimeras, both TAT and D-dimer were significantly low, when compared to HbA/FIIWT mice: D-dimer levels were reduced in HbS/FIIlox/- mice relative to FII-sufficient sickle (HbS/FIIWT) mice, consistent with decreased baseline thrombin generation (Figure 2B); and as would be expected with a diminution in baseline FII, there was approximately two-fold reduction in TAT complexes in
HbS/FIIlox/- mice versus HbS/FIIWT mice (Supplemental Figure 7). In HbA/FIIWT mice, TAT levels were variable, though generally quite low. When compared to HbA/FIIWT mice, only a fraction of HbS/FIIWT mice exhibited high TAT levels, possibly reflecting episodic sickle cell-associated thrombin generation events. As a result, the average TAT levels of HbS/FIIWT animals were not statistically different from the HbA/FIIWT mice (Supplemental Figure 7). TAT levels in non-transplanted Berkeley sickle mice also showed similar patterns, with some mice showing high TAT levels and others not, and were also not significantly different compared to Berkeley HbA animals (data not shown).

Reduction in FII Levels in SCD Decreases Inflammation and Endothelial Cell Dysfunction. As expected, diminution of FII did not alter the RBC parameters between HbS/FIIWT and HbS/FIIlox/- mice, with both HbS chimeras showing RBC features consistent with classic sickle cell anemia. RBC parameters were normal in HbA/FIIWT and HbA/FIIlox/- mice (Supplemental Table 1). However, HbS/FIIlox/- mice had significant reduction in the SCD-associated leukocytosis (specifically neutrophilia and monocytosis) relative to HbS/FIIWT chimeras. Further, the SCD-associated thrombocytosis, present in the HbS/FIIWT mice was also significantly diminished in the HbS/FIIlox/- animals (Figure 3A-D). As anticipated, no change in the otherwise normal leukocyte, neutrophil or platelet counts was seen in HbA/FIIWT versus HbA/FIIlox/- animals, although monocyte numbers were lower in HbA/FIIlox/- mice. There was no significant difference in the lymphocyte count between these animals (Supplemental Figure 8).

This diminution of leukocytosis FII in HbS/FIIlox/- animals was also accompanied by significantly reduced IL-6, a systemic marker of inflammation, and reduced levels of the endothelial cell activation marker sVCAM, as compared to HbS/FIIWT mice (Figure 3E-F). These markers of inflammation and endothelial dysfunction were at low levels in HbA/FIIWT mice and remained unchanged in HbA/FIIlox/- animals.

Reduction in FII Levels in SCD Protects Against Sickle Nephropathy. To assess the impact of lowered FII on the cumulative organ damage associated with SCD, we examined its effect on sickle nephropathy, a serious complication that results in progressive albuminuria in 50-70% of adults and renal failure and mortality in approximately 30% of SCD patients. The nephropathy of SCD is characterized by both a tubular dysfunction, that manifests early as hyposthenuria, and glomerular damage, which manifests as glomerular hypertrophy, expansion of the mesangium, thickening of capillary loops and glomerular...
basement membrane, significant albuminuria and focal segmental glomerulosclerosis (FSGS), all of which result in progressive nephron loss and renal insufficiency.\textsuperscript{57-59} Consistent with the renal histopathological changes in SCD individuals, HbS/FII\textsuperscript{WT} mice show similar glomerular lesions, and all of these glomerular pathologies were significantly reduced in HbS/FII\textsuperscript{lox/-} mice (Figure 4A-E). Tubular pathology was also improved in HbS/FII\textsuperscript{lox/-} mice (Figure 4F). Semi-quantitative histological scoring of glomerular, vascular, and tubular changes was performed by the study pathologist who was blinded to the mouse genotypes. We observed a remarkable improvement in the kidney pathology scores (Figure 4G-J). There were minimal glomerular changes in HbA/FII\textsuperscript{WT} mice, deemed to be secondary to irradiation for HCT, that were also improved in HbA/FII\textsuperscript{lox/-} mice (Supplemental Figure 9).

Functionally, HbS/FII\textsuperscript{WT} mice had hyposthenuria and significant albuminuria (Figure 4K-L). Consistent with the improved histology, HbS/FII\textsuperscript{lox/-} mice showed a highly significant decrease in albuminuria. Although hyposthenuria was also significantly improved, this difference was not as pronounced as the improvement in albuminuria. These results indicate that procoagulant function significantly contributes to the renal, and specifically the glomerular pathology in SCD, and lowering FII in mice with SCD protects against development of sickle nephropathy.

**Reduction in FII results in Improved SCD-Associated Cardiopulmonary Pathologies.**

Pulmonary hypertension (PHT) and ventricular diastolic dysfunction are recognized complications of SCD and have been shown to be associated with coagulation activation, endothelial dysfunction, and inflammation.\textsuperscript{14,34} While the incidence of right heart catheterization proven PHT in SCD adults is now estimated at 6-10%,\textsuperscript{60,61} we\textsuperscript{54} and others\textsuperscript{62} have commonly observed development of PHT in SCD mice with increasing age. We have previously shown that right ventricle (RV) enlargement and associated increased RV mass (as determined by cardiac MRI [CMR] or physical mass) correlates with increased RV pressures on cardiac catheterization in Berkeley SCD mice.\textsuperscript{54} We therefore assessed the RV in HbS/FII\textsuperscript{lox/-} and HbS/FII\textsuperscript{WT} mice. CMR was done in two each of HbS/FII\textsuperscript{WT} and HbS/FII\textsuperscript{lox/-} mice, and one HbA/FII\textsuperscript{WT} control. RV wall thickness was increased in HbS/FII\textsuperscript{WT} mice, as compared to the HbA/FII\textsuperscript{WT} control (Figure 5A). In addition, RV and LV end diastolic volumes were increased in the presence of normal ejection fraction, indicative of ventricular dilatation. These features were less pronounced in the HbS/FII\textsuperscript{lox/-} mice compared to HbS/FII\textsuperscript{WT} mice, although the animal numbers were limited (Figure 5B-C). As in humans with SCD, systolic function was normal in HbS chimeras (Supplemental Figure 10). RV and LV weights confirmed the CMR data in
larger numbers of animals, showing a significant decrease in the RV/LV+Septum ratio in HbS/FIIlox/− mice as compared to HbS/FIIWT mice (Figure 5D).

PHT is also characterized by proliferation of smooth muscles and thickening of tunica media of pulmonary arteries/arterioles. In mice, instead of the classic concentric smooth muscle thickening seen in humans and rats, increased number of cells expressing α-smooth muscle actin (α-SMA) is observed. Lung sections of HbS/FIIlox/− mice showed decreased α-SMA staining around the blood vessels as compared to HbS/FIIWT mice (Figure 5E). In addition, increased α-SMA was also seen around the airways in HbS/FIIWT mice relative to HbS/FIIlox/− mice. The latter is a feature of airway reactivity, another known association with SCD. As anticipated PHT and other associated pulmonary pathologies were absent in HbA chimeras.

Additionally, lung histology revealed presence of foamy alveolar macrophages, thickened arterial/arteriolar tunica media, and increased lymphocyte and macrophage infiltration (Figure 6A-C). Edema around the blood vessels and vascular congestion was also pronounced in HbS/FIIWT mice. In contrast, all of these pathologies were remarkably reduced in HbS/FIIlox/− mice (Figure 6A-C). These changes were absent in both the HbA chimeric controls (Supplemental Figure 11).

Together, these data indicate that increased thrombin generation contributes to the cardiopulmonary pathologies in SCD; and consistent with a decrease in circulatory markers of inflammation, pulmonary inflammation is also reduced with diminution of circulating FII.

**Reduced FII Levels Protect Against Hepatic Inflammation and Coagulative Necrosis.**

The hepatic pathophysiology in SCD results from ischemia, vascular occlusion and acute hepatic sequestration. In HbS/FIIWT mice, multifocal areas of coagulative hepatic necrosis (Figure 7A-D) were observed. Necrotic areas were infiltrated with lymphocytes and macrophages in their periphery, and marked congestion within hepatic sinusoids with shrinkage of hepatocytes were observed (Figure 7A-D). Examination of the hepatic vasculature revealed massively engorged blood vessels filled with sickle RBCs and likely represents sludging of the blood flow in the liver vasculature. In some areas, these vascular engorgements were associated with accompanying necrosis, suggesting vascular blockade (Figure 7B-C and F-H). These occlusive events were significantly reduced in HbS/FIIlox/− mice. However, there was no difference in microvascular density between HbS/FIIlox/− and HbS/FIIWT mice. Indeed, in
HbS/FII\textsuperscript{lox/-} mice, areas of focal hepatic necrosis were fewer and more limited, and completely absent in a few animals. Additionally, reduced hepatic inflammatory infiltrates were seen. We also stained liver sections with CD31, an endothelial cell marker, and show that additionally, vessels were congested with reactive endothelium in HbS/FII\textsuperscript{WT} liver sections, which was not a prominent feature in HbS/FII\textsuperscript{lox/-} liver sections (Figure 7F-H). Consistent with the diminished microscopic evidence of hepatic damage, plasma alanine transaminase activity was also significantly lower in HbS/FII\textsuperscript{lox/-} mice compared to HbS/FII\textsuperscript{WT} mice (Figure 7E). No hepatic histopathological changes or elevation in transaminase activity was observed in HbA chimeras, regardless of FII levels (Figure 7E; Supplemental Figure 12).

Discussion

In this study we show that FII plays a significant role in inflammation and multi-organ pathologies in mice with SCD. Consistent with prior work,\textsuperscript{50} we found that even in SCD mice, reducing the circulating FII to 10% of normal was associated with a relatively mild prolongation in the PT and reduced markers of FIIa activity, but was not associated with spontaneous bleeding. Moreover, these mild increases in PT are well below clinical PT targets for safe and effective anticoagulation. Yet, there was an impressive reduction in inflammation or associated reactive changes: there was significantly diminished (i) leukocytosis, specifically neutrophilia and monocytosis, (ii) circulating IL-6, (iii) inflammatory infiltrates in lung, liver and kidneys, (iv) vascular dysfunction (sVCAM) and (v) thrombocytosis. In addition, there was marked improvement in cardiac, renal, pulmonary and hepatic histology and function: amelioration of RV hypertrophy and dilatation, albuminuria and hyposthenuria. Of note, this reduction in inflammation and organ pathologies was not due to a change in the RBC parameters, suggesting no correlation of the inflammation with the underlying chronic hemolytic anemia or RBC turnover (reticulocytosis) with reduced circulating FII. These findings also suggest that reduction in FII is unlikely to limit hemolysis and free hemin-induced events, such as TLR-4 activation,\textsuperscript{68} although this has not been formally excluded. Rather, diminution of FII likely limited the vaso-occlusive manifestations of SCD and the secondary inflammation. An ultimate entirely objective indicator of the benefit of this intervention was the significantly improved survival of Berkeley sickle mice carrying low levels of FII.

A connection between sickle RBC, the hemostatic system and SCD pathobiology has been long-speculated. Vaso-occlusions are the hallmark of SCD, and thrombotic changes secondary
to sickling-induced vascular damage and sludging of blood flow are also innate to its pathophysiology. Consequently, the potential significance of procoagulant function in SCD has been recognized for decades. Prior studies of the anticoagulant vitamin K antagonist (VKA), warfarin, given during acute vaso-occlusive events (VOE) suggested no effect on the duration of VOE in the short-term, and showed a modest reduction in VOE (from 1.3 VOE/year to 0.9 VOE/year) when given long-term. However the authors acknowledged that the anticoagulation was poorly controlled and therefore, the intervention had associated bleeding episodes in some patients. VKAs have the disadvantage of lowering all vitamin K-dependent factors, including the anti-coagulant and anti-inflammatory protein C. Thus, based on these early findings, the benefits, of targeting the coagulation system, in general, remained uncertain, and any long-term benefits and liabilities of interventions at the level of a selected procoagulant have remained entirely unknown.

The present study unambiguously establishes, for the first time, that intervention at the level of a single key hemostatic factor, FII, can significantly ameliorate SCD organ pathologies and improve survival, at least in mice. FII-specific ASO provides complementary proof-of-principle of the potential utility of pharmacological intervention. A recent high-profile clinical trial using a FXI-specific ASO gapmer established their utility in safely attenuating postoperative venous thromboembolism. While first-generation ASOs have been associated with proinflammatory effects, second-generation ASO gapmers are devoid of these side effects due to their advanced chemistries, high sequence specificity and comprehensive screening strategies. Based on the present findings, it will be of keen interest to now also explore long-term benefits of interventions of this type or direct thrombin or FXa inhibitors in future studies of SCD. While FII-specific ASO gapmers may have potential short-term utility in venous thromboembolism, their long-term use for the prevention of chronic organ damage in SCD may have compliance issues. In this regard, recent studies of SCD in mice intervening at the level of tissue factor (TF), the premiere initiator of the coagulation system, suggested suppressing TF activity reduced endothelial cell activation markers (sVCAM) and inflammatory markers (IL-6). Yet no such benefits were reported with regard to either of these metrics in mice treated with the oral FIIa inhibitor, dabigatran in the short duration of 10 days. Our report demonstrates that intervention at the level of FII in sickle mice can mirror findings made previously with TF blocking antibodies in reducing circulating sVCAM and IL-6. However, the seeming discrepancy with prior dabigatran studies could simply reflect the distinct experimental observation periods (young adults studied for 10 days vs. mice studied for 10-12 months) and the known progressive nature...
of sickle cell pathologies. It would not be surprising that some benefits of limiting FII become fully manifest over long time frames in sickle mice by reducing the cumulative tissue damage.

Recent studies have examined the components of the coagulation system in human subjects and in mouse models of SCD. Increased TF expression secondary to sickling and SCD-induced vascular endothelial/tissue damage is one likely driver of procoagulant activation. Recent evidence suggests that besides coagulation system activation from vascular endothelial/tissue damage, sickle RBC membrane damage and PS exposure (plus PS-positive RBC microparticles) may be a significant driver of chronic thrombin generation and coagulation system activation, especially given their exceptional mass and turnover in SCD. Lowering FII in mice with SCD would be anticipated to limit the chronic procoagulant response to diminish both vaso-occlusion and inflammation. In addition to this, benefits of thrombin suppression could be gained at the level of both reduced platelet activation and reduced reactive changes leading to thrombocytosis. Although we did not examine platelet activation, our data suggests that part of the thrombocytosis in the HbS/FIIlox-/- mice was reactive, secondary to the chronic inflammation that results from increased thrombin generation.

While the precise mechanism(s) tying FII to SCD have not yet been formally established, at least two primary proteolytic targets of FIIa are strong candidates to support SCD-induced thrombo-inflammatory disease. First, fibrin(ogen), a known modifier of inflammatory diseases, may provide a local cue supporting leukocyte activation, especially macrophages, via the αMβ2 ligand, located on the γ-chain of fibrin(ogen). The availability of Fibγ390-396A mice carrying a mutant form of fibrinogen lacking the primary αMβ2 binding motif, but supporting clotting function, provide an attractive means for testing the concept that this thrombin target is coupled to SCD pathophysiology without imposing a hemorrhagic risk. The viability of mice lacking other thrombin substrates, such the fibrin-stabilizing transglutaminase FXIII, provides an opportunity for more detailed mechanistic studies. If indeed dampening the proinflammatory effect of fibrin(ogen) offers benefit in SCD, the wholesale absence of clotting function is likely to be distinctly counterproductive, as the complete absence of fibrin(ogen) in an established, albeit far milder, model of SCD (transgenic SAD mice) resulted in increased mortality. A second, and not mutually exclusive target of FIIa that may be mechanistically coupled to SCD outcome is protease activated receptor-1 (PAR-1). Proteolytic activation of PAR-1 on endothelial cells results in a spectrum of functional changes that could be linked to SCD pathobiology, including changes in adhesive properties, the elaboration of hemostatic proteins, and changes in vascular
permeability. While short-term studies in PAR-1-deficient mice with SCD did not show significant benefits, longer term studies may be needed to better define any PAR-1 and/or PAR-4 involvement.

Increased TF expression in monocytes and endothelium and monocyte-TF bearing microparticles have been reported in patients with SCD and been associated with SCD pathologies including increased peak pulmonary artery pressures; however, the reports have been controversial, with some studies showing an association and others not. Studies on mouse models of SCD lend credence to the clinical associations while also illuminating causality. Here, we show that a genetically-imposed chronic deficit in prothrombin, leading to a relatively modest increase in PT is a major modifier of inflammation and organ pathology in SCD mice. A prothrombotic state is known to predispose to pulmonary microemboli and pulmonary vascular pruning in non-SCD associated PHT. We show that this also occurs in SCD, where reduction in procoagulant function prevents development of PHT. In addition, we show a pleiotropic amelioration of multi-organ dysfunction. Of note, the remarkable improvement in glomerulopathy (reduction in albuminuria and FSGS), lung and liver pathology implies that this is a primarily microvascular effect, since these tissues are rich in capillaries. Reduced thrombin-mediated endothelial cell activation, as evidenced by plasma sVCAM levels (and thereby reduced RBC and WBC endothelial cell adhesion) and leukocytosis in HbS/F11ox⁻ mice likely reduces microvascular blockade, as was observed in liver sections. While Gavin et al have shown that direct inhibition of thrombin acutely via antithrombin III or hirudin reversed the accelerated experimentally induced thrombus formation in large vessels subjected to light/dye injury induced cerebral thrombosis in sickle mice, we show that chronic reduction in FIIa also limits microvascular thrombosis and endothelial dysfunction, impeding chronic end-organ damage. Its effect on stroke prevention, however, could not be elucidated, as SCD mouse model is not a good model of unprovoked stroke. Overall, our studies establish that prothrombin diminution imparts substantial long-term benefit to multiple organs in SCD mice under normoxic day to day conditions, likely from reduced vascular occlusions, at least in the liver. It will be of added interest to explore whether short-term benefits are also gained in SCD mice when subjected to hypoxia or other secondary challenges (e.g., TNFα).

Several human trials with anticoagulants and antiplatelet agents have been conducted, but have mostly been non-randomized non-placebo controlled studies in small cohorts of patients. These studies have been limited by complex endpoints such as SCD-associated pain,
which have thus far yielded mixed results. Nevertheless, these studies have shown that some of the anticoagulant and antiplatelet agents can be safely given to patients with SCD without inordinate risk of bleeding.\cite{45,82-89} Two placebo controlled studies of acencoumarol and tinzaparin have been performed,\cite{90,91} of which the large randomized placebo-controlled trial by Qari et al has shown a significant effect of tinzaparin, a low molecular weight heparin, in reducing VOE.\cite{91} However, except for one anecdotal report on healing of leg ulcers published nearly 60 years ago, none of the clinical trials have attempted to examine the effect of reduced procoagulant function on end-organ damage in SCD, which worsens asymptotically over long periods and only becomes clinically apparent when overt organ failure ensues. We show, for the first time, that long-term intervention at the level of a single hemostatic factor, prothrombin, is sufficient for a system-wide anti-inflammatory effect, reduced endothelial cell activation, and amelioration of organ pathology and dysfunction and significantly improved survival in SCD mice, without hemorrhagic events. Our studies open a new paradigm of targeting excessive thrombin generation or activity as novel means to limit multi-organ damage in SCD. The availability of new orally bioavailable FIIa, FXa and PAR1 inhibitors designed to suppress selected procoagulants suggest that translation of these results may be possible in the near future.
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Author Contributions:
PIA, AL, MS and ESM performed the experiments and plotted the data, SKS scored and interpreted tissue histology, JW performed and analyzed CMR, JD, PM, PIA, BM and ESM designed the experiments and interpreted the overall experimental data, PIA, PM, ESM and JD wrote the manuscript, all authors provided their comments on the manuscript.

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Brett P. Monia is an employee of Isis Pharmaceuticals. All other authors declare no competing financial interest.
Main Figure legends

Figure 1 Reduction of circulating FII using antisense oligonucleotides (ASO) reduces early mortality in Berkeley sickle mice. (A) Western blot analysis showing levels of circulating FII protein in representative Berkeley sickle mice administered either control ASO (n=4, Lane 1-4) or FII-specific ASO (n=4, Lane 6-9) beginning at 3 weeks of age and sacrificed for FII analysis at 15 weeks of age. The plasma FII levels in representative untreated FII^WT (Lane 5) and untreated FII^lox/- (Lane 10) mice are also shown as additional controls for comparison. (B) Prothrombin times of Berkeley sickle mice administered control ASO or FII ASO beginning at 3 weeks of age and sacrificed for plasma analysis at 15 weeks of age. Each symbol represents an individual animal and the bar indicates the median values. Data were analyzed by Mann-Whitney U-test; **P<.01. (C) Kaplan-Meier survival analysis of cohorts of Berkeley sickle mice administered either FII ASO (n=20) or control ASO (n=20) beginning at 3 weeks of age and continued on weekly ASO treatments until 15 weeks of age. Equal numbers of males and females were enrolled for either control ASO or FII-specific ASO administration. Among the sickle mice that had early mortalities, the ratio of males to females was 1:1. Comparison of survival curves using Log-rank (Mantel-Cox) test; *P<.05.

Figure 2 Sickle chimeras with 10% circulating FII exhibit a modest increase in prothrombin time and diminished evidence of coagulation activation. (A) PT values in FII^WT mice [with 100% circulating FII] and FII^lox/- mice [with ~10% FII levels], and cohorts of FII^WT and FII^lox/- mice transplanted with either bone marrow from donor Berkeley mice expressing HbA (HbA/FII^WT and HbA/FII^lox/-) or donor Berkeley sickle mice expressing HbS (HbS/FII^WT and HbS/FII^lox/-). (B) D-dimer levels in HbS/FII^WT, HbS/FII^lox/-, HbA/FII^WT and HbA/FII^lox/- mice. The bar indicates the median. Each symbol represents an individual animal. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year after transplantation. Cohorts were analyzed by Mann-Whitney U-test and statistical significance between FII^WT and FII^lox/- chimeras is indicated by asterisks. **P<.01, *P<.05.
Figure 3 Sickle chimeras with genetically-imposed reductions in circulating FII exhibit significant decreases in inflammation. Total (A) white blood cell (WBC), (B) neutrophil, (C) monocyte, and (D) platelet counts in HbS/FIIWT, HbS/FIIlox/−, HbA/FIIWT and HbA/FIIlox/− mice are shown. (E) Plasma IL-6 and (F) plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) levels in HbS/FIIWT, HbS/FIIlox/−, HbA/FIIWT and HbA/FIIlox/− cohorts. Each symbol represents an individual animal. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year post transplantation. sVCAM levels in female mice trended to be lower compared to males within each group. In male mice, sVCAM levels HbS/FIIWT vs. HbS/FIIlox/− mice were 17 965 ± 3406 ng/mL vs. 12 870 ± 338 ng/mL respectively. In female mice, sVCAM levels in HbS/FIIWT vs. HbS/FIIlox/− mice were 13 009 ± 1361 ng/mL vs. 10 914 ± 937 ng/mL respectively. The differences between the two genders in the different groups were not statistically significant. Cohorts were analyzed by Mann-Whitney U-test and statistical significance between FII WT and FIIlox/− chimeras is indicated by asterisks. **P<.01, *P<.05, ***P<.001.

Figure 4 Sickle chimeras with low FII levels are protected against kidney damage and sickle nephropathy. (A-F) Representative hematoxylin and eosin (H&E) kidney sections comparisons between HbS/FIIWT (left panel) and HbS/FIIlox/− (right panel) mice showing (A) ischemia, inflammatory cell infiltration and renal scarring (10X objective) (B-C) Focal segmental glomerulosclerosis (FSGS) and glomerular atrophy (shown by arrows) in HbS/FIIWT glomeruli compared to HbS/FIIlox/− glomeruli. (D-E) Periodic acid Schiff’s (PAS) staining showing glomerular basement membrane thickening in HbS/FIIWT glomeruli compared to HbS/FIIlox/− glomeruli. (F) Representative H&E stained sections of renal tubules showing tubular loss, inflammatory cell infiltration (indicated by dotted lines) and regenerative tubules. (B-F) 60X objective (G-J) Semi-quantitative assessment of histologic features: Histology scores ranged from 0-5, where 0 represented normal kidney morphology, 1 represented changes in less than 15 % of glomerular area, 2: 20-30 % of the glomerular area and 3: 40-50 % of the glomerular area and 4: approximately 60-70 % of glomerular area, 5: severe changes in most all glomeruli. (K) Urine albumin and (L) urine osmolality values. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year post transplantation. Each symbol represents an individual animal. Statistical analyses on the histological scores were done by Mann-Whitney U-test. Statistical significance between
HbS/FII\textsuperscript{WT} (n=6) and HbS/FII\textsuperscript{lox/-} (n=7) mice is indicated by asterisks ****\(P<.0001\), ***\(P<.01\), **\(P<.01\), *\(P<.05\).

**Figure 5** Sickle chimeras with genetically reduced FII levels have improved cardio-pulmonary pathology and function. (A) Cardiac MRI on representative Hba/FII\textsuperscript{WT}, HbS/FII\textsuperscript{WT} and HbS/FII\textsuperscript{lox/-} mice at 1 year following bone marrow transplantation. A representative view of the ventricles is shown with RV in the same plane, which is indicated by the red arrows. (B-C) LV and RV end diastole volumes in chimeric mice; Symbols represent the value of each individual mouse and the bars denote the average. (D) RV hypertrophy is measured as a ratio of RV wall weight/ left ventricle (LV) + Septum weights (Fulton Index) in HbS/FII\textsuperscript{WT} (n=12), HbS/FII\textsuperscript{lox/-} (n=15), Hba/FII\textsuperscript{WT} (n=6), Hba/FII\textsuperscript{lox/-} (n=7) mice. Cohorts were analyzed by Mann-Whitney U-test and statistical significance between FII\textsuperscript{WT} and FII\textsuperscript{lox/-} chimeras is indicated by asterisks **\(P<.01\), *\(P<.05\). (E) \(\alpha\)-Smooth muscle actin staining in lung tissue. Images are representative of 5-7 mice/group. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year after transplantation.

**Figure 6.** Sickle chimeras with 10% of normal FII levels have diminished pulmonary pathology. (A-C) Representative lung sections of a HbS/FII\textsuperscript{WT} mice (left panels) compared to that of HbS/FII\textsuperscript{lox/-} mice (right panels) showing: (A) Increased inflammatory infiltrate in HbS/FII\textsuperscript{WT} lungs compared to HbS/FII\textsuperscript{lox/-} lungs, 10X objective (B) Increased alveolar macrophages in HbS/FII\textsuperscript{WT} lungs compared to HbS/FII\textsuperscript{lox/-} lungs, 40X objective (C) Increased edema around blood vessels in HbS/FII\textsuperscript{WT} lungs versus its lack in HbS/FII\textsuperscript{lox/-} lungs, 40X objective. Black arrows indicate the pathology described in each panel. Images are representative of 6-9 mice/experimental group. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year post transplantation.

**Figure 7.** Sickle chimeras with genetically reduced FII levels have improved hepatic morphology, function and reduced vascular occlusions. Representative H&E liver sections from HbS/FII\textsuperscript{WT} (left panel A-D) and HbS/FII\textsuperscript{lox/-} (right panel A-D) mice 1 year following marrow transplant viewed at various magnifications (A. 10X, B. 20X, C. 40X and D. 60X objectives) showing multifocal areas of coagulative hepatic necrosis with occasionally associated massively congested blood vessels (shown by arrows in 7C) in HbS/FII\textsuperscript{WT} mice. Areas of necrosis are
marked by dotted lines (A-B, D) and infiltrating leukocytes are marked by arrow heads (D) in HbS/FII<sup>WT</sup> mice. In HbS/FII<sup>lox/-</sup> mice, the areas of coagulative necrosis were minimal with comparatively fewer congested blood vessels seen. (F-H) CD31 staining of liver vascular endothelial cells showing vascular congestion in HbS/FII<sup>WT</sup> mice (shown by arrows) along with prominent rounded endothelial cells (H). These changes were minimal in HbS/FII<sup>lox/-</sup> mice. No differences were observed in the microvascular density (MVD) in liver sections from HbA/FII<sup>WT</sup> and HbA/FII<sup>lox/-</sup> mice. Panel F-G: 40X objective, panel H: 100X objective. Images are representative of 9 mice/experimental group. (E) Alanine transaminase (ALT) activity in the serum of HbS/FII<sup>WT</sup>, HbS/FII<sup>lox/-</sup>, HbA/FII<sup>WT</sup> and HbS/FII<sup>lox/-</sup> mice. Each symbol represents an individual animal and lines represent median values. Mice used as transplant recipients were of similar ages (8-10 weeks) with equal numbers of males and females distributed among the experimental groups. Fully chimeric sickle or normal mice were followed for a year after transplantation. Cohorts were analyzed by Mann-Whitney U-test and statistical significance between FII<sup>WT</sup> and FII<sup>lox/-</sup> chimeras is indicated by asterisks *P < .05. n.s. = not significant.
References


Figure 3

A

WBCs ($10^3/\mu L$)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA

B

Neutrophils ($10^3/\mu L$)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA

C

Monocytes ($10^3/\mu L$)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA

D

Platelets ($10^3/\mu L$)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA

E

IL-6 (pg/mL)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA

F

sVCAM-1 (ng/mL)

FII$^{WT}$ FII$^{lox/-}$

FII$^{WT}$ FII$^{lox/-}$

HbS HbA
Figure 4

A

HbS/FII\textsuperscript{WT}  HbS/FII\textsuperscript{lox/-}

B

C

D

E

F

G

H

I

J

K

L

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Figure 6

HbS/FII^{WT}  HbS/FII^{lox/-}

A

B

C
Genetic diminution of circulating prothrombin ameliorates multi-organ pathologies in sickle cell disease mice

Paritha I. Arumugam, Eric S. Mullins, Shiva Kumar Shanmukhappa, Brett P. Monia, Anastacia Loberg, Maureen A. Shaw, Tilat Rizvi, Janaka Wansapura, Jay L. Degen and Punam Malik