

GENE THERAPY

Silencing of PAI-1 using siRNA-lipid nanoparticles reduces thrombosis and prolongs life span in murine models

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KEY POINTS

- siRNA targeting PAI-1, encapsulated in lipid nanoparticles, lowers PAI-1 levels in age-associated disease models with no overt toxicity.
- A reduction in circulating PAI-1 levels reduced thrombosis in young and aged mice and prolonged life span in a fast-aging mouse model.

Plasminogen activator inhibitor 1 (PAI-1) is an inhibitor of fibrinolysis, thereby promoting blood clot stabilization. PAI-1 contributes to thrombosis, diet-induced obesity, and age-associated diseases, such as diabetes, cancer, and Alzheimer disease. Circulating PAI-1 level increases with age, contributing to the increased thrombotic risk in age-related diseases. In contrast, partial PAI-1 deficiency protects patients from cardiovascular morbidity and extends life span. Decreasing circulating PAI-1 levels has both experimental and therapeutic value. RNA gene therapy can regulate the levels of target proteins, including those not amenable to traditional small-molecule or antibody-based therapies. Here, we developed a therapeutic approach to induce long-lasting PAI-1 knockdown in vivo with small interfering RNA (siRNA)-lipid nanoparticles (siPAI-1). One dose of siPAI-1 resulted in 90% knockdown of plasma PAI-1 and lasted 10 days after administration with no overt toxicity. siPAI-1 decreased thrombus weight after complete ligation of the inferior vena cava (IVC) in young and aged mice and increased survival in aged mice 4 days post-IVC ligation. Hepatic PAI-1 mRNA expression in diet-induced obese mice was >10 times higher than in healthy mice and was exponentially correlated with body weight. One dose of siPAI-1 in obese mice resulted in 70% knockdown of circulating PAI-1. Furthermore, siPAI-1 normalized the supraphysiologic concentration of PAI-1 in aged mice and prolonged life span in a fast-aging mouse model. Thus, siRNA-mediated PAI-1 knockdown represents a long-term antithrombotic approach and effective strategy to limit pathologic impact of PAI-1 in aging and age-related diseases.

Introduction

Age-related diseases account for >1.4 million deaths annually in the United States, with thrombosis being a primary cause of death among older adults, affecting 1 in every 100 individuals over the age of 45 years.¹⁻³ An important accelerator of thrombosis is plasminogen activator inhibitor 1 (PAI-1), a circulating protein in the blood that impedes clot degradation (fibrinolysis) by inhibiting tissue and urokinase plasminogen activators (tPA and uPA, respectively),^{4,5} both of which mediate the conversion of plasminogen to plasmin.⁵ Upon vascular injury, circulating PAI-1 concentration increases, leading to inhibition of fibrinolysis and contributing to clot stabilization,

but it also increases the risk of thrombosis.⁶ Elevated PAI-1 concentrations are correlated with thrombosis risk and are observed in related conditions such as disseminated intravascular coagulation, which is often observed in older patients with COVID-19.⁶⁻⁹ Notably, in patients with COVID-19, PAI-1 is linked with disease severity and elevated morbidity.⁹ Moreover, PAI-1 acts as a mediator of cellular senescence; its levels rise during aging and the progression of multiple age-associated diseases, such as atherosclerosis, obesity, Alzheimer disease, diabetes, and cancer.¹⁰⁻¹⁴

Healthy PAI-1 concentrations in humans range from 21.0 ± 7.2 ng/mL and with age they typically increase up to

50 ng/mL.^{15,16} Patients with complete PAI-1 deficiency (<1 ng/mL) may experience prolonged bleeding after severe trauma, including spontaneous intracranial, mucocutaneous, joint, and oral bleeding.¹⁷⁻¹⁹ However, those with partial PAI-1 deficiency (>1 ng/mL), including heterozygote individuals (*SERPINE1*^{-/+}), do not experience excessive risk of bleeding²⁰ but instead have reduced cardiovascular morbidity and evidence of increased life span.¹¹ Thus, PAI-1 has emerged as a promising therapeutic target for modulating aging and age-associated pathologies, including thrombosis.^{21,22}

Multiple small-molecule, antibody, and nanobody inhibitors for PAI-1 have been developed.^{4,23-27} These inhibitors have been tested in various mouse and rat disease models of thrombosis, Alzheimer disease, atherosclerosis, and multiple cancer types, with each case demonstrating promising results.^{24,28} However, PAI-1 inhibitors face specificity challenges due to the inherent conformational plasticity of PAI-1, which exists in active, latent, and cleaved forms. TM5614 is the only orally bioavailable small-molecule PAI-1 inhibitor that has reached clinical trials.²⁹ In the United States, TM5614 is currently in a phase 2 clinical trial for high-risk patients with severe COVID-19, but in Japan, it has recently completed a randomized trial in patients with mild-to-moderate COVID-19.^{30,31} Furthermore, in Japan, TM5614 is in a phase 2 clinical trial for the treatment of malignant melanoma.³² MDI-2517 is a novel small-molecule inhibitor that has recently entered the clinic and is currently in phase 1 to assess safety and tolerability. No PAI-1 inhibitor has yet received US Food and Drug Administration approval for therapeutic use.^{5,33}

RNA gene therapy represents an alternative approach to regulate the levels of target proteins, including those unsuitable for traditional small-molecule or antibody-based approaches.^{34,35} RNA interference approaches are an alternative strategy to previously explored inhibitors.³⁶⁻³⁹ Small interfering RNA (siRNA) are short, noncoding RNA molecules that can be designed to specifically degrade an mRNA of interest, thereby suppressing the production of specific proteins for long durations (weeks to months).⁴⁰ Lipid nanoparticles (LNP) are approved by the US Food and Drug Administration as nonviral nucleic acid delivery system used in the Pfizer/BioNTech/Acutas and Moderna mRNA COVID-19 vaccines.^{41,42} siRNA-LNP agents can be used to both degrade hepatic mRNA and modulate the concentration of its corresponding protein in the blood.^{37-39,43,44} LNP were first approved in Onpatro, an siRNA targeted for hereditary transthyretin amyloidosis.⁴⁰ Patients who received Onpatro every 3 weeks for >6 years have maintained improvement in polyneuropathy symptoms without overt toxicity.⁴⁵ LNP preferentially accumulate in the liver, a tissue in which PAI-1 is synthesized.^{16,46} PAI-1 is also synthesized in endothelial cells, megakaryocytes, smooth muscle cells, fibroblasts, monocytes, adipocytes, endometrium, peritoneum, mesothelial cells, and cardiac myocytes, making it unclear whether an approach based on siRNA-LNP could sufficiently decrease circulating PAI-1 level.¹⁶ For example, the higher concentrations of circulating PAI-1 in patients with obesity are attributed to overexpression by the adipose tissue.^{13,47,48} In this study, we developed a long-lasting siRNA-LNP that decreases circulating PAI-1 levels, providing both an experimental and therapeutic tool to control PAI-1 in age-associated diseases.

Methods

A detailed description of all the experimental procedures and statistical analysis are available in the supplemental Methods (available on the *Blood* website).

Animals

Murine studies were performed in accordance with approved protocols from the animal care and use committees of affiliated institutions.

siRNA-LNP formulation

siRNA targeting PAI-1 or luciferase were encapsulated in LNP as previously described.⁴³

mRNA quantification and analysis of PAI-1 levels

For mRNA quantification, the livers were processed as previously described.⁴⁹ The total PAI-1 concentration was analyzed with a mouse total PAI-1 enzyme-linked immunosorbent assay kit (IMSPA11KTT; Innovative Research).

IVC ligation

Complete inferior vena cava (IVC) ligation was performed under isoflurane as described in the supplemental Methods.³⁹

Mouse models of bleeding

The bleeding models were lateral vein transection (TVT), tail tip transection (TTT), and a 6-hour tail-bleeding test, and they were performed using procedures previously described.^{50,51}

Results

Circulating PAI-1 can be potently knocked down with siRNA-LNP in mice

We evaluated whether siRNA designed against PAI-1 mRNA encapsulated in LNP could achieve knockdown of circulating PAI-1 protein. *In silico*, 6 siRNA sequences were designed to target different regions of the PAI-1 mRNA. Sequences were encapsulated in LNP and screened in mice. The optimal siRNA sequence targeting mouse PAI-1 encapsulated in LNP (siPAI-1) was then selected for future experiments (data not revealed). Mice were injected with either siPAI-1 or an siRNA control with no target mRNA in mice (siLuc) at 3 mg of siRNA per kilogram of body weight (3 mg/kg). PAI-1 protein was measured in plasma and hepatic tissue, and PAI-1 mRNA was measured in hepatic tissue. Three days after injection, male and female mice injected with siPAI-1 at a dose of 3 mg/kg had significantly lower circulating plasma PAI-1 level. The concentration of PAI-1 in siLuc control male mice was 2.2 ± 0.3 ng/mL compared with 0.3 ± 0.1 ng/mL ($P < .0001$) in male mice injected with siPAI-1 (Figure 1A). To evaluate whether the LNP itself modulates PAI-1 protein levels, mice were injected with phosphate-buffered saline (PBS) and blood was collected 3 days after injection. There were similar circulating PAI-1 levels in mice injected with PBS (1.6 ± 0.1 ng/mL) or siLuc (2.3 ± 0.3 ng/mL; $P = .17$). Similarly to male mice, female mice injected with siPAI-1 (1 ± 0.1 ng/mL) had lower circulating PAI-1 levels 3 days after injection compared with siLuc-treated mice (2 ± 0.3 ng/mL; $P = .02$; Figure 1B). Due to the interanimal variability in PAI-1 levels in female mice, all subsequent studies

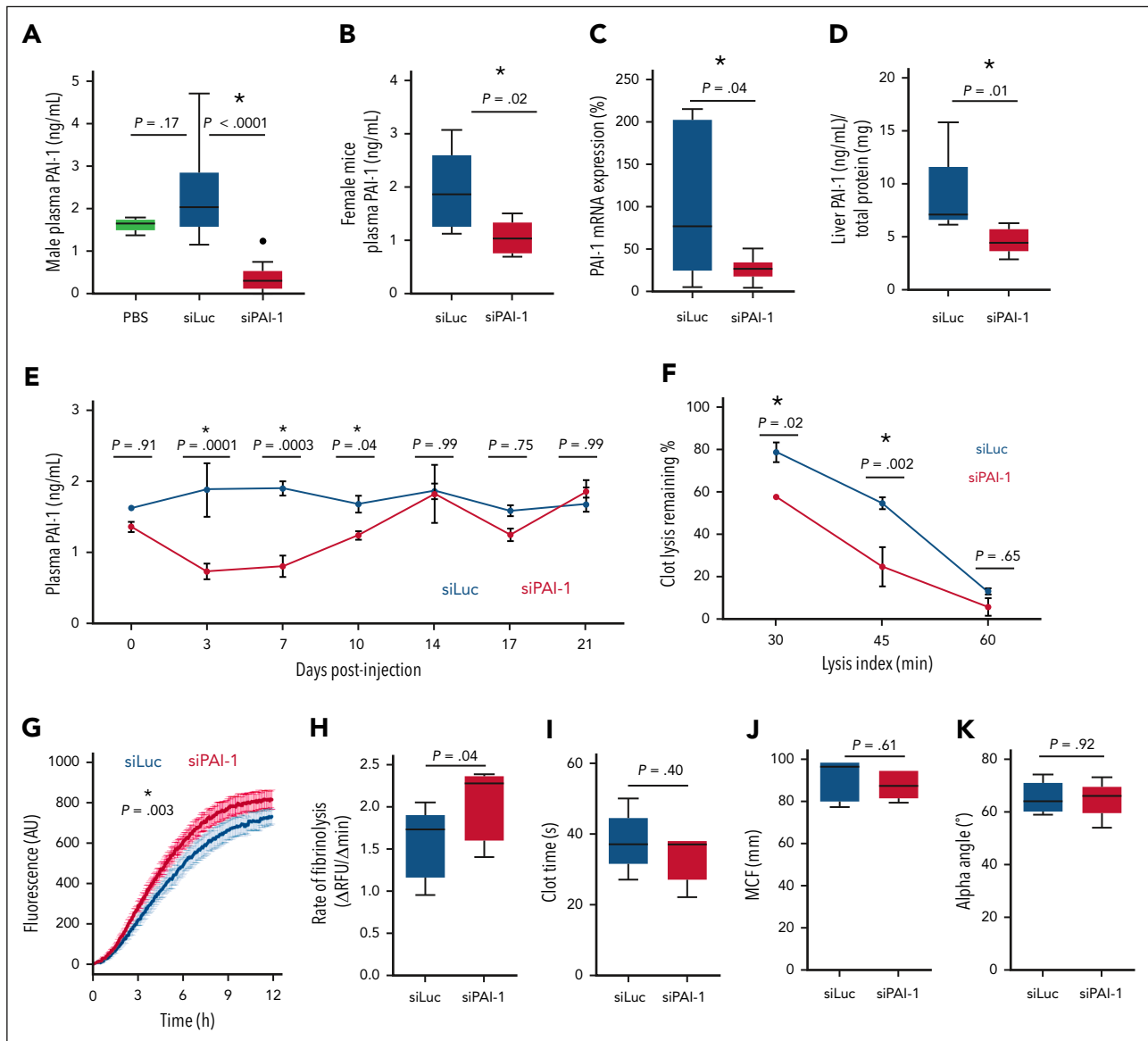


Figure 1. Circulating PAI-1 can be knocked down with siRNA-LNP in mice. Mice kept with standard animal care and housing were injected with either siPAI-1 (red), siLuc (blue) control or PBS (green). (A) Total plasma PAI-1 levels analyzed with an enzyme-linked immunosorbent assay (ELISA) in male mice. (B) Total plasma PAI-1 levels in female mice. (C) Hepatic PAI-1 mRNA levels standardized to the siLuc-injected male mice. (D) Hepatic PAI-1 protein normalized to the concentration of total hepatic protein 3 days after injection in male mice. (E) Total plasma PAI-1 protein levels analyzed at baseline (2 days before injection) and at 3, 7, 10, 14, 17, and 21 days after injection in a separate cohort of male mice. (F) Fibrinolysis in the presence of tPA assessed with ROTEM of the collected blood. (G) Cleavage of a fluorescent substrate by plasmin in clotted plasma with added tPA (1nM). (H) Rate of fibrinolysis, determined from data in panel G. (I-K) Coagulation assessed with ROTEM, measuring clot time (I), MCF (J), and α -angle (K). n = 3 to 15; * $P < .05$. MCF, maximum clot firmness.

used male mice.^{52,53} The relative amount of mRNA in the liver of mice injected with siPAI-1 ($26\% \pm 6\%$) was also significantly decreased compared with mice injected with siLuc ($100\% \pm 37\%$; $P = .04$; Figure 1C). PAI-1 protein concentration was significantly lower in hepatic tissue from siPAI-1-injected mice (4.6 ± 0.6 ng/mL) compared with siLuc-treated mice (8.7 ± 1.8 ng/mL; $P = .01$; Figure 1D).

To assess the duration of PAI-1 knockdown on siPAI-1 administration, mice were injected with siPAI-1 or siLuc and blood sampled at 3, 7, 10, 14, 17, and 21 days after injection. Plasma PAI-1 levels were significantly lower in siPAI-1-treated mice 3, 7, and 10 days after injection compared with the siLuc-treated control mice at each time point (Figure 1E). Mice injected with

siPAI-1 had similar PAI-1 levels to control siLuc mice at 14, 17, and 21 days after injection.

To test whether PAI-1 knockdown in mice altered coagulation and fibrinolysis, rotational thromboelastometry (ROTEM) was performed on whole blood from mice injected with siPAI-1 or siLuc. To assess fibrinolysis ex vivo, human tPA (500 ng/mL) was added. Blood from mice injected with siPAI-1 displayed faster fibrinolysis compared with siLuc-treated mice 7 days after injection. Measuring the percentage of clot remaining at 30 and 45 minutes after clot initiation (LI30 and LI45), siPAI-1-injected mice had an LI30 of $58\% \pm 1\%$ and an LI45 of $25\% \pm 9\%$, whereas, mice injected with siLuc had an LI30 of $79\% \pm 5\%$ ($P = .02$) and an LI45 of $55\% \pm 3\%$ ($P = .002$; Figure 1F).

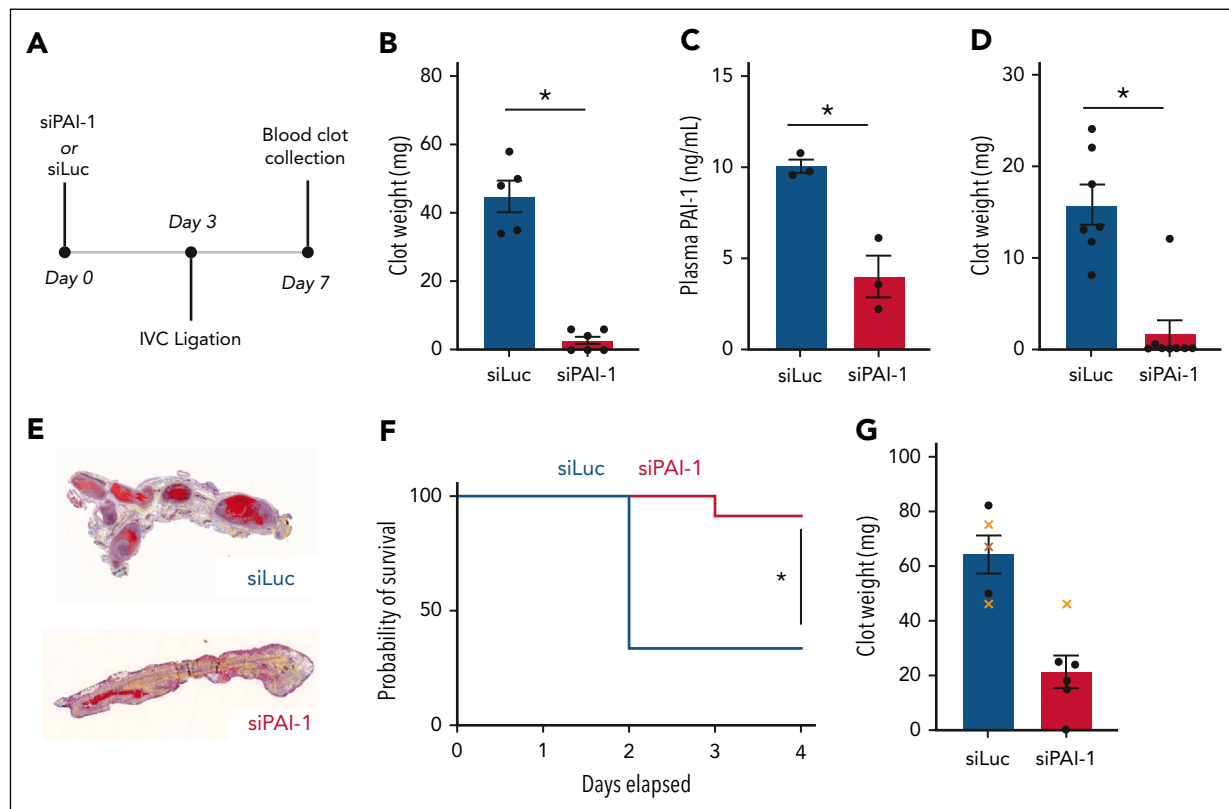


Figure 2. siPAI-1 decreases thrombosis in both young and aged mice and increases survival in aged mice in the IVC stasis model of venous thrombosis. (A) Workflow of the experiment, in which mice were injected with either siPAI-1 (red) or siLuc (blue) and then their IVCs were completely ligated after 3 days. Panel 2A was partially created with BioRender.com. Ferraresso F (2026) <https://BioRender.com/1vrh01f> (B) Weight of the clot and IVC segment in which the clot was present in young mice (8-10 weeks) 4 days after ligation. (C) Total plasma PAI-1 protein levels 4 days after ligation in young adult mice. (D) Weight of the clot in young mice (8-10 weeks) 4 days after ligation. (E) Representative trichrome stain and hematoxylin and eosin (H&E) stain cross-sectional images of the IVC 4 days after ligation. IVC slides were magnified x20. (F) Survival of aged mice (80-81 weeks). (G) Clot weight for aged mice 4 days after ligation (black dot) and clot weight for aged mice that died before 4 days (yellow cross). $n = 5$ to 8 ; $*P < 0.05$; error bars represent mean \pm standard error of the mean (SEM).

To determine the impact on plasmin generation, a plasmin-generation assay was performed using clotted blood plasma and measuring the cleavage of a fluorescent substrate for plasmin. Mice injected with siPAI-1 had significantly higher plasmin activity ($2.1 \pm 0.2 \Delta$ relative fluorescence units [RFU]/min) than siLuc-injected mice ($1.5 \pm 0.1 \Delta$ RFU/min; $P = .04$; Figure 1G-H). The blood coagulation profile was assessed for potential risk of altered coagulation after PAI-1 knockdown, including α -angle, clot time, and maximum clot firmness using ROTEM. No significant differences were observed between siPAI-1- and siLuc-injected mice in these parameters (Figure 1I-K).

siPAI-1 decreases venous thrombosis in both young adult and aged mice and increases survival rates in aged mice in a model of deep vein thrombosis

Due to the ability of siPAI-1 to enhance fibrinolysis, we assessed whether siPAI-1 could decrease thrombus formation. In both young adult and aged mice (9-10 and 80-82 weeks, respectively), the IVC was completely ligated 3 days after infusion of siPAI-1 or siLuc. The clot and the IVC segment containing the clot were collected 4 days after ligation (7 days after infusion; Figure 2A). Young adult mice injected with

siPAI-1 had significantly smaller thrombi (3 ± 1 mg of clot and affected IVC segment) than siLuc-injected mice (45 ± 10 mg; $P < .001$; Figure 2B) and significantly lower plasma PAI-1 concentrations at the end point (4 ± 1 ng/mL) than siLuc-injected mice (10 ± 0.4 ng/mL; $P = .03$; Figure 2C). Similarly, if only the clot was weighed, siPAI-1 (2 ± 2 mg) had significantly smaller thrombi than siLuc-treated mice (16 ± 2 mg; $P < .001$; Figure 2D). After extraction of the thrombi, the IVC were visualized with trichrome hematoxylin and eosin staining. Mice injected with siPAI-1 had little-to-no visual clots within the vessel (Figure 2E). No mortality occurred in young adult mice in the IVC thrombosis model.

In the aged mice, the siLuc-injected mice had a significantly lower chance of survival at 4 days after IVC ligation (33%) than mice injected with siPAI-1 (83%; $P = .02$; Figure 2F). Furthermore, 4 of the 6 siLuc-injected mice died between days 2 and 3 after ligation, whereas 1 of 6 siPAI-1-injected mice died 3 days after ligation. Due to the high mortality rate, blood samples were not collected for evaluation of PAI-1 knockdown. Thrombi were collected 4 days post-ligation for mice that survived to the study end point and within 6 hours from death for mice that died before the study end point. siPAI-1-injected mice developed smaller clots (16 ± 5 mg) than mice injected with siLuc (66 ± 16 mg; $P = .001$; Figure 2G).

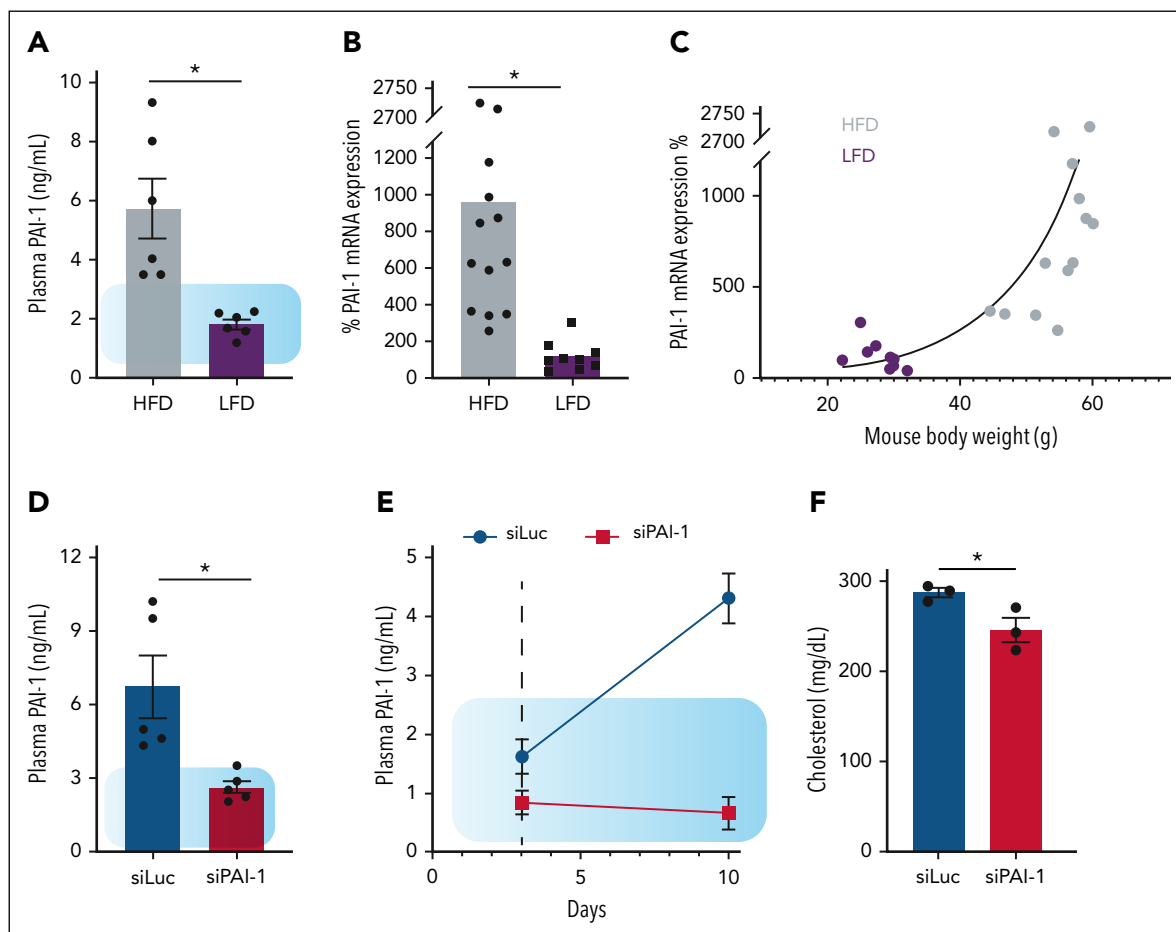


Figure 3. Hepatic PAI-1 mRNA expression is elevated in obese mice and can be normalized with siPAI-1. (A) Total plasma PAI-1 levels of mice on HFD or LFD. (B) Hepatic PAI-1 mRNA expression levels of mice on HFD or LFD. (C) Hepatic PAI-1 mRNA expression with respect to mouse body weight at end point. (D) Total plasma PAI-1 levels of wild-type (WT) obese mice on an HFD 7 days after receiving siLuc or siPAI-1. (E) Total plasma PAI-1 levels at days 3 and 10 of ApoE^{-/-} mice injected with siPAI-1 or siLuc twice (injections on days 0 and 7). The gray dotted line represents the initiation of a HFD (n = 6). (F) Cholesterol levels of ApoE^{-/-} mice 3 days after receiving siLuc or siPAI-1. The shaded blue area represents normal range of PAI-1.⁵⁷ n = 3 to 12; *P < .05; error bars represent mean ± SEM. HFD, high-fat diet; LFD, low-fat diet.

siPAI-1 normalizes pathologically high PAI-1 levels in obese and hypercholesteremic mice

PAI-1 levels are elevated in obesity, as is the risk of thrombosis. Thus, we explored whether siPAI-1 could knock down PAI-1 in obese mice, effectively evaluating the role of hepatic-derived PAI-1 in obesity. Circulating PAI-1 protein levels in wild-type mice fed a high-fat diet were significantly higher than mice fed a low-fat diet (5.8 ± 1 ng/mL vs 1.2 ± 0.1 ng/mL, respectively; $P = .003$; Figure 3A). Hepatic mRNA PAI-1 levels were also significantly higher in mice fed a high-fat diet when compared relative to mice fed a low-fat diet ($961\% \pm 229\%$; $P < .001$; Figure 3B). The hepatic mRNA PAI-1 levels in high-fat diet fed mice were exponentially correlated to the mouse body weight ($R^2 = 0.82$; Figure 3C). As the mRNA expression and circulating PAI-1 protein levels are significantly higher in obese mice, we tested whether siPAI-1 could still knock down circulating PAI-1 protein. Obese mice injected with siPAI-1 had significantly lower circulating PAI-1 levels (2.7 ± 0.3 ng/mL) than siLuc-injected mice (6.7 ± 3 ng/mL; $P = .01$; Figure 3D).

Due the ability of siPAI-1 to reduce circulating PAI-1 in obese mice, we evaluated the role of hepatic PAI-1 expression in proatherosclerotic mice (ApoE^{-/-}) to assess the siPAI-1 impact on

cholesterol levels. High cholesterol levels (>240 mg/dL) are a risk factor for thrombosis and other heart diseases, and a reduction in circulating cholesterol of ~15% significantly decreases the risk of cardiovascular diseases.⁵⁴ Mice were injected twice, 7 days apart, with either siPAI-1 or siLuc, and plasma was collected 3 days after each injection. LNP require apoE to transfect hepatocytes, so due to the apoE deficiency in these mice, LNP were incubated with human recombinant apolipoprotein E (apoE) before injection. 3 days after the first injection, mice were switched to a high-fat and high-cholesterol diet for the remainder of the study to induce the proatherosclerotic hypercholesteremic phenotype, which includes elevated cholesterol level. 10 days after the first injection, mice injected with siPAI-1 had significantly lower circulating PAI-1 levels (0.7 ± 0.3 ng/mL) than siLuc-injected mice (4.3 ± 0.4 ng/mL; $P < .001$; Figure 3E). There were significantly lower (14%) cholesterol levels in the siPAI-1-injected mice than siLuc-injected mice (246 ± 14 mg/dL vs 287 ± 5 mg/dL; $P = .02$; Figure 3F).

siPAI-1 sustainably knocks down elevated PAI-1 levels in aged mice and prolongs life span

To assess the feasibility of repeated injections of siPAI-1 and knockdown over time, aged mice (80–82 weeks) were injected

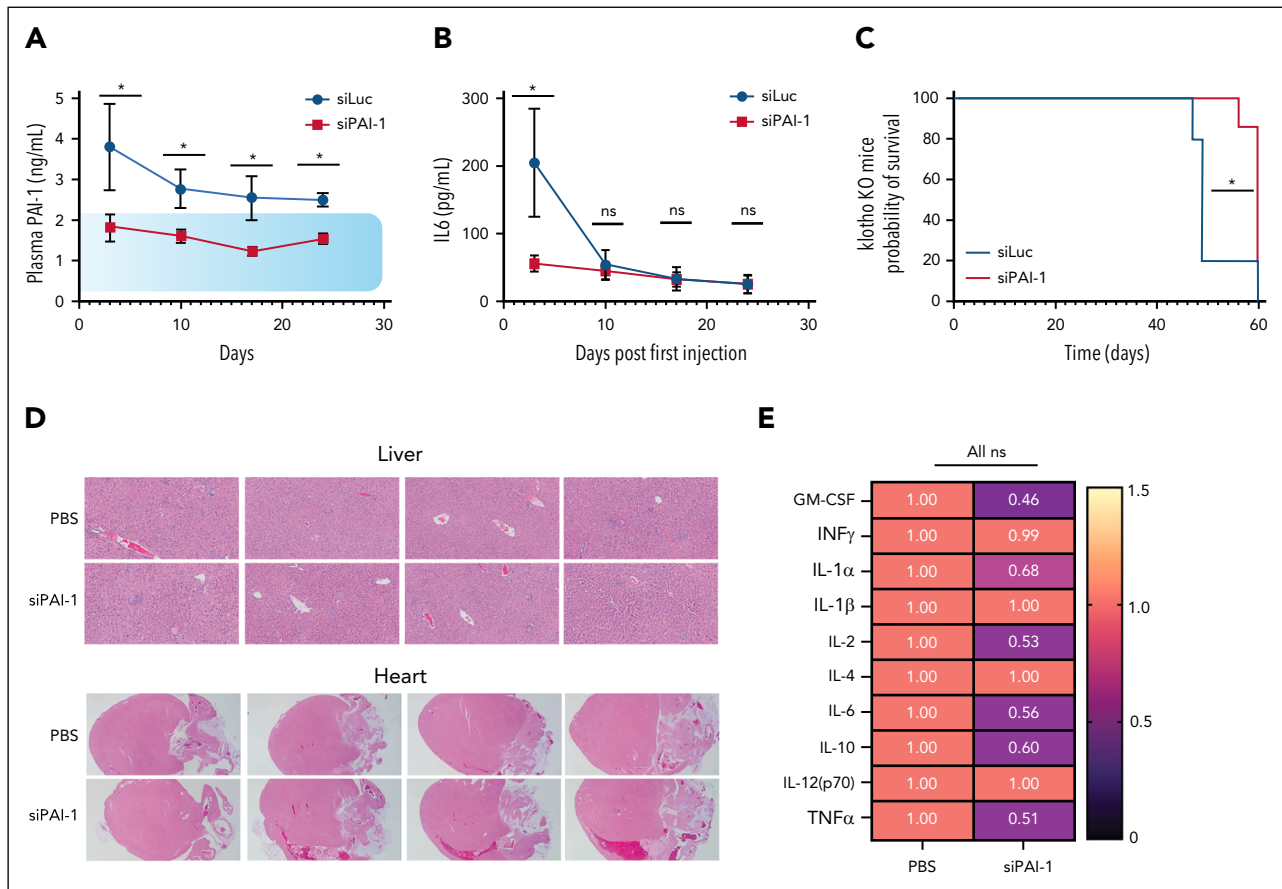


Figure 4. Repeated injections of siPAI-1 consistently knock down circulating PAI-1 levels and prolong life span in a fast-aging mouse model. (A-B) Aged WT mice (80–82 weeks) administered siPAI-1 or siLuc every 7 days. Total plasma PAI-1 (A) and serum IL-6 (B) 3 days after each injection. (C) Probability of survival of fast-aging *Klotho*^{-/-} mice injected every 7 days starting at the age of 3 weeks. (D-E) Aged WT mice (starting at the age of 70 weeks) injected every 7 days for 6 months. Images are H&E stains of the heart and liver at end point, in which each column is a separate mouse. The liver was magnified at $\times 10$ and the heart at $\times 1.5$ (D). Heat map shows representative serum cytokine levels standardized to the PBS-injected mice (E). The shaded blue area represents normal range of PAI-1.⁵⁷ $n = 4$ to 6; ns, * $P < .05$; error bars represent mean \pm SEM. GM-CSF, granulocyte-macrophage colony-stimulating factor; $\text{INF}\gamma$, interferon gamma; IL-1 α , interleukin 1 alpha; TNF- α , tumor necrosis factor alpha; KO, knockout; ns, not significant.

every 7 days for 1 month and plasma and serum were collected 3 days after each injection. siPAI-1-injected mice displayed consistently significantly lower PAI-1 concentrations compared with siLuc-injected mice. Throughout the month, mice injected with siLuc exhibited higher PAI-1 levels relative to siPAI-1-treated mice (Figure 4A).⁵⁵ In mice treated with siPAI-1, mean PAI-1 levels ranged from 1.22 ± 0.10 ng/mL to 1.81 ± 0.30 ng/mL, whereas in mice treated with siLuc, levels varied from 2.50 ± 0.20 ng/mL to 3.80 ± 1.10 ng/mL. Serum was assessed for potential off-target effects and immune activation. No differences were observed in interleukin-6 serum levels (Figure 4B) and the other 9 cytokine levels tested between siPAI-1- and siLuc-treated mice (data not shown).

Based on the ability of siPAI-1 to induce several beneficial changes on age-associated factors, such as thrombosis and cholesterol, and based on the known extended life span of partial PAI-1-deficient mice and humans, we assessed the potential of PAI-1 to prolonging life span in *Klotho*^{-/-} mice, a fast-aging mouse model.^{6,7,10,11} *Klotho*^{-/-} mice exhibit accelerated degeneration of multiple age-sensitive traits similar to humans, such as ectopic calcification, skin atrophy, muscle atrophy, osteoporosis, arteriosclerosis, and pulmonary emphysema.⁵⁶

Klotho^{-/-} mice were injected with siPAI-1 every 7 days starting at 3 weeks of age. Mice injected with siPAI-1 had a 20% longer life span ($P < .05$) than mice injected with siLuc (Figure 4C).

To assess long-term toxicity, mice were injected weekly for 6 months with siPAI-1 or PBS as control. At study end point, serum was collected to measure the concentration of 10 cytokines, and the heart and liver were collected for histological staining. A veterinary pathologist evaluated the histological stains of the heart and liver of mice treated with siPAI-1 and PBS, and no pathologic abnormalities were observed between the treatment groups (Figure 4D). There were no significant differences in the cytokine levels between siPAI-1- and PBS-treated mice (Figure 4E). siPAI-1-treated mice had overall lower cytokine levels than PBS-treated mice. No cardiac fibrosis was observed in the heart samples collected from siPAI-1-treated mice.

siPAI-1 does not elicit overt toxicity in vivo

We evaluated acute and fundamental markers of toxicity to begin understanding the therapeutic potential of siPAI-1. To assess acute toxicity, we analyzed the levels of alkaline

phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase, and blood urea nitrogen 5 hours post-injection in young adult mice. No significant changes were observed between siPAI-1- and PBS-injected mice (Figure 5A-D; $P = .42$, $P = .26$, $P = .17$, and $P = .89$, respectively). Although aspartate aminotransferase and ALT levels of some mice were comparatively higher than control mice receiving PBS, they were within or near normal ranges. There were no increases in other toxicity markers, such as albumin, bile acids, creatinine, total bilirubin, blood urea nitrogen, γ -glutamyl transferase, and globulin, suggesting no overt toxicity (data not shown). To assess hepatocellular injury, ALT levels were assessed 1, 3, and 7 days after injection. No significant changes were observed between siPAI-1- and siLuc-injected mice, and ALT levels

remained within normal ranges (Figure 5E).⁵⁷ White blood cell count, red blood cell count, and platelet count were analyzed 7 days after injection and were all within normal range and comparable to PBS-injected mice (Figure 5F-H). To assess the biodistribution of PAI-1 knockdown, PAI-1 protein was measured in several nonhepatic tissues that express PAI-1, the heart, vein (IVC), a large vein (IVC), and white fat. Mice treated with siPAI-1 (11 ± 2 ng/mL; PAI-1 per mg total protein) had significantly lower PAI-1 levels in the IVC than siLuc-treated mice (23 ± 5 ng/mL PAI-1 per mg total protein; $P = .002$), but no changes in the heart and adipose tissue (Figure 5I). Low to undetectable PAI-1 levels were observed in the adipose tissue across both treatment groups, likely due to the young age and low body weight of the mice.

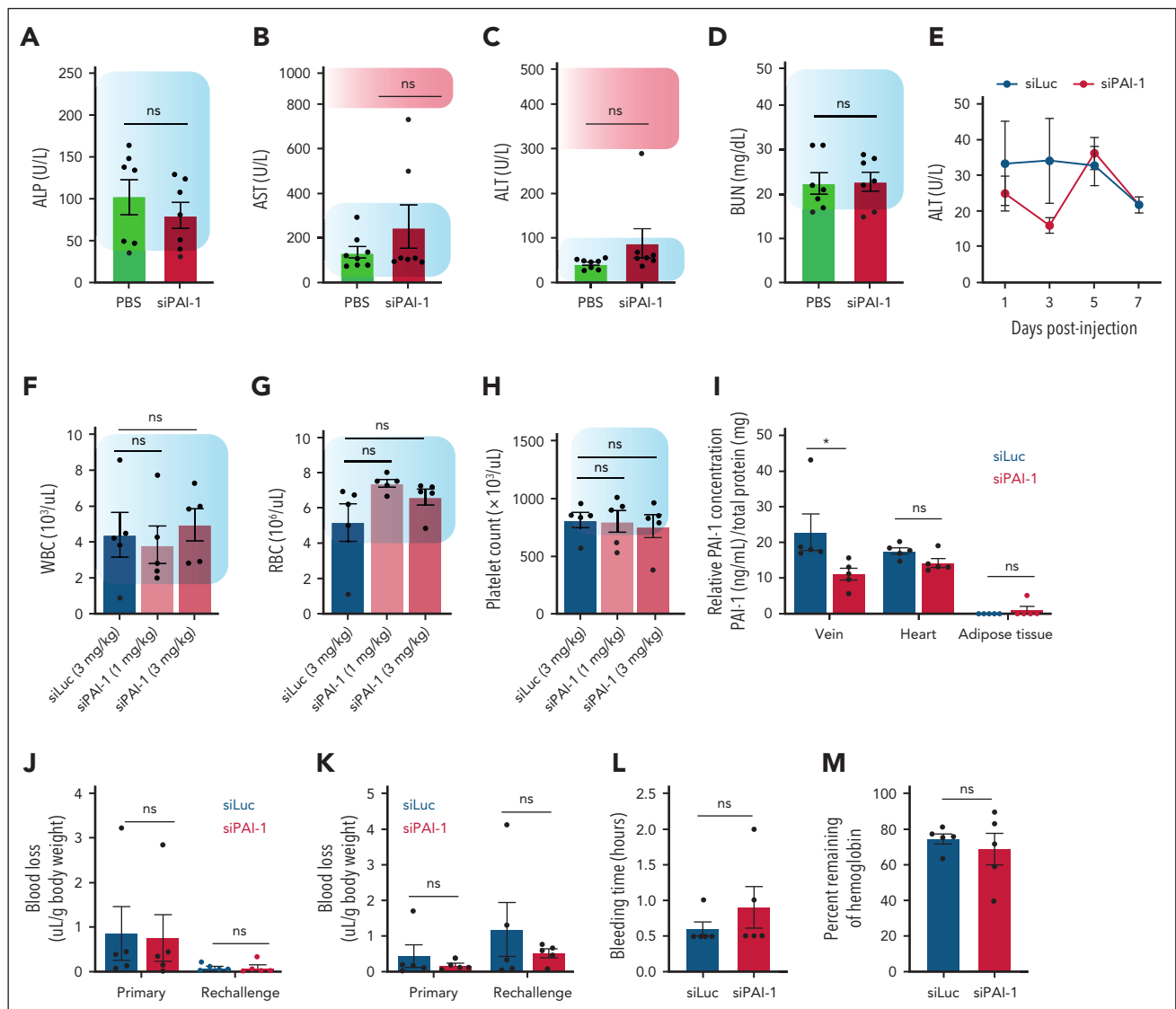


Figure 5. PAI-1 knockdown with siPAI-1 does not elicit overt toxicity or increased bleeding in mice. (A-D) Mice receiving an injection of siPAI-1 (red) or PBS (green) as a control, measuring serum levels of ALP (A), AST (B), ALT (C), and BUN (D) 5 hours after injection. (E) Serum ALT levels of mice injected with siPAI-1 or siLuc (blue) at 1, 3, and 7 days after injection. (F-H) Blood cell counts of mice that received an injection of siPAI-1 or siLuc at either 1 or 3 mg/kg, including WBC (F), RBC (G), and platelets (H) measured 5 hours after injection. (I) The relative amount of PAI-1 protein in a vein (IVC), heart, and adipose tissue, normalized to the concentration of total hepatic protein, 3 days after injection. (J-M) Bleeding assessments in mice treated with siPAI-1 or siLuc 3 days after injection. Primary and rechallenge blood loss of mice subjected to TTT (J) and TTT (K); bleeding time (L) and percentage of hemoglobin remaining (M) after a 6-hour tail-bleeding injury model. The shaded blue area represents normal range of biomarker, and the shaded red area represents toxic range. $n = 5$ to 7 ; $*P < .05$; error bars represent mean \pm SEM. ALP, alkaline phosphatase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; ns, not significant; RBC, red blood cell; WBC, white blood cell.

To assess the bleeding risk associated with knocking down PAI-1 in mice, 3 bleeding injury models were used: TVT injury, TTT, and a 6-hour tail-bleeding test. In both the TVT and TTT injury models, there were no significant differences in blood loss or bleeding time between siPAI-1- and siLuc-treated mice during either the primary bleed or a rechallenge bleed (Figure 5J-K). In the 6-hour tail-bleeding challenge, there was no statistical difference in bleeding time between siPAI-1-treated (0.9 ± 0.3 hours) and siLuc-treated (0.6 ± 0.1 hours; $P = .35$) mice. In addition, there was no significant difference in the change in circulating hemoglobin concentration. siPAI-1-treated mice had $68\% \pm 9\%$ of their baseline hemoglobin at the end of the 6-hour tail-bleeding challenge and siLuc-treated mice had $74\% \pm 3\%$ ($P = .56$; Figure 5L-M).

Discussion

These results demonstrate that after an IV injection of siPAI-1, circulating PAI-1 concentrations significantly decrease with no signs of overt toxicity. In the last 2 decades, many attempts have been made to develop an in vivo PAI-1 inhibitor.⁵ siPAI-1 represents, to our knowledge, the first approach that provides feasible long-term in vivo inhibition of PAI-1. Knocking down PAI-1 at the RNA level resulted in reduced circulating PAI-1 activity and antigen concentrations. siPAI-1 can therefore be applied to study the role of PAI-1 in various PAI-1-related diseases. The impact of siPAI-1 on fibrinolysis was evaluated using ROTEM with whole blood and a plasmin-generation assay with plasma. In the ROTEM experiments, supra-physiologic concentrations of tPA (500 ng/mL) were used to generate a clot and subsequently achieve fibrinolysis. Consistent with these results, the plasmin-generation assay performed on platelet-poor plasma using physiologically relevant concentrations of tPA also confirmed a difference in fibrinolysis between treatment groups. siRNA-LNP agents have the advantage that they primarily deliver their cargo to the liver, thus enabling the study of hepatic PAI-1 contribution.⁵⁸ We observed lower PAI-1 levels in the IVC of siPAI-1-treated mice, but we do not believe that this reflects direct LNP delivery to the vessel and inhibition of PAI-1 synthesis in endothelial cells. The reduction in protein content in the IVC may be due to reduced scavenging of circulating PAI-1 that was produced in hepatocytes. Circulating PAI-1 can complex with uPA, which is internalized through the uPA receptor on endothelial cells.⁵⁹ However, future studies will be required to investigate this mechanism. Aside from the IVC vessel, other nonhepatic tissues known to synthesize PAI-1, such as the adipose tissue and the heart, demonstrated no reduction in PAI-1 protein levels after siPAI-1 administration. Although we do not anticipate significant changes in biodistribution over time after administration compared with siRNA-LNP that are used clinically,⁶⁰ additional studies will be required to characterize siRNA clearance and biodistribution pharmacokinetic profiles.

The relationship between the site of PAI-1 synthesis and disease is unknown.⁶¹ One aspect of our study evaluated the relationship between hepatic PAI-1 and diet-induced obesity, prevalent in older populations. The increase in PAI-1 expression in obesity has been assumed to be due to expression in adipose tissue, although hepatic PAI-1 concentrations in obese mice were statistically higher compared with normal mice. The hepatic PAI-1 mRNA expression was correlated to the mouse

body weight, thus implicating a hepatic PAI-1 contribution to the increased circulating levels of PAI-1 in obesity. These results are alike humans in which PAI-1 mRNA expression from liver biopsies correlated with the person's body mass index but not with the PAI-1 mRNA expression in the adipose tissue.⁶² However, the adipose tissue could still contribute to the rise in PAI-1 concentrations in obesity, because the knockdown in obese mice is lower than in young healthy mice.

Because atherosclerosis is an age-associated disease closely related with obesity, we evaluated whether siPAI-1 normalizes circulating PAI-1 levels in a proatherosclerotic mouse model, ApoE^{-/-} mice on a Western diet. The potential benefits of lowering PAI-1 levels in proatherosclerotic mice were observed in circulating cholesterol levels, because siPAI-1-injected mice had significantly lower cholesterol levels than controls. These findings are consistent with the mechanism that PAI-1 sequesters tPA in hepatocytes, thus permitting lipidation of apolipoprotein B and secretion of very-low-density lipoprotein in the blood, which in turn increase cholesterol levels.⁶³

This study applied siPAI-1 to determine the role of PAI-1 in an adult and aged mouse IVC thrombosis model 4 days after ligation. Similar to mice in ferric chloride thrombosis models previously tested with oral PAI-1 inhibitor,⁵³ the clots were smaller in adult mice injected with siPAI-1 compared with the controls.⁶⁴ Future studies will need to determine the temporal role of PAI-1 in clot resolution in the context of partial PAI-1 knockdown. In aged mice, the survival of the mice after ligation was significantly improved with PAI-1 knockdown. Two days after ligation is the transition point between acute and chronic thrombosis, when the thrombus burden is the highest. All the mice that died before the study end point also died during this transition period. This transition causes an increase in inflammation that may have contributed to the early death of the mice. The relatively smaller thrombus burden combined with the inhibition of PAI-1-driven expression of inflammatory mediators potentially contributed to the increased survival of the siPAI-1-injected mice. Because the control mice had significantly decreased survival after ligation, there is a potential for survivor bias when assessing the thrombosis measurements. The magnitude of thrombus reduction observed by siPAI-1 in our study is greater than that reported in previous studies using PAI-1 knockout mice. These differences may reflect compensatory mechanisms that develop in germ line knockouts, particularly given that PAI-1 is synthesized by various cell types. By specifically targeting hepatic PAI-1 with siPAI-1, we preserve contributions from other sources of PAI-1, which may be acting through distinct mechanisms. In addition, IVC ligation can alter hepatic blood flow, potentially affecting coagulation and fibrinolytic gene expression and thereby influencing clot resolution, which may differ between complete PAI-1 knockout and partial hepatic PAI-1 knockdown. Future studies will have to investigate mechanistic differences between genetic knockout of PAI-1 and partial PAI-1 knockdown. Furthermore, it remains unclear whether platelets acquire PAI-1 exclusively from megakaryocytes during their formation or also scavenge it from the circulation. Platelet-associated PAI-1 levels vary substantially across species, and many mouse strains, including C57BL/6J, exhibit significantly lower levels than in humans.⁶⁵ Future investigations in large animal models will be required to define the contribution and

regulation of platelet-derived PAI-1 after siPAI-1 administration. Because PAI-1 plays a role in senescence and proliferation, humans who have low levels of PAI-1 on average live 10% longer and are protected from cardiovascular morbidity. The potential of siPAI-1 was evaluated in a fast-aging mouse model, *Klotho*^{-/-} mice.¹⁰⁻¹² siPAI-1 injected mice lived 20% longer compared with control mice, thus providing proof of concept that siPAI-1 has an effect in aging. PAI-1 is a key mediator of p53 and interleukin-6 signaling, implicating it in the regulation of senescence and age-associated inflammation.¹² However, additional studies will be required to elucidate the specific mechanisms by which siPAI-1 contributes to extending life span.

Similar to humans, mice have significant sex differences in endogenous PAI-1 concentrations, thus not allowing direct comparisons between sexes.⁶⁶ Treatment with siPAI-1 achieved significant knockdown of circulating PAI-1 concentrations in both male and female mice; however, female mice exhibited a greater interanimal variability. Females have much greater variability of circulating PAI-1 than males.^{52,53} To minimize experimental variability, all subsequent studies were performed in male mice.

Furthermore, in this study, we assessed bleeding phenotype associated with decreased PAI-1 concentrations in 3 mouse bleeding injury models. Although there were no detectable differences in bleeding time or blood loss between mice treated with siPAI-1 or siLuc, future studies will investigate the safety and tolerability of siPAI-1 in large animal models, such as canine or swine bleeding models that have more similarities to the fibrinolysis system in humans.^{38,67} Although humans with complete PAI-1 deficiency exhibit excessive bleeding, we do not expect siPAI-1 to cause a bleeding phenotype because ~1 ng/mL is considered sufficient to maintain hemostasis.^{18,68} Furthermore, because siPAI-1 does not modulate PAI-1 protein levels in the heart or fat of mice, circulating concentrations are unlikely to drop below 1 ng/mL. Furthermore, we evaluated long-term toxicity by administering weekly injections of siPAI-1 or PBS to mice for 6 months. No pathologic abnormalities were detected in the liver or heart, and no signs of inflammation were observed in the cytokine analysis. Future studies will have to be performed to evaluate long-term tolerability in larger animal models.

siPAI-1 represents a mechanistically distinct approach compared with existing small-molecule and antibody-based PAI-1 inhibitors, such as TM5614 and MDI-2517. Whereas these agents transiently modulate PAI-1 activity for ~6 hours, siPAI-1 acts at the mRNA level, enabling a sustained and long-term reduction in PAI-1 expression for up to 10 days. Advances in siRNA delivery may enable the duration of knockdown to be extended even further in the future.⁶⁹ Current clinical PAI-1 inhibitors may reduce PAI-1 levels by ~60% to 80%, which is comparable to the reduction observed with siPAI-1. Furthermore, the thrombosis outcomes we observed with siPAI-1 are consistent with those with existing PAI-1 inhibitors.^{16,70,71}

In conclusion, PAI-1 concentration increases in various age-associated diseases, and siPAI-1 can be administered to decrease this elevation, normalizing the concentration. Decreasing PAI-1 levels could potentially have significant benefits for older adults, both by decreasing thrombosis and enhancing healthy aging. Furthermore, siPAI-1 can be applied as a research tool to address knowledge gaps of the role of PAI-1 in

pathophysiologic processes. Overall, siPAI-1 has potential as both a research tool and a therapeutic for a wide range of diseases.

Acknowledgments

This work was supported by the American Heart Association (Collaborative Science Award, 952422), the National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH) (grant R01HL166382), and the Canadian Institutes of Health (doctoral award 187577 [F.F.]; postdoctoral fellowships MFE181897 [W.S.H.] and MFE414357 [C.L.]). This research was also supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases, NIH (grant R01DK135649 [J.P.L.]) and the NHLBI/NIH (grant R01HL102035 [Q.S.]), support from the US Department of Agriculture National Institute of Food and Agriculture, and the Albert C. and Lois E. Dehn Endowment to Michigan State University for Veterinary Medicine (Pathobiology and Diagnostic Investigation [J.P.L.]). This research was also supported by the Shared Resource Facilities of the Versiti Blood Research Institute, Milwaukee, Wisconsin; the University of British Columbia Centre for Blood Research, Vancouver, Canada; and the Histology Core at Children's Hospital of Wisconsin, Milwaukee, Wisconsin.

Authorship

Contribution: F.F. designed and performed experiments, analyzed and interpreted data, made the figures, and wrote the initial draft of the manuscript; C.W.S., W.S.H., M.S., T.H.Y.C., Z.W., W.D., L.M.K., H.L., G.G.R., C.L., and Y.Z. designed and performed experiments, analyzed and interpreted data, and edited the manuscript; L.J.J. and A.W.S. provided intellectual input on hypothesis and experimental design and edited the manuscript; P.R.C., M.R.D., J.P.L., M.J.F., Q.S., H.Y., A.L.G., M.P., and Z.Z. helped design the experiment, interpreted data, and edited the manuscript; and C.J.K. designed experiments, interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure: C.J.K., P.R.C., A.W.S., F.F., and L.J.J. are directors, shareholders, and/or cofounders of companies developing RNA therapies, including Syrina Therapeutics Inc (F.F., C.J.K., and P.R.C.), SeraGene Therapeutics, Inc (C.J.K., P.R.C., A.W.S., and L.J.J.), NanoVation Therapeutics, Inc (C.J.K. and P.R.C.), and Acuitas Therapeutics, Inc (P.R.C.). Z.Z., W.D., and H.L. are shareholders and cofounders of Milwaukee Therapeutics Inc. C.J.K., P.R.C., F.F., Z.Z., W.D., H.L., A.W.S., and L.J.J. have filed intellectual property on RNA-based therapies with the intention of commercializing these inventions. The remaining authors declare no competing financial interests.

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Footnotes

Submitted 7 May 2025; accepted 21 December 2025; prepublished online on *Blood* First Edition 26 January 2026. <https://doi.org/10.1182/blood.2025029834>.

Data are available from the corresponding author, Christian J. Kastrup (ckastrup@versiti.org), on request.

The online version of this article contains a data supplement.

There is a [Blood Commentary](#) on this article in this issue.

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