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Reversing PAI-1 deficiency in blood using mRNA lipid nanoparticles

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Plasminogen activator inhibitor-1 (PAI-1) deficiency is a rare disorder that causes moderate to severe bleeding and cardiac fibrosis, caused by mutation in the *SERPINE-1* gene and no detectable circulating PAI-1 protein. There are currently no therapies that can effectively replace PAI-1 because the protein has a short half-life. An alternative approach to using recombinant protein is to endogenously increase circulating PAI-1 levels using mRNA therapy. Delivering mRNA encoding PAI-1 to the liver, a major site of PAI-1 synthesis, using lipid nanoparticles (mPAI-1) is a potential approach to increase circulating PAI-1 protein. Here, we developed mPAI-1, which induced expression of PAI-1 *in vivo* upon intravenous administration. In both wild-type (WT) mice and PAI-1 knockout mice, mPAI-1 induced supraphysiological circulating PAI-1 and inhibited fibrinolysis when measured *ex vivo*. In WT mice, plasma PAI-1 levels increased in a dose-dependent manner between 0.1 and 1 mg of mRNA per kg of body weight, peaking at 6 h post-injection and returning to baseline by 48 h. There was consistent production of PAI-1 after repeat dosing of mPAI-1 in the same mice. Expression of PAI-1 using mRNA-based approaches has the potential to be a preventive therapy for bleeding and cardiac fibrosis for PAI-1-deficient patients.

INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) deficiency is a rare genetic disorder characterized by impaired regulation of fibrinolysis, which leads to abnormal bleeding.[1] PAI-1 protein contributes to blood clot stabilization during hemostasis by creating a complex with tissue plasminogen activator (tPA), inhibiting tPA-mediated fibrinolysis.[2,3] PAI-1 was initially identified in the early 1980s, and the first patient with a bleeding diathesis caused by PAI-1 deficiency was reported in 1989.[2] The phenotype was subsequently studied in a large family of Old Order Amish descent in Indiana in which PAI-1 deficiency causes excessive bleeding, especially following trauma or surgery.[1,2] Additional cases have been reported in North America, Europe, and Asia, but the prevalence of PAI-1 deficiency remains largely unknown as diagnosis is complicated by both its rarity and the limitations of current laboratory testing.[2] Diagnosis of PAI-1 is complicated because it has a low concentration in blood of 5–20 ng/mL and accurate assays for PA1-1 activity are lacking.[1,2,4] PAI-1 has a diurnal variation with higher values in the morning and nadir in the afternoon.[5] Diagnosis is determined when PAI-1 activity and antigen levels are low to undetectable (below 1 IU/mL), are accompanied by a bleeding phenotype, and a pathogenic mutation in *SERPINE1* is found.[1,3]

While PAI-1 deficiency is inherited equally by both sexes in an autosomal recessive pattern, diagnosis is more frequent in females due to menorrhagia and complications during pregnancy and childbirth.[3] Patients with PAI-1 deficiency also experience other mild to moderate bleeding symptoms such as easy bruising with hematomas, and excessive bleeding post trauma and post medical procedures.[1] Most bleeding events can be prevented or managed with short-acting antifibrinolytic agents such as tranexamic acid (TXA); however, the treatment regimen requires frequent administration of approximately 1 g of TXA three times per day.[1] Many people cannot maintain this regimen for the long periods required to sustain therapeutic levels for prophylactic use. Patients with PAI-1 deficiency may also experience life-threatening bleeds including joint and soft tissue bleeding events, intracranial hemorrhage, and internal bleeding following surgery or trauma.[1] Severe bleeds commonly require fresh-frozen plasma transfusions to increase PAI-1 activity or intravenous tranexamic acid to rebalance fibrinolysis.[1] In addition to bleeding complications, PAI-1 deficient patients can develop cardiac

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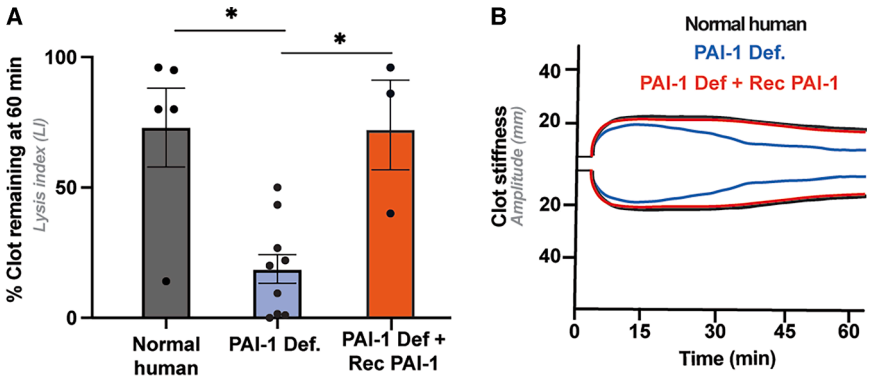

Figure 1. Excessive fibrinolysis in human PAI-1-deficient plasma can be corrected with recombinant PAI-1 protein

(A) The percentage of clot remaining (also known as lysis index, LI) at 60 min (LI60) in normal human plasma (black), in PAI-1-deficient human plasma (blue) or PAI-1-deficient plasma with recombinant PAI-1 (400 ng/mL, red). (B) Representative ROTEM curves of data in (A), showing clots forming and lysing in the presence of tPA (180 ng/mL). n = 4–9, $^*p < 0.05$, ns, not significant. Error bars represent mean ± SEM.

fibrosis, which may result in heart failure.[6,7] Cardiomyocyte PAI-1 plays a critical role in regulating the balance between degradation and deposition of extracellular matrix (ECM) proteins.[8] Deficiency in PAI-1 protein leads to excess ECM protein deposition, resulting in cardiac muscle scarring and stiffening of the ventricular walls.[8] There is currently no targeted preventive treatment for cardiac fibrosis in PAI-1 deficiency, contributing to the early mortality rates of these patients. There has been one death reported due to sudden heart failure in a young man with complete PAI-1 deficiency and cardiac fibrosis.[6]

Recombinant PAI-1 protein has been investigated as a therapeutic option to address hematological and cardiac complications in PAI-1 deficiency, but its short half-life of approximately 30 min limits its potential for prophylactic use.[9] Furthermore, bolus intravenous injection of a high amount of recombinant PAI-1 treatment may pose a risk of inducing thrombosis due to the initial peak concentration following administration.[9] An alternative approach to recombinant PAI-1 treatment is to endogenously increase circulating levels of PAI-1 through hepatic-targeted mRNA therapy, which could be longer acting with a more stable pharmacokinetic profile. mRNA encapsulated in lipid nanoparticles (LNPs) represents a promising class of novel therapeutics that can modulate blood coagulation by leveraging the liver as an endogenous bioreactor for protein expression.[10] LNPs are a viable delivery system for RNA, as shown by Food and Drug Administration (FDA)-approved applications such as ONPATTRO, the first small interfering RNA (siRNA) LNP therapy, and the COVID-19 mRNA vaccines.[11–13] LNPs injected intravenously naturally accumulate in the liver, a major organ for synthesis of most coagulation factors, including PAI-1.[14–17] The use of LNPs to deliver mRNA encoding PAI-1 offers a potential solution to the short-lived effects of PAI-1 protein by providing sustained protein expression and therapeutic benefit. Current mRNA therapeutic approaches express protein for 0.5–2 days in the liver, but longer-acting forms of mRNA are being developed that may extend the duration of efficacy in the future.[10,17–19] An mRNA PAI-1-LNP may offer a more effective treatment for bleeding episodes and could also represent a viable treatment option for managing cardiac fibrosis in these patients, offering long-term benefits with repeat dosing.

RESULTS

Excessive fibrinolysis in human PAI-1-deficient plasma can be corrected by the addition of recombinant PAI-1 protein

To determine whether protein replacement with mRNA is a viable therapeutic approach for PAI-1-deficient patients, we first tested if the excessive fibrinolytic characteristics of PAI-1-deficient plasma can be overcome with recombinant PAI-1 protein. We tested this in blood *ex vivo*, where limitations in the circulation time of PAI-1 recombinant protein is less relevant. Fibrinolysis was examined using rotational thromboelastometry (ROTEM), which measures viscoelastic properties in blood to assess clot formation and lysis.[20] Normal and PAI-1-depleted human plasma were analyzed *ex vivo* with ROTEM with the addition of tPA. In the absence of tPA no fibrinolysis was expected, and PAI-1-depleted plasma and normal human plasma exhibited no fibrinolytic differences within 60 min following the initiation of the clot, as measured by the lysis index at 60 min after initiation of clotting (LI60). In the presence of tPA, PAI-1-depleted plasma had significantly higher fibrinolysis (20% ± 6% LI60) compared with normal plasma (73% ± 15% LI60, $p < 0.05$), whereas PAI-1-depleted plasma spiked with recombinant PAI-1 protein (400 ng/mL) corrected this difference (72% ± 17% LI60, $p < 0.05$ compared with PAI-1-depleted plasma without recombinant protein) (Figures 1A and 1B). While one replicate of normal human plasma had high fibrinolysis, we expect this was due to variability in the fibrinolysis assay, as pooled normal plasma was used in each replicate.

Circulating PAI-1 levels can be increased with mRNA-LNP in mice

We examined whether mRNA encoding PAI-1 encapsulated in LNPs can overexpress PAI-1 in the liver and increase plasma PAI-1 concentrations. The PAI-1 coding sequence was uridine depleted, and mRNA was encapsulated in LNPs. Wild-type (WT) mice were intravenously injected with either mRNA PAI-1 LNP (mPAI-1) at 0.5 mg of mRNA per kg of mouse body weight (mg/kg) or PBS as a vehicle control. Plasma was analyzed for total protein PAI-1 levels 24 h post-injection (Figure 2A). Mice treated with mPAI-1 had significantly higher PAI-1 levels compared with mice treated with PBS (7.14 ± 0.9 ng/mL mPAI-1 vs. 1.5 ± 0.1 ng/mL, $p < 0.05$).

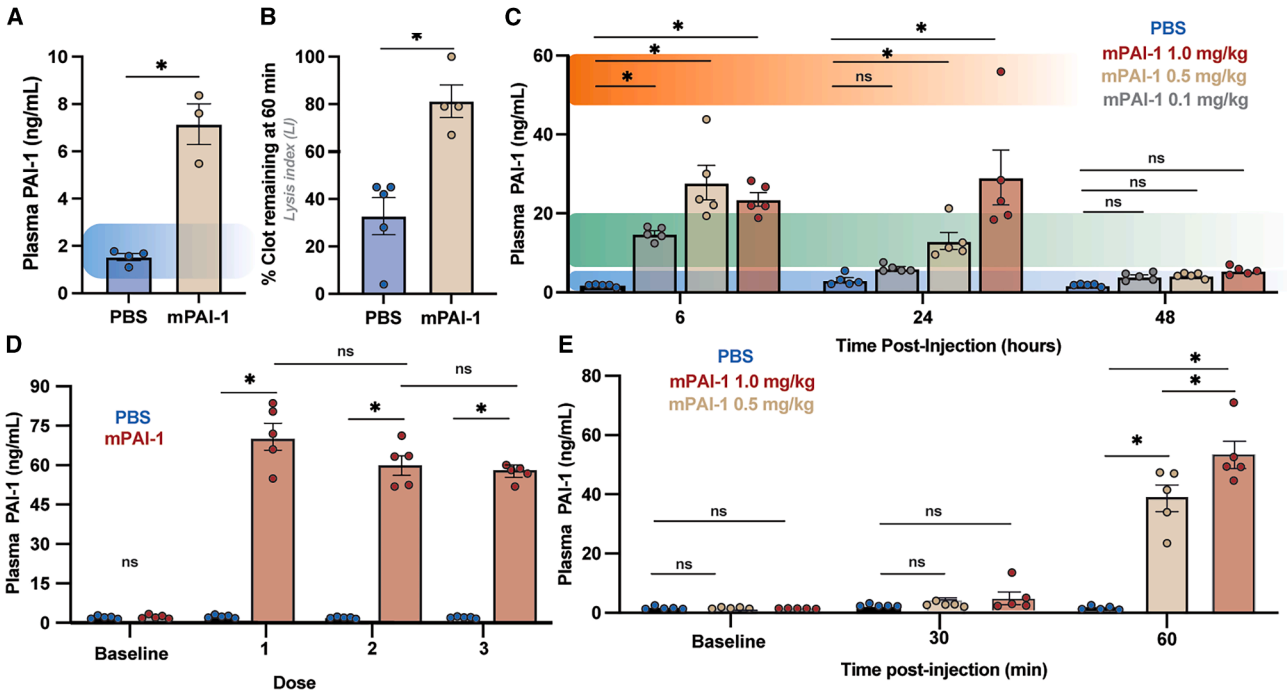

Figure 2. Increased expression of circulating PAI-1 in mice following intravenous injection of mPAI-1

(A) WT mouse plasma PAI-1 concentrations following administration of mRNA PAI-1 LNP (mPAI-1) at 0.5 mg/kg or 0 mg/kg (PBS) 24 h post-injection. Healthy normal mouse PAI-1 levels indicated by blue shaded region.[21] (B) Percentage of clot remaining, measured by ROTEM in the presence of tPA, in blood collected from mice administered PBS or mPAI-1 at 0.5 mg/kg 24 h post-injection. (C) WT mouse plasma PAI-1 concentrations following administration of PBS (blue) or mPAI-1 at 0.1 mg/kg (gray), 0.5 mg/kg (brown), or 1 mg/kg (red) at 6, 24, and 48 h. Healthy normal mouse PAI-1 levels indicated by blue shaded region, healthy human PAI-1 levels indicated by green shaded region, and prothrombotic human PAI-1 levels indicated by red shaded region. (D) Mice were injected with mPAI-1 or saline every 7 days for 3 weeks. Plasma PAI-1 concentrations in WT mice 6 h post-injection of mPAI-1 (red) or PBS (blue). (E) WT mouse plasma PAI-1 concentrations 30 and 60 min post-injection of PBS (blue) or mPAI-1 at 0.5 mg/kg (brown), or 1 mg/kg (red). n = 4–5 mice, $^*p < 0.05$, ns, not significant. Baseline = 2 days before injection. Error bars represent mean ± SEM.

To test if increasing circulating PAI-1 concentrations in mice alters fibrinolysis, whole blood from WT mice treated with mPAI-1 was assessed by ROTEM. Blood from mice treated with mPAI-1 had significantly less fibrinolysis (LI60 of 81% ± 7%) compared with mice treated with PBS (33% ± 8%, $p < 0.05$) (Figure 2B).

To assess the longevity and dosing regimen of mPAI-1, mice were injected with mPAI-1 at 0, 0.1, 0.5, or 1 mg/kg dose and plasma PAI-1 protein concentrations were assessed 6, 24, and 48 h post-injection (Figure 2C). Mice treated with mPAI-1 at 0.5 and 1 mg/kg had significantly higher PAI-1 levels at 6 h (28 ± 4, 24 ± 2 ng/mL, $p < 0.05$) and 24 h (13 ± 2 ng/mL, 29 ± 7 ng/mL, $p < 0.05$) post-injection compared with mice treated with PBS (1.7 ± 0.1, 3 ± 0.6 ng/mL, respectively). Mice treated with mPAI-1 at 0.1 mg/kg had significantly higher PAI-1 levels 6 h post-injection (15 ± 1 ng/mL, $p < 0.05$) compared with PBS, but similar levels at 24 h post-injection. All treated mPAI-1 mice had similar PAI-1 levels at 48 h post-injection compared with mice treated with PBS.

To assess the feasibility of repeat injections of mPAI-1, mice were injected every 7 days for a 3-week period with mPAI-1 at 1 mg/kg or saline as a control. Plasma was collected 6 h following each injection. The circulating PAI-1 levels were similar after each dose (dose 1: 70 ± 5 ng/mL, dose 2: 60 ± 4 ng/mL, dose 3: 59 ± 1 ng/mL; $p > 0.05$) (Figure 2D). Mice treated with mPAI-1 had significantly higher PAI-1 levels compared with mice treated with saline after each dose (Figure 2D).

To determine the feasibility of mPAI-1 as a potential treatment for acute breakthrough bleeds, where PAI-1 is needed quickly, PAI-1 plasma concentrations were determined 30 and 60 min post-injection. Mice treated with mPAI-1 at 0.5 and 1 mg/kg showed significant increases of plasma PAI-1 60 min post-injection but not 30 min post-injection (Figure 2E). There was a statistically significant dose-dependent difference in circulating PAI-1 between 0.5 mg/kg and 1.0 mg/kg at 60 min post-injection (Figure 2E).

Physiological levels of circulating PAI-1 in PAI-1$^{-/-}$ mice can be achieved with mPAI-1 LNP

We examined whether mPAI-1 can express PAI-1 in complete PAI-1 knockout (PAI-1$^{-/-}$) mice. PAI-1$^{-/-}$ mice were intravenously injected with mPAI-1 at a dose of 1 mg/kg or 200 μL of saline as a vehicle control and blood was collected at 6 and 24 h post-injection (Figure 3A). PAI-1$^{-/-}$ mice treated with mPAI-1 had significantly

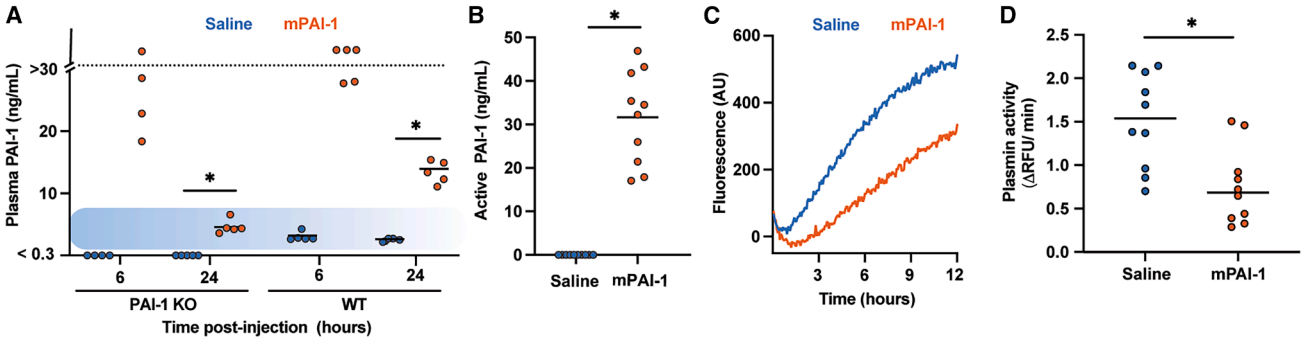

Figure 3. mPAI-1 generates circulating PAI-1 protein in PAI-1$^{-/-}$ mice

(A) PAI-1$^{-/-}$ and PAI-1$^{+/+}$ mouse plasma PAI-1 concentrations following administration of mRNA PAI-1 LNP (mPAI-1) at 1 mg/kg or saline at 6- and 24-h post-injection. Healthy normal mouse PAI-1 levels indicated in blue shaded region.[21] Accurate limit of detection of assay indicated by gray dashed line. (B) Active state PAI-1 plasma concentrations of PAI-1$^{-/-}$ mice 6 h post-injection of mPAI-1 at 1 mg/kg (red) or saline (blue). (C) Fluorescence curve of a substrate over time cleaved by plasmin in pooled plasma collected from PAI-1$^{-/-}$ mice 6 h after mice were injected with mPAI-1 at 1 mg/kg (red) or saline (blue). (D) Plasmin activity, determined from data such as in (C), where each marker is plasma from each individual mouse. n = 5–10 mice. *$p < 0.05$, ns, not significant.

higher PAI-1 levels at both time points (>25, 5 ± 1 ng/mL vs. <0.3 ng/mL, $p < 0.05$). In parallel to this study, PAI-1$^{+/+}$ mice were administered saline or mPAI-1 at a dose of 1 mg/kg and plasma PAI-1 protein concentration was analyzed 6 and 24 h post-injection (Figure 3A). PAI-1$^{+/+}$ mice treated with mPAI-1 had significantly higher PAI-1 levels at both time points (>30, 13 ± 1 ng/mL vs. 3 ± 0.3, 1 ± 0.2 ng/mL). Some of the values in these analyses were outside of the dynamic range of the assay; therefore, they are presented as larger or smaller than a specific value.

To assess functionality of the PAI-1 expressed in PAI-1$^{-/-}$ mice, active state PAI-1 concentrations were determined as well as plasmin activity in a plasmin generation assay. Active state PAI-1 is PAI-1 that can bind uPA in an ELISA assay.[22] PAI-1$^{-/-}$ mice treated with mPAI-1 had detectable active PAI-1 levels (30 ± 4 ng/mL; $p < 0.05$) compared with mice treated with saline that had undetectable levels of active PAI-1 6 h post-injection (Figure 3B). 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 mPAI-1 were still able to generate plasmin but had significantly less plasmin activity (1.5 ± 0.2 ΔRFU/min; $p < 0.05$) compared with saline-injected mice (0.75 ± 0.2 ΔRFU/min; $p < 0.05$) (Figures 3C and 3D).

DISCUSSION

Currently, there are no targeted therapeutic options for PAI-1-deficient patients. Bleeding events can be only partially mitigated with repeated frequent administrations of common anti-fibrinolytics, and cardiac fibrosis progression is typically only monitored, as no approved prophylactic treatment exists.[1,2] Due to the lack of preventive treatment for these patients, cardiac fibrosis can result in heart failure and mortality in young adults.

The addition of recombinant PAI-1 protein effectively mitigated the excessive fibrinolytic activity observed in PAI-1-deficient plasma. Recombinant PAI-1 has been considered as a potential therapeutic but has never reached the clinic due to its very short half-life of 30 min,[9] necessitating extremely frequent administration, which limits its clinical feasibility, thus hindering its therapeutic potential.[9]

Here, we developed a novel agent, mRNA encoding PAI-1 encapsulated in LNP (mPAI-1), which offers a potential solution to the current standard of care for breakthrough bleeds in PAI-1-deficient patients. Current standard of care includes administration of TXA to rebalance fibrinolysis as well as transfusion of fresh-frozen plasma to provide circulating PAI-1. mPAI-1 has the potential to enable a direct, and fast approach to increase circulating PAI-1 following acute bleeds. While mPAI-1 may potentially be useful for aspects of PAI-1 deficiency, TXA will still be advantageous as it is administered orally and is expected to have better patient adherence in various contexts such as for prophylactic bleeding. The implications of mPAI-1 as a potential therapeutic extends beyond bleeding management. PAI-1 deficiency is implicated in the pathogenesis of cardiac fibrosis, suggesting that mPAI-1 could provide a novel research tool for studying the role of circulating PAI-1 in fibrotic processes. mPAI-1 has the potential to offer a therapeutic benefit for the prevention of cardiac fibrosis, particularly if future mRNA-based approaches with long half-lives are utilized.

mPAI-1 offers a distinct advantage over current treatment options by enabling endogenous production of PAI-1 protein, which provides extended therapeutic effects and reduces the need for continuous administration, without the potential for inhibitory antibody production commonly developed in protein replacement therapies. Furthermore, mPAI-1 treatment restored PAI-1 levels to physiological and supraphysiological concentrations in PAI-1 knockout mice, underscoring its utility in managing PAI-1 deficiency. The increased circulating PAI-1 protein is functional and plasma collected from mice exhibited significantly decreased plasmin generation, demonstrating the potential of mPAI-1 to stabilize clots in PAI-1 deficiency.

mPAI-1 could be used as a preventive treatment for patients undergoing high-risk procedures or anticipated trauma, commonly seen in athletes or certain occupations, as well as for chronic conditions like heavy menstrual bleeding in PAI-1-deficient women. Furthermore, due to the short LNP circulation time and rapid onset of mRNA expression, exogenous protein expression can be observed as early as 30 min post-administration. Thus, mPAI-1 also could be suitable during acute bleeding events such as those occurring during surgical procedures or trauma. The implications of mPAI-1 as a potential therapeutic extends beyond bleeding management. PAI-1 deficiency is implicated in the pathogenesis of cardiac fibrosis, suggesting that mPAI-1 could provide a novel research tool for studying the role of circulating PAI-1 in fibrotic processes and potentially offer a therapeutic benefit for the prevention of cardiac fibrosis. We investigated the feasibility of repeated injections of mPAI-1 over several weeks; however, a long-term study will have to be conducted to evaluate the immunogenicity of mPAI-1, and whether it can be administered regularly or as just an "on-demand" treatment. Onpattro, an FDA-approved siRNA-LNP therapeutic with a similar LNP formulation to mPAI-1 has been administered to patients every 3 weeks for more than 6 years with no over toxicity.[23,24] Future advances in mRNA therapy that extend the half-life of mRNA to weeks or months could make mPAI-1 feasible for long-term prophylactic use.

While the results showed significant increases in circulating PAI-1 protein following mPAI-1 administration, these concentrations were outside the physiological range of normal mouse PAI-1. Mice have 5- to 10-fold lower levels of PAI-1 (~2 ng/mL) compared with humans (5–20 ng/mL), thus relative increases of PAI-1 in mice are not directly comparable to humans. In humans, thrombosis is associated with PAI-1 concentrations of more than 50–80 ng/mL.[25–27] In the plasminogen activation assay, blood plasma from mPAI-1 treated mice had less fibrinolysis than PAI-1 KO mice, but all samples had some degree of fibrinolysis. Thus, because fibrinolytic shutdown did not occur, the potential for thrombosis is less than if there was complete fibrinolytic shutdown. Additional experiments with large animal models such as swine will have to be performed to assess potential thrombosis risk and determine optimal dosage. In this study, WT mice were used to determine the time scale of expression and dose-dependence of mPAI-1, while PAI-$1^{-/-}$ mice were administered mPAI-1 at the highest dose (1 mg/kg). While we do not expect qualitative differences in the response to mPAI-1 between WT and PAI-$1^{-/-}$ mice, future studies will have to be performed.

Future studies will investigate the therapeutic potential of mPAI-1 in bleeding management as well as in a cardiac fibrosis animal model. In this study, we were unable to evaluate bleeding phenotypes in our PAI-$1^{-/-}$ mouse model as they do not have an excessive bleeding phenotype compared with WT mice.[3] Unlike humans, anti-fibrinolysis does not usually decrease bleeding in mice.[28] Consequently, further studies will require the use of alternative animal models to assess the physiological presentation of PAI-1 deficiency and the therapeutic efficacy of mPAI-1 in clinical scenarios. Additionally, while our results demonstrated increased circulating PAI-1 levels after mPAI-1 administration, these increases were not specific to cardiomyocytes. Further investigations are necessary to elucidate the potential of mPAI-1 to reverse cardiac fibrosis, especially in the absence of cardiomyocyte-specific PAI-1 expression. We expect that translation of this approach to humans will require utilizing mRNA that has longer half-lives than those used here, such as circular RNA.

In summary, this study establishes the foundation for an mRNA-based PAI-1 replacement therapy as a versatile therapeutic modality for PAI-1-deficient patients. mPAI-1 addresses significant limitations of conventional protein replacement therapies, offering sustained effects and broader applicability.

MATERIALS AND METHODS

mRNA synthesis

Messenger RNA (mRNA) for encapsulation was synthesized in bulk by *in vitro* transcription. Briefly, plasmid DNA template encoding a CleanCap AG bacteriophage T7 promoter site and uridine-depleted, codon-optimized mouse PAI-1 coding sequence was linearized with SapI enzyme. RNA was produced by *in vitro* transcription reactions containing CleanCap AG reagent and N^1methylpseudouridine-5′-triphosphate (TriLink BioTechnologies, San Diego, CA). DNA template was digested by DNAseI and purified using an RNeasy Kit (Qiagen, Toronto, ON, Canada) prior to enzymatic tailing using an A-Plus Poly(A) Polymerase Tailing Kit (CellScript, Madison, WI). mRNA was purified a final time and integrity monitored by bioanalyzer (Agilent Technologies, Santa Clara, CA) before encapsulation into LNP.

mRNA-LNP formulation

N^1methylpseudouridine mRNA encoding PAI-1 was encapsulated in LNP as previously described.[29] mRNA was dissolved in sodium acetate buffer (pH 4) and lipids were dissolved in pure ethanol. The lipid solution consisted of ALC-0315, DSPC, cholesterol, and PEG-DMG, (Avanti Lipids) at a 50:10:38.5:1.5% molar ratio. The mRNA and lipid solutions were combined at an amine-to-phosphate (N/P) ratio of 6. The LNPs were dialyzed overnight against Dulbecco's phosphate-buffered saline (PBS) in 500-fold volume excess. To determine mRNA concentration and encapsulation efficiency, RiboGreen assay (Quant-IT Ribogreen RNA Assay Kit, ThermoFisher) was performed. The Malvern Zeta Particle Sizer was used to determine the size and polydispersity index (PDI) of the particles. The LNPs were diluted to a final concentration of 0.1 mg mRNA per mL in 10% sucrose and 10 mM L-Histidine buffer and frozen at −80°C prior to intravenous injection.

Mice

All murine studies were conducted in accordance with institutional animal care guidelines, approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) (Protocol #AUA00007758) and the University of Ottawa Heart Institute (Protocol #2909). Male WT*C57BL/6J* mice (Jackson Labs, Bar

Harbor, ME, stock # 000664), between ages 6 and 11 weeks were used in wild-type mouse studies. PAI-1$^{-/-}$ littermates B6.129S2-Serpine1tm1Mlg/J (Jackson Labs, Bar Harbor, ME, Strain #002507), between ages 6 and 10 weeks were used.[30]

Mice injections and plasma extraction

Mice were administered LNPs intravenously by retro-orbital or tail vein injection at doses of 1, 0.5, or 0.1 mg mRNA per kg body weight (mg/kg). Non-terminal blood draws were collected either retro-orbitally under isoflurane anesthesia or by tail-nick. Blood samples taken at endpoint were collected via cardiac puncture. Blood was collected into a pipette tip or syringe containing sodium citrate (0.32% final) for retro-orbital and cardiac puncture blood collection or in heparin coated tubes for tail-nick blood collection. Plasma was separated from whole blood by spinning at 1,500 × *g* for 10 min at room temperature.

Analysis of PAI-1 levels in plasma

The plasma PAI-1 concentration was analyzed with a mouse total PAI-1 ELISA kit (IMSPAI1KTT, Innovative Research, Novi, MI) and the active plasma PAI-1 concentration was analyzed with a mouse active PAI-1 ELISA kit that measured binding of active PAI-1 to urokinase-type plasminogen activator (uPA) (IMSPAI1KTA, Innovative Research, Novi, MI) following the manufacturer's guidelines. Alterations to the manufacturer's protocol included diluting plasma 1:1 for each sample and increasing primary antibody incubation time to 1.5 h.

Fibrinolysis analysis *ex vivo*

Rotational thromboelastometry (ROTEM) (Rotem Delta, Werfen S.A., Spain) was performed according to the manufacturer's instructions. All reagents were first warmed to 37°C. Each test was allowed to proceed for 1.5 h. For mouse experiments, whole blood was mixed with 20 µL of 0.2 M $CaCl_2$, 20 µL EXTEM reagent containing tissue factor (Werfen S.A., Spain), and recombinant mouse tPA (AB92715, Abcam) at a final concentration of 350 ng/mL. For human sample experiments, 300 µL of human plasma (pooled normal plasma from George King Bio-Medical Inc, or PAI-1 depleted plasma (PAI-DP) from Affinity Biologicals) was mixed with 20 µL of 0.2 M $CaCl_2$, 20 µL EXTEM reagent containing tissue factor (Werfen S.A., Spain), recombinant human tPA (AB92637, Abcam) at 180 ng/mL and in the absence or presence of purified recombinant PAI-1 protein (1786-PI-010, R&D Systems) at 400 ng/mL.

Plasminogen activation assay in plasma-derived clots

Plasmin generation in plasma-derived clots was assessed using a high-throughput fluorescent assay as previously described with specific modifications.[31] Plasma samples were diluted 1:1 with HEPES buffer (25 mM HEPES, 137 mM NaCl) and clots were formed in 96-well plates by incubating 30 µL of diluted plasma with 4 U/mL thrombin and 66.6 mM $CaCl_2$ at 37°C for 1 h. Following clot formation, plasmin generation was initiated by adding 1 nM tissue plasminogen activator (tPA) and 1 µM plasmin-specific fluorescent substrate (Boc-Glu-Lys-Lys-AMC) to reach a total reaction volume of 100 µL. Fluorescence measurements were performed at 37°C using an EnVision microplate reader. Negative control absorbance values were subtracted at each time point to correct for background signal before calculating the plasmin activity rate. Due to the low tPA concentration, fibrinolysis did not initiate immediately from when the assay began, thus plasmin activity presents as a negative value between 0 and 3 h. The slope of each curve was calculated from different linear time windows for individual mice.

Statistical analysis

The statistical analysis was completed using GraphPad Prism (Version 10.0.3). The F-test was performed to confirm the standard deviation (SD) between groups was not statistically significant. Comparisons between the mean of two groups were performed with a one-tailed unpaired parametric t test and two-way analysis of variance (ANOVA) was used to compare two datasets over time. Welch's t test or Welch's two-way ANOVA were used respectively if the SD between groups was significantly different. Significance was designated at p values <0.05.

DATA AVAILABILITY

Further details of this study are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

F.F. designed and performed experiments, analyzed and interpreted the data, made the figures, and wrote the article; C.W.S., K.B., S.M.P., L.K., M.P., and T.H.S.C. helped perform experiments, analyze the data, and edit the article; P.R.C., S.G., A.S., and E.E.M. helped design experiments, analyze data, and edit the article; and C.J.K. designed experiments, interpreted the data, and wrote the article.

DECLARATION OF INTERESTS

C.J.K., P.R.C., F.F., and K.B. are directors, shareholders, and/or co-founders of companies developing RNA therapies, including SeraGene Therapeutics, Syrina Therapeutics, NanoVation Therapeutics, and Acuitas Therapeutics. C.J.K., P.R.C., F.F., and K.B. have filed intellectual property on RNA-based therapies with the intention of commercializing these inventions.

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