Molecular Therapy
Methods & Clinical Development
Original Article

siRNA-mediated reduction of a circulating protein in swine using lipid nanoparticles

Massimo F. Cau,[1,2,8] Francesca Ferraresso,[1,3,4,8] Monica Seadler,[4,5] Katherine Badior,[4] Youjie Zhang,[4] Laura M. Ketelboeter,[4] Geoffrey G. Rodriguez,[4] Taylor Chen,[4] Matteo Ferraresso,[4] Amanda Wietrzny,[4] Madelaine Robertson,[1,3] Amber Haugen,[4] Pieter R. Cullis,[3] Marc de Moya,[5] Mitchell Dyer,[4,6] and Christian J. Kastrup[1,3,4,5,7]

[1]Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T 1Z4, Canada; [2]School of Biomedical Engineering, University of British Columbia, Vancouver, BC V6T 1Z4, Canada; [3]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada; [4]Versiti Blood Research Institute, Milwaukee, WI 53226, USA; [5]Department of Surgery, Division of Trauma and Acute Care Surgery, Medical College of Wisconsin, Milwaukee, WI 53226, USA; [6]Department of Surgery, Division of Vascular and Endovascular Surgery, Medical College of Wisconsin, Milwaukee, WI 53226, USA; [7]Departments of Biochemistry, Biomedical Engineering, and Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

Genetic manipulation of animal models is a fundamental research tool in biology and medicine but is challenging in large animals. In rodents, models can be readily developed by knocking out genes in embryonic stem cells or by knocking down genes through *in vivo* delivery of nucleic acids. Swine are a preferred animal model for studying the cardiovascular and immune systems, but there are limited strategies for genetic manipulation. Lipid nanoparticles (LNPs) efficiently deliver small interfering RNA (siRNA) to knock down circulating proteins, but swine are sensitive to LNP-induced complement activation-related pseudoallergy (CARPA). We hypothesized that appropriately administering optimized siRNA-LNPs could knock down circulating levels of plasminogen, a blood protein synthesized in the liver. siRNA-LNPs against plasminogen (siPLG) reduced plasma plasminogen protein and hepatic plasminogen mRNA levels to below 5% of baseline values. Functional assays showed that reducing plasminogen levels modulated systemic blood coagulation. Clinical signs of CARPA were not observed, and occasional mild and transient hepatotoxicity was present in siPLG-treated animals at 5 h post-infusion, which returned to baseline by 7 days. These findings advance siRNA-LNPs in swine models, enabling genetic engineering of blood and hepatic proteins, which can likely expand to proteins in other tissues in the future.

INTRODUCTION

Genetic manipulation of animal models is a fundamental research tool in biology and medicine.[1] One potent strategy for genetically manipulating small animals includes *in vivo* delivery of nucleic acids,[2] but this technique is challenging in large animal models that are necessary for clinical trials of new therapies.[3,4] The FDA approved nucleic acid therapeutics including the small interfering RNA (siRNA) therapeutic Onpattro (Alnylam)[5] for hereditary transthyretin-mediated amyloidosis, and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) messenger RNA (mRNA) vaccines (Pfizer/BioNTech and Moderna)[6] were tested in non-human primates (NHPs), as they are the closest species to humans. Nucleic acid therapeutics for the bleeding disorders hemophilia A and B and von Willebrand disease are commonly tested in dogs deficient in coagulation factor VIII/IX or von Willebrand factor, which are susceptible to the spontaneous joint bleeds observed in humans with these diseases.[7–9] Both NHPs and dogs have a long lifespan and are useful for performing repeated dose studies to determine long-term toxicity of nucleic acid therapies.[3] However, using these models is costly,[10,11] carries ethical issues,[12] and may not be appropriate for all diverse applications of nucleic acid delivery. An expanded repertoire of techniques is needed to support genetic modification and testing of new therapies in large animals.

Swine (*Sus scrofa domesticus*) have similar anatomical size and structure, immunology, genome, and physiology to humans.[13] Swine are considered the gold-standard animal model to study the human cardiovascular system and are also widely used for studying the immune, respiratory, musculoskeletal, reproductive, neurologic, and ophthalmologic systems.[13] However, there are few strategies for performing genetic manipulation in swine.[14] Lipid nanoparticles (LNPs) administered intravenously efficiently deliver siRNA to knock down circulating proteins synthesized in the liver,[15,16] but swine are sensitive to LNP-induced complement activation-related pseudoallergy (CARPA).[17,18] CARPA occurs when the components of LNPs interact with and activate circulating complement proteins in the blood, leading to a systemic immune response.[17,18] The physicochemical characteristics of LNPs that are associated with CARPA include the presence of polyethylene glycol (PEG), surface charge, and the tendency of the LNP to aggregate.[18] Faster infusion rates also increase the risk of CARPA, which is

Received 31 October 2023; accepted 22 April 2024;
https://doi.org/10.1016/j.omtm.2024.101258.

[8]These authors contributed equally

Correspondence: Christian J. Kastrup, Versiti Blood Research Institute, Milwaukee, WI 53226, USA.
E-mail: ckastrup@versiti.org

mild and transient in most cases but can be life-threatening.[19] An estimated 30%–40% of humans develop CARPA,[19] and swine display many similar symptoms upon LNP delivery, including skin changes, laboratory and hematological abnormalities, and cardiopulmonary distress.[18] Severe CARPA was observed in swine using previous liposomal formulations at therapeutically relevant doses.[18,20]

Limited reports of the *in vivo* delivery of nucleic acids to knock down protein expression in swine have been published, mainly focusing on alternative carriers to LNPs and adeno-associated vectors (AAVs). Recently, intravenous delivery of biomimetic peptide-polymer nanocomplexes[21] containing siRNA against the Hippo signaling pathway protein Sav1 improved cardiac function and promoted myocardium regeneration in a swine model of ischemia/reperfusion-induced myocardial infarction. In swine models of Huntington's disease, the delivery of AAVs containing the CRISPR-Cas9 system reduced the expression of mutant huntingtin (HTT), with modest replacement of wild-type HTT.[22] Moreover, AAV-mediated delivery of CRISPR-Cas9 in a swine model of Duchenne's muscular dystrophy produced a shortened dystrophin protein and ameliorated pathology.[23] However, testing AAVs in large animal models is limited by their immunogenicity, which makes repeated systemic dosing difficult,[24] as well as the high costs and technical challenges associated with scaled-up manufacturing.[25]

Significant advances in engineering and optimizing LNP chemistry have generated clinically approved LNPs with excellent safety profiles and efficacy at low doses; only transient CARPA was observed in swine that received the Pfizer/BioNTech COVID-19 mRNA-LNP vaccine.[26] Our previous work demonstrated that siRNA-LNPs containing the ionizable cationic lipid ALC-0315, a component of the Pfizer/BioNTech vaccine,[27,28] exhibited increased potency in mice[29] compared to formulations containing another ionizable cationic lipid, DLin-MC3-DMA (MC3), which is used in Onpattro.[30,31] We also showed that intravenously delivering siRNA-LNPs targeting the expression of the circulating blood protein plasminogen resulted in safe and effective protein knockdown in mice and in dogs.[9] Plasminogen is synthesized in the liver where LNPs naturally accumulate, and decreasing plasminogen levels is not associated with major adverse effects.[32] Here, we tested if potent LNPs carrying siRNA would safely knock down the expression of plasminogen in swine, which would be the first demonstration of using siRNA-LNPs to genetically manipulate the levels of circulating proteins in this animal species. We hypothesized that delivering these siRNA-LNPs combined with a carefully designed infusion protocol would be well tolerated without clinical signs of CARPA and effectively knock down circulating levels of plasminogen. We demonstrate a safe and effective approach for genetically manipulating swine models *in vivo* and creating a transient model of a protein deficiency.

RESULTS

siRNA-LNP-mediated knockdown of a circulating protein in swine

We examined whether protein knockdown occurs following an intravenous injection of siRNA-LNPs *in vivo* in swine. Initially, we screened three siRNA sequences targeting porcine plasminogen *in vitro* (siPLG1–3). Plasminogen is a protein we have knocked down previously with siRNA-LNPs in mice and dogs because it is a therapeutic target for bleeding disorders.[9] siRNA sequences were encapsulated in LNPs and then incubated for 24 h in porcine hepatocyte cell culture at a dose of 3 μg/mL. All three siPLG sequences significantly knocked down plasminogen mRNA levels (32% ± 8%, 56% ± 11%, and 26% ± 6%, respectively, $p < 0.05$) standardized to hepatocytes treated with scramble siRNA (scRNA) (100% ± 20%) (Figure 1A). Selected siPLG sequences from the *in vitro* screen were encapsulated in LNPs, and swine were treated with either siPLG or scRNA at 0.3 mg per kg of body weight (mg/kg) or phosphate-buffered saline (PBS). Plasminogen protein and mRNA levels were measured in the plasma and hepatic tissue. At 7 days post-infusion, swine treated with siPLG had significantly lower circulating plasminogen levels in plasma (7 ± 5 μg/mL, $p < 0.05$) compared to baseline values and compared to swine treated with PBS or scRNA (176 ± 5, 184 ± 4, and 161 ± 9 μg/mL, respectively) (Figure 1B) as measured by ELISA. Plasma plasminogen levels were also analyzed through western blot (Figure 1C), which showed a similar level of protein knockdown in siPLG-treated swine between baseline and 7 days (0.9 ± 0.2 and 0.06 ± 0.01, respectively, $p < 0.05$) (Figure 1D). There seemed to be an increase in band density in the PBS group between days 0 and 7, which is likely due to experimental variability; the difference was not statistically significant. The data from the ELISA, which is a more quantitative measurement, confirms that plasminogen concentrations were similar at both time points in the PBS group. Plasminogen mRNA levels in the hepatic tissue of siPLG-treated swine were significantly decreased (3% ± 0.5%, $p < 0.05$) at 7 days standardized to swine treated with PBS (100% ± 18%) (Figure 1E). To determine if siRNA-LNP knockdown is dose dependent, swine were administered siPLG at two additional doses of 0.1 ($N = 3$ animals) and 0.03 mg/kg ($N = 3$ animals), and circulating plasminogen levels were measured using ELISA. At 7 days post-infusion, plasma levels of plasminogen in the 0.1 mg/kg group were significantly lower compared to baseline (83 ± 27 and 172 ± 18 μg/mL, respectively, $p < 0.05$). Swine that received 0.03 mg/kg siPLG had lower plasminogen levels compared to baseline (134 ± 8 and 172 ± 18 μg/mL, respectively), but this effect did not reach statistical significance (Figure 1F). To account for possible sex differences affecting the knockdown, an additional group of male ($N = 3$) swine was administered the 0.1 mg/kg siPLG dose and compared to female ($N = 3$) swine receiving the same dose. There were no differences between groups at baseline. At 7 days, circulating plasminogen levels between male and female animals were similar (61% ± 8% and 48% ± 9% of baseline levels, respectively) (Figure 1G).

Knockdown of plasminogen can be detected with a functional assay for fibrinolysis

We examined whether plasminogen knockdown in swine could be functionally detected as a change in systemic blood coagulation via rotational thromboelastometry (ROTEM) analysis. Baseline ROTEM analysis parameters were similar between groups. ROTEM analysis on plasma samples at 7 days post-infusion showed significant

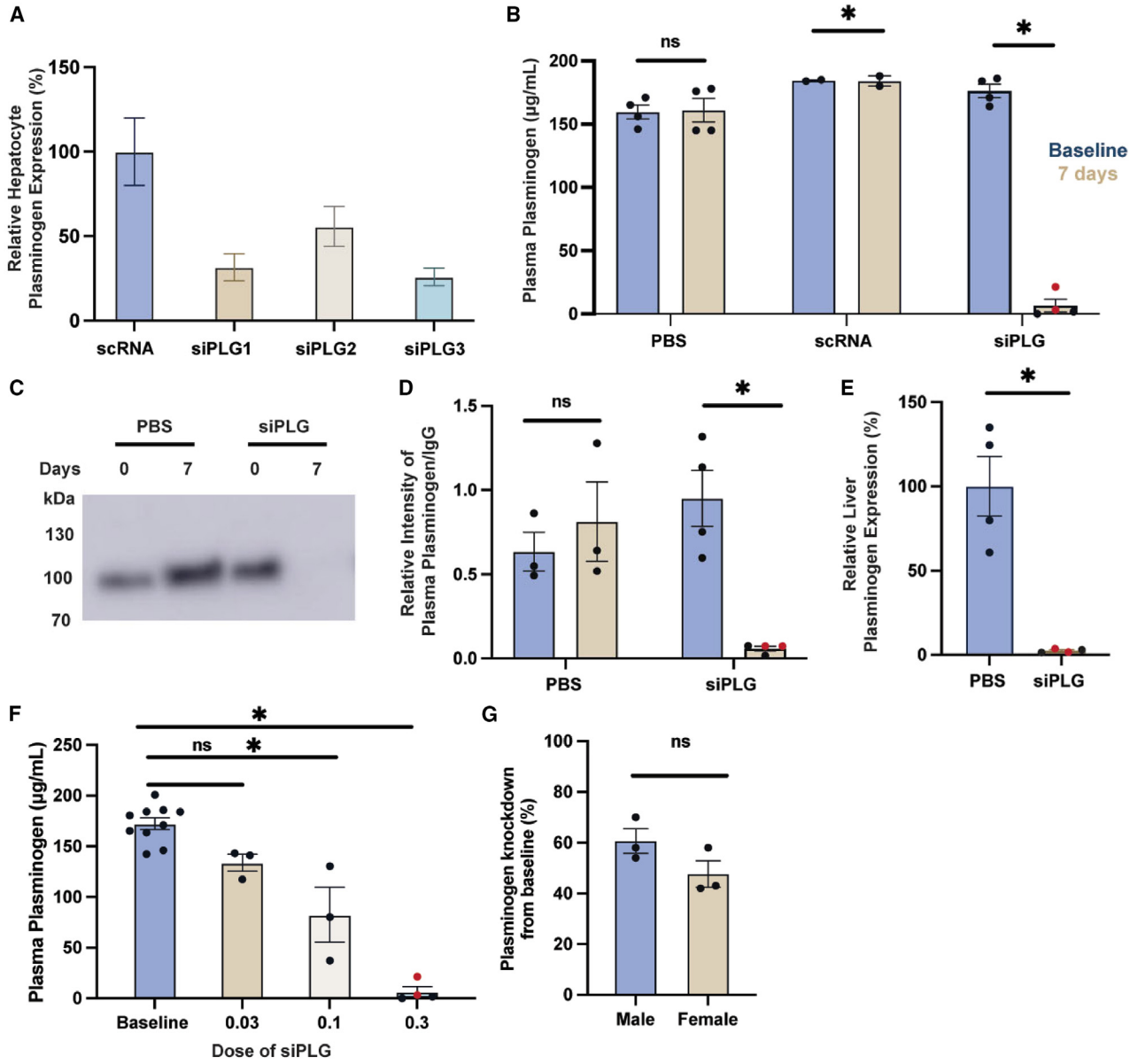

Figure 1. A circulating blood protein can be knocked down by intravenously injecting siRNA-LNPs in swine

(A) Three siRNA sequences against plasminogen were encapsulated in LNPs and screened for their potency at knocking down plasminogen mRNA in swine hepatocytes *in vitro* ($N = 1$ well of cells/sequence). (B) Plasma levels of circulating plasminogen following siPLG, scRNA, and PBS control infusion measured using ELISA. (C) Plasma levels of circulating plasminogen following siPLG, scRNA, and PBS control infusion measured using western blot. (D) Densitometry analysis of western blot gel for quantifying protein concentrations. (E) Relative differences in plasminogen mRNA expression from harvested liver samples and analyzed using qPCR. (F) Plasma levels of circulating plasminogen at 7 days following siPLG administration at three different doses, measured using ELISA. (G) Plasma levels of circulating plasminogen at 7 days in male and female swine, which received 0.1 mg/kg siPLG. $N = 3$–4, $^*p < 0.05$, ns, not significant. Error bars represent mean ± SEM. Red markers denote data from siPLG3 administration.

differences in fibrinolysis of swine treated with siPLG or PBS (Figures 2A and 2B). Swine treated with siPLG had significantly inhibited fibrinolysis after 30 min (LI30 [percentage of clot remaining at 30 min]) (100% ± 0% of clot remaining, $p < 0.05$) compared to PBS-treated swine (1% ± 1%, $p < 0.05$) in the presence of tissue plasminogen activator (tPA), a fibrinolytic protein (Figure 2C). The time to form a clot (clotting time [CT]) and the rate of clotting (alpha angle [α angle]), measured in both the presence and absence of tPA, were similar for siPLG- or PBS-treated swine (Figures 2D and 2E). The maximum clot firmness (MCF) in swine treated with siPLG was not significantly different in the presence and absence of tPA (20 ± 3 and 20 ± 3 mm, respectively). In swine treated with PBS, the

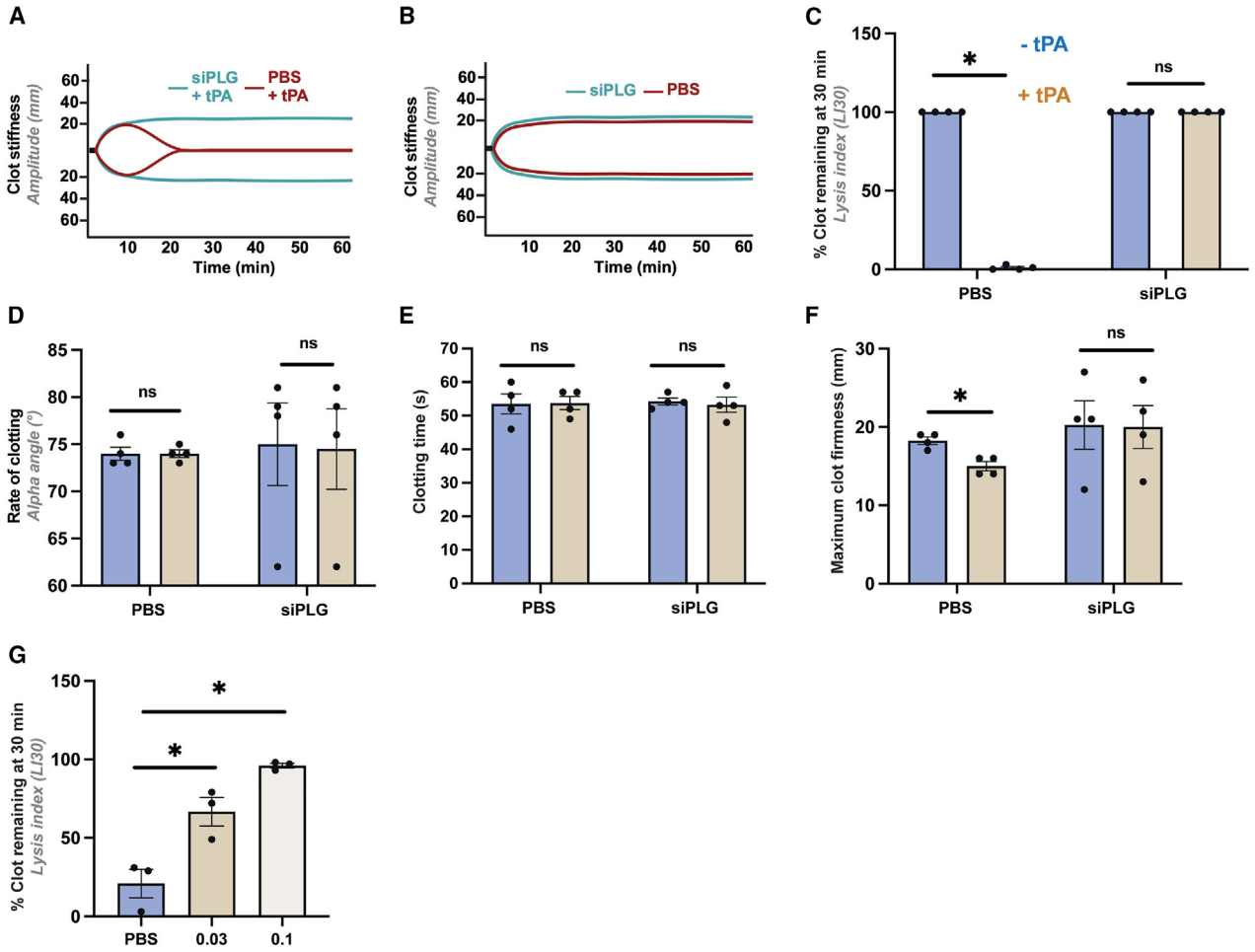

Figure 2. Knockdown of plasminogen can be detected functionally

(A) Representative ROTEM analysis curve at 7 days post-infusion showing normal blood clot formation and inhibited fibrinolysis in the presence of tPA in siPLG animals only. (B) Representative ROTEM analysis curve showing normal blood clot formation and no fibrinolysis within 60 min in both siPLG and PBS groups in the absence of tPA. (C) The percentage of clot remaining at 30 min (also known as the clot lysis index, LI30) in the presence (light brown) or absence (blue) of tPA. (D) The rate of clot formation (α angle) of both groups in the presence and absence of tPA. (E) Clotting time values of both groups in the presence and absence of tPA. (F) Maximum clot firmness values indicating mechanical clot stiffness of both groups in the presence and absence of tPA. (G) Clot lysis index values in the presence of tPA in swine administered siPLG at two different doses or PBS. N = 3–4 animals, 1 blood sample/time point/animal *$p < 0.05$, ns, not significant. Error bars represent mean ± SEM.

MCF was significantly decreased in the presence of tPA compared to the absence of tPA (15 ± 1 and 18 ± 0.5 mm, respectively, $p < 0.05$) (Figure 2F). These data show the complete inhibition of fibrinolysis and no effect on clot formation in siPLG-treated swine, which is consistent with reduced circulating levels of plasminogen. To determine if there was a dose-dependent effect of siPLG on fibrinolysis, ROTEM analysis was performed on blood samples from animals that received lower doses of siPLG of 0.1 and 0.03 mg/kg. There were no differences in CT, MCF, or α angle ROTEM values across all groups at any time point. At 7 days, the LI30 in the presence of tPA was significantly higher in both the 0.1 and 0.03 mg/kg groups compared to the PBS group (96% ± 1.5%, 67% ± 16%, and 21% ± 16%, respectively, $p < 0.05$) (Figure 2G). The LI30 data for the 0.1 and 0.03 mg/kg doses of siPLG could not be displayed on the same graph and directly compared to the 0.3 mg/kg group (Figure 2C), as ROTEM analysis was performed using whole blood rather than plasma and required a different concentration of tPA. However, examining the LI30 values achieved by all three doses of siPLG demonstrates the dose-dependent functional inhibition of fibrinolysis.

siRNA-LNPs displayed a low risk of CARPA and other toxicities

Similar to the FDA-approved siRNA-LNP therapy Onpattro,[33] swine received dexamethasone, diphenhydramine, and famotidine 30 min prior to infusion of either PBS, siPLG, or scRNA. Swine were closely monitored for signs of CARPA at the time of infusion and for the following 2 h. The importance of the slow infusion protocol and the premedication regimen used in this study was highlighted by one animal that erroneously did not receive premedication and

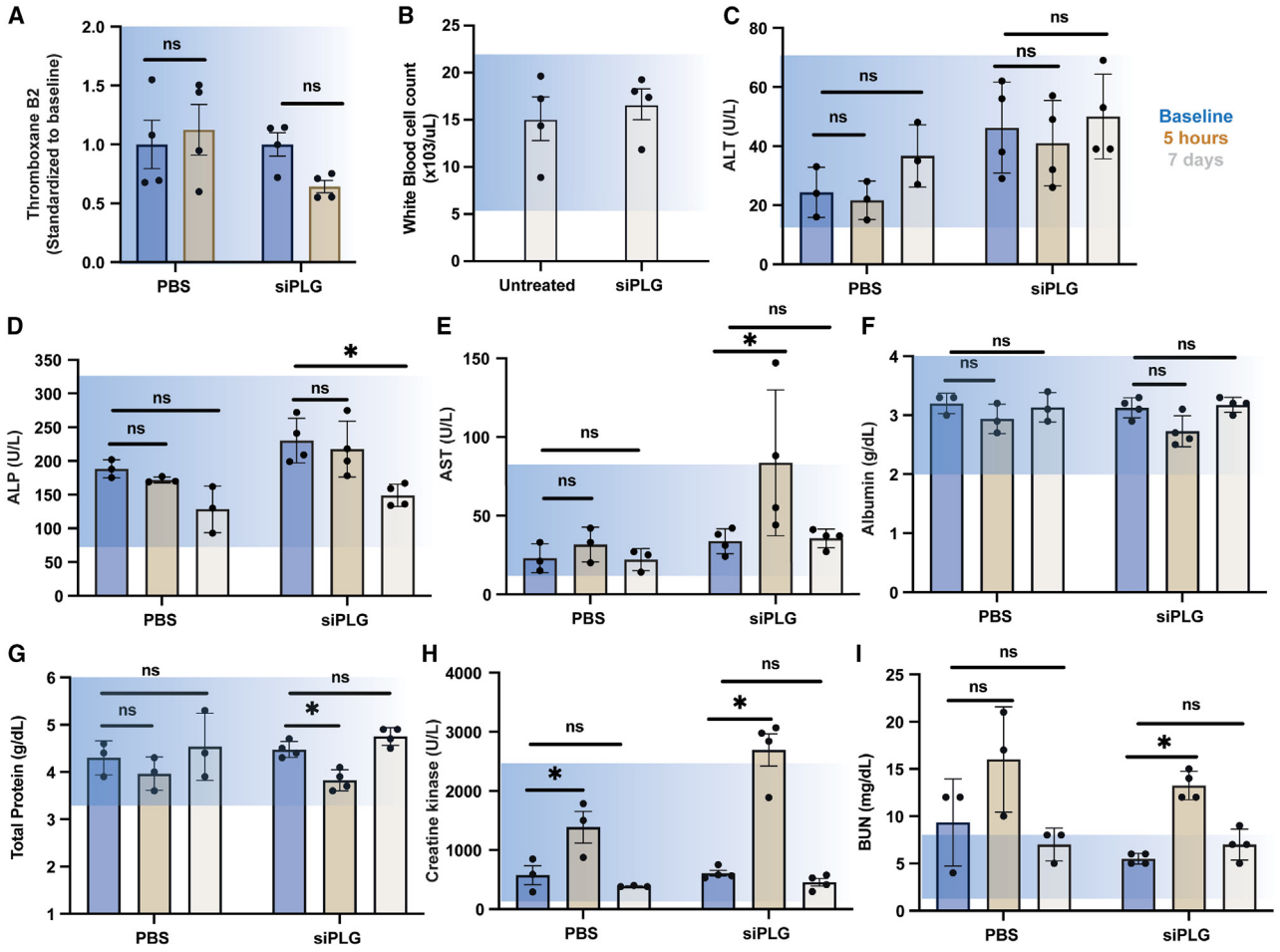

Figure 3. siRNA-LNPs produce mild transient hepatotoxicity without signs of CARPA

(A) Thromboxane B2 levels in siPLG and PBS groups at baseline and 5 h post-injection. (B) White blood cell count in siPLG group at 7 days compared to untreated (anesthesia-only) control swine. (C–I) Serum levels of ALT, ALP, AST, albumin, total protein, creatine kinase, and BUN at baseline, 5 h post-injection, and 7 days in siPLG and PBS groups. $N = 3–4$ animals, 1 sample/animal/time point, $^*p < 0.05$, ns, not significant. Where $N = 3$, blood samples for analysis could not be recovered from one animal in the experimental group because not enough blood was available. Blue zones represent previously published normal ranges for the specific marker.

received a bolus injection of siRNA-LNPs rather than slow infusion. This animal showed symptoms consistent with CARPA and was humanely euthanized. However, we did not perform a necropsy or collect blood samples from this animal to confirm markers of CARPA in the blood, and this experimental protocol was not repeated for ethical reasons. There were no observed clinical signs of toxicity and no significant changes in skin discoloration, temperature, or heart rate. At 5 h and 7 days post-infusion, blood draws were performed to assess toxicity. Thromboxane B2 levels, a known marker of CARPA, were comparable to baseline levels in siPLG- and PBS-treated swine at 5 h (Figure 3A). To monitor for potential signs of inflammation, white blood cell (WBC) count was analyzed, and levels were within a normal range[34] and comparable to untreated swine (Figure 3B). Untreated swine underwent anesthesia but did not receive any further injections; this control group was included in specific analyses when blood samples in the PBS group were not available. Serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assessed to determine liver toxicity; no significant changes were observed between levels at baseline, 5 h, and 7 days in both siPLG- and PBS-treated swine (Figures 3C and 3D). Serum levels of aspartate aminotransferase (AST) transiently increased at 5 h post-infusion from baseline in the siPLG-treated swine (84 ± 23 and 34 ± 4 U/L, respectively, $p < 0.05$); however, they were similar to baseline by 7 days (35 ± 3 U/L) (Figure 3E). Furthermore, two of the four swine administered siPLG had AST levels within the normal range.[35] A drop in total protein levels at 5 h post-injection was observed in both treatment groups; however, this drop was only significant for siPLG-treated swine (Figure 3G). For both PBS and siPLG treatment groups, there was a significant increase in creatine kinase and blood urea nitrogen (BUN) levels at 5 h post-injection, which returned to baseline levels by 7 days. This increase was within normal ranges[36] and was likely due to the

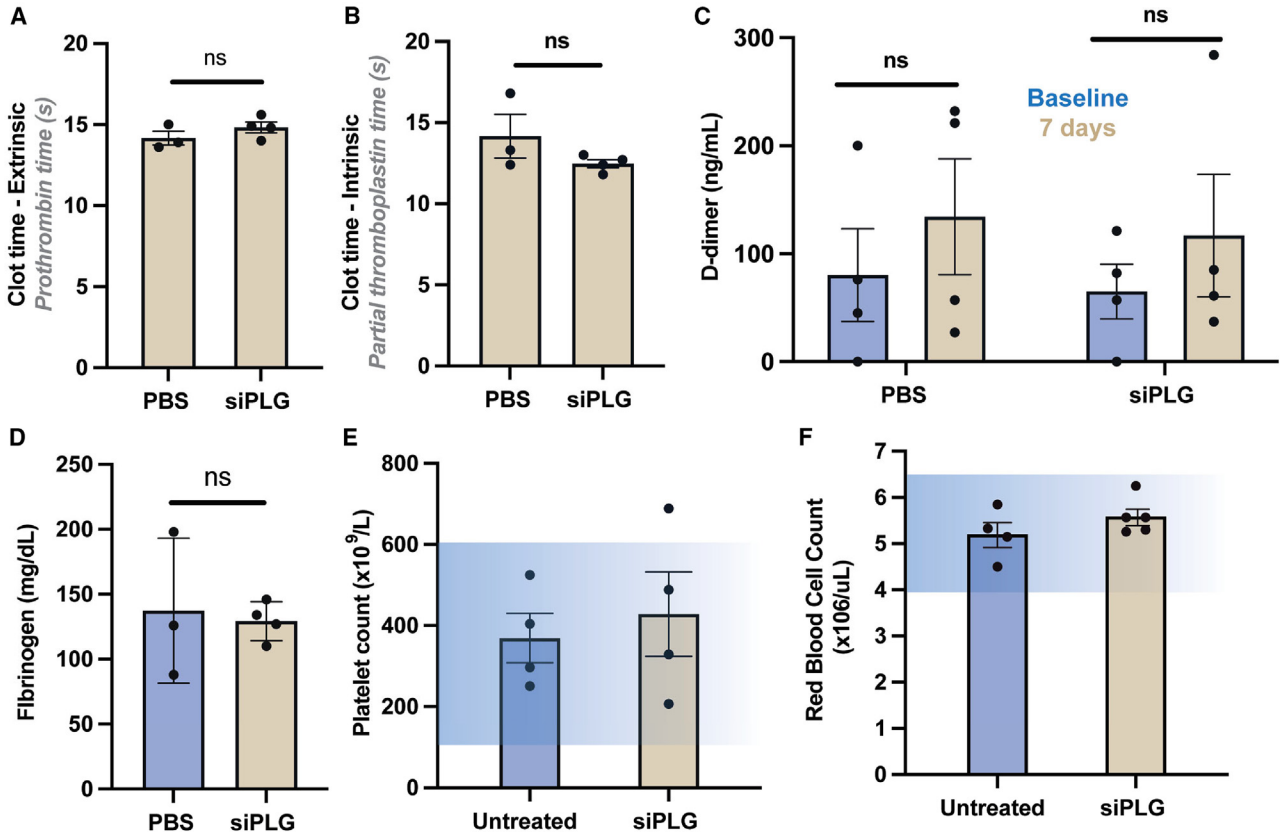

Figure 4. Plasminogen can be specifically knocked down without affecting other components of blood coagulation

(A and B) Prothrombin time and partial thromboplastin time of blood samples from siPLG and PBS groups at 7 days. (C) D-dimer measurements for thrombosis at baseline and 7 days post-injection. (D–F) Fibrinogen levels, platelet counts, and red blood cell counts at endpoint. *N* = 3–4 animals, 1 sample/animal/time point, ns, not significant. Where *N* = 3, blood samples for analysis could not be recovered from one animal in the experimental group because not enough blood was available. Blue zones represent previously published normal ranges for the specific marker.

premedication, anesthesia, or catheter placement for LNP infusion (Figures 3H and 3I). There was no increase in other serum toxicity markers such as albumin (Figure 3F), gamma-glutamyl transferase (GGT), globulin, and creatinine.

siPLG was specific without off-target effects on other components of the blood coagulation system

The blood coagulation profile of the swine, including prothrombin time, partial thromboplastin time, fibrinogen level, platelet count, and red blood cell (RBC) count, were analyzed to assess any potential risk for thrombosis and altered coagulation following siPLG injection. For the WBC, platelet, and RBC measurements, blood samples from PBS control animals were not available, and the siPLG animals were compared to an untreated control group (*N* = 4 animals). Untreated control animals underwent anesthesia only, and were recovered and monitored for 7 days. There were no significant differences between siPLG pigs and the respective controls at baseline or 7 days in any of the analyzed parameters (Figures 4A, 4B, and 4D–4F). D-dimer levels were also analyzed, as they are a clinical standard test to assess potential thrombosis risk and were comparable between PBS and siPLG pigs (Figure 4C). The scRNA group was not included in this analysis, as plasminogen levels in this group were similar to PBS control animals and suggested a low potential for modulating systemic blood profiles.

DISCUSSION

Currently, there are few strategies for performing genetic manipulation in swine models. *In vivo* strategies rely on viral vectors or alternative routes of administration such as direct injection into tissues for targeting specific proteins and disease states.[37–50] Breeding transgenic swine mirrors the genetic knockout models utilized extensively in small animal species, but this process is time consuming, costly, and technically challenging.[13,51–54] Systemically delivered LNPs naturally target the liver, which produces 85%–90% of the total circulating blood protein volume[55] and thus contains numerous protein targets important in human health and disease. Here, we demonstrated a cost-friendly, convenient, and high-fidelity approach for *in vivo* genetic manipulation of swine, using plasminogen as a representative circulating protein. Plasminogen is secreted from the liver into systemic circulation at a concentration of 180 μg/mL (2 μM).[56] Studies

investigating PLG$^{-/-}$ animals have demonstrated that the most common adverse effect of plasminogen deficiency is ligneous conjunctivitis of the mucous membranes.[57] In humans with plasminogen deficiency, ligneous conjunctivitis can be treated with an infusion of purified plasminogen protein.[58,59] As an inactive zymogen, plasminogen is converted to the protease plasmin during and after fibrin blood clot formation and degrades clots to restore flow through vessels in the process known as fibrinolysis. Inhibiting fibrinolysis has a stabilizing effect on blood clots, whereas unchecked fibrinolysis results in weak clots that are prone to rupture or embolization.[56] Clinically, inhibiting plasminogen by administering the antifibrinolytic drug tranexamic acid is beneficial for decreasing bleeding in patients with bleeding disorders; it does not cause bleeding or thrombosis in healthy people.[60] A single injection of siPLG had extensive and dose-dependent functional effects on blood coagulation, as measured by ROTEM, inhibiting fibrinolysis in the presence of tPA. The presence of tPA did not affect clot formation or the rate of clot formation, but the MCF was significantly decreased in the PBS group. This finding is consistent with increased levels of plasminogen in the PBS group compared to the siPLG group, as fibrinolytic proteins act to oppose blood clot formation.[61,62]

In the short term, siPLG could enable long-term studies of antifibrinolytic therapy in swine, as well as therapeutic targeting of plasminogen in disease models where plasminogen has been implicated, such as asthma, multiple sclerosis, and many bleeding disorders.[63–65] For example, siPLG reduced and sustained circulating plasminogen levels at below 50% with minimal toxicity in healthy dogs.[9] In dogs deficient in coagulation factor VIII, which is a model for testing new therapeutics for hemophilia A, siPLG reduced the frequency of bleeding events over a 4 month period.[9] Dogs received an injection of siPLG every 3 weeks to maintain low plasminogen levels, demonstrating a long period of knockdown.[9] Long term, the ability to selectively knock down circulating blood proteins has many potential applications. For example, in cardiovascular research, siRNA-LNPs could be applied to standard swine models investigating the pathophysiology of trauma and coagulopathy,[66] atherosclerosis,[67] ventricular dysfunction,[68–70] and tissue repair and regeneration following myocardial infarction.[71–74] Using juvenile swine allowed us to maximize the number of animals and siRNA groups tested, as there are significant costs associated with purchasing and formulating siRNA and lipid reagents for large animals. Differences in the ages of the animals would not be expected to change the effectiveness of the siRNA, as this has not been observed in clinical studies testing Onpattro in humans.[75] No differences in plasminogen knockdown between sexes were observed in this study using both male and female animals. As sex is an important biological variable, it warrants additional research when developing future gene therapies and genetic tools. Sex differences between female and male swine include an increased inflammatory response to bacterial challenge[76] and superior hemodynamics and reduced cardiac injury during hemorrhage.[77] The blood coagulation analyses, including ROTEM, fibrinogen, and D-dimer measurements, suggest that siPLG is specific for plasminogen, and there were no off-target effects on the gene expression of other coagulation or fibrinolytic factors. However, more comprehensive analyses using tools such as RNA sequencing are needed. Lastly, we did not monitor animals to determine the duration of siPLG knockdown; this is important to completely characterize the siRNA-LNP approach, as several swine models are long-term recovery models.[69] It is expected that the siRNA begins to degrade plasminogen mRNA within hours of administering siRNA-LNPs, with the effects lasting up to 4 weeks in hepatocytes *in vivo*.[78] The endpoint of 7 days chosen in this study provides an acceptable starting period for evaluating knockdown and toxicity toward future widespread applications of siRNA-LNPs in swine models.

CARPA was likely mitigated by using optimized LNPs,[17,18,20,79] prophylaxis with immunosuppressive drugs,[33,79–84] and slow infusion rates,[79–81,83] which are factors known to influence disease severity. Pretreatment with immunosuppressive drugs was appropriate in this study, as it represents the standard of care that human patients undergoing siRNA-LNP therapy with Onpattro receive.[33] Thromboxane B2 levels[18,83,85,86] and WBC counts at different time points were similar to baseline, and there were no changes in skin tone or other known clinical signs of CARPA. However, additional studies are needed to measure the levels of complement protein activation in blood and blood pressure monitoring to quantitatively determine hemodynamics following the LNP infusion. Plasminogen can bind the complement proteins C3, C3b, and C5[87] and decrease complement activation *in vitro*, but it is unlikely that plasminogen levels in the blood affected the development of CARPA in this study. siRNA begins to knock down plasminogen expression hours after administration, while CARPA develops minutes after exposure to LNPs. The anesthesia protocol, premedication, and intravenous infusion protocol may have contributed to mild organ toxicity[88–90] in both the siPLG and PBS groups, as transient increases were observed in creatine kinase and BUN levels, respectively, and transient decreases were observed in total protein levels. However, general anesthesia is commonly utilized during serial blood sampling in swine[91] and in other large animals such as NHPs.[92] Albumin levels in the siPLG group were reduced at 5 h compared to the control groups, which could be attributed to the mild and transient liver toxicity in these animals, as the liver is the site of albumin synthesis.[93] Inflammation and surgery are also known to decrease albumin levels,[94] and these may have also contributed, although no major signs of inflammation or distress were observed during monitoring. The transient increase in AST levels in swine after administering siPLG is similarly observed in other animal models[95,96] and human studies[97] that tested the *in vivo* delivery of siRNA.

In conclusion, a single injection of siRNA-LNPs knocked down a circulating blood protein to <5% of physiologic levels, producing functional effects on blood behavior, with an acceptable preliminary safety profile. These findings open new avenues for low-cost genetic manipulation in swine with the ability to modulate nearly all of the systemic plasma proteome and represents a major advance in lipid-based nucleic acid delivery *in vivo*. The ability of LNPs to transfect

extrahepatic tissues is rapidly growing,[98,99] and along with this capability, the range of proteins that can be readily knocked out in large animals will also expand.

MATERIALS AND METHODS

Formulating siRNA-LNP

siRNA (Integrated DNA Technologies, Coralville, IA, USA) targeting plasminogen or scRNA was encapsulated in LNPs as previously described.[29] Briefly, siRNA was dissolved in sodium acetate (pH 4) and combined with a lipid solution at an amine-to-phosphate ratio of 3. The lipid solution consisted of ALC-0315, DSPC, cholesterol, and PEG-DMG (Avanti Lipids, Alabaster, AL, USA) at a 50%:10%:38.5%:1.5% molar ratio. The LNPs were dialyzed overnight against Dulbecco's PBS at pH 7.4 in 1,000-fold volume excess. To determine siRNA concentration and encapsulation efficiency, the RiboGreen assay (Quant-IT RiboGreen RNA Assay Kit, Thermo Scientific, Waltham, MA, USA) was performed. Encapsulation efficiency was analyzed by comparing the signals in LNP samples with or without Triton X-100 detergent. Cholesterol content was measured using the Cholesterol E Assay Kit (Wako Chemicals, Mountain View, CA, USA). The LNPs were diluted to a final concentration of 0.03, 0.1, and 0.3 mg siRNA per mL in PBS (pH 7.4) prior to intravenous injection.

In vitro siRNA-LNP transfection

Primary swine hepatocytes (Xenotech, Kansas City, KS, USA) were seeded at 1×10^5 cells per well and transfected following 4 h of incubation at 37°C. Cells were transfected with the 3 different siRNA-LNP (siPLG1–3) sequences at a dose of 3 μg/mL of siRNA or siRNA against luciferase as a control (Integrated DNA Technologies). The three sequences tested corresponded to nucleotide bases 738–763 for siPLG1, 1089–1114 for siPLG2, and 2458–2483 for siPLG3 on the plasminogen mRNA sequence for swine (*Sus scrofa domesticus*) (GenBank: DQ530369.1).[100] The following day, cells were lysed, and the RNA was extracted using the PureLink RNA Mini Kit (Thermo Scientific) following the manufacturer's protocol.

Quantifying plasminogen mRNA levels

Quantitative PCR (qPCR) was performed using the SYBR Green Master Mix (Thermo Scientific) and DNA primers against plasminogen (F: 5'-GAGAACTACTGCCGTAACC-3'; R: 5′-CAGATACTCCGTGGATGTC-3′). Plasminogen expression was quantified using the ΔΔCt method relative to the expression of the housekeeping gene (B-actin) (F: 5′-GTCCACCTTCCAGCAGAT-3'; R: 5′-CAGTCCGCCTAGAAGCAT-3′). Primers were synthesized by Integrated DNA Technologies, and the data were collected using the QuantStudio6.

Injecting siRNA-LNP in swine

Swine studies were approved by and performed in accordance with the Medical College of Wisconsin Animal Care Committee protocol #AUA00007759. Landrace-Yorkshire cross swine (Wilson's Prairie View Farm, North Prairie, WI, USA) aged 3–5 weeks and weighing between 8 and 15 kg were used in the study. Swine were anesthetized using an intramuscular injection of tiletamine/zolazepam and xylazine, followed by intubation and maintenance of anesthesia with 1%–5% isoflurane in 100% oxygen. An incision was made parallel to the femoral vessels, and the femoral vein was circumferentially exposed. 2-0 silk sutures were placed proximally and distally, and a 4F catheter was placed into the vein using a modified Seldinger technique. A baseline blood sample was collected followed by administration of famotidine, diphenhydramine, and dexamethasone, which is based on clinical protocols for reducing the risk of CARPA when administering Onpattro in human patients.[33] After 30 min to establish baseline, siRNA-LNPs or PBS was slowly infused over 1 h. Following the infusion, the catheter was removed, the silk sutures were tied down, and the groin was closed in two layers using Vicryl sutures. Swine were awakened from anesthesia and allowed to recover. All animals that received siPLG *in vivo* received siRNA sequence siPLG1 except for 2 of 4 animals in the 0.3 mg/kg dose group, which received siPLG3. As 0.3 mg/kg was the first dose of siPLG tested *in vivo*, two different sequences were tested to compare the relative efficacies of each sequence, but knockdown in these animals produced similar results of reducing plasminogen levels to <5%. siPLG1 and siPLG3 animals were pooled to produce one group ($N = 4$) at 0.3 mg/kg for statistical comparisons to controls.

Collecting serum, plasma, and liver from swine

At 5 h post-injection, swine were anesthetized, and blood was collected from the femoral vein branch. At 7 days post-injection, blood was collected via cardiac puncture under isoflurane anesthesia, and the liver was surgically excised. Blood was collected into a syringe containing sodium citrate (0.32% final), and plasma samples were obtained by spinning the whole blood twice at $1{,}500 \times g$ for 10 min. Blood was collected without sodium citrate and was allowed to clot for 30 min before centrifuging as described above to obtain serum. Animals were euthanized using a pentobarbital-based euthanasia solution administered via intravenous injection followed by bilateral pneumothorax.

mRNA extraction and quantification in tissues

Tissues were homogenized in Trizol (Thermo Scientific), and RNA was isolated by precipitating the homogenized tissue in phenol-chloroform, washing in ethanol, and diluting in water. Reverse transcription on the extracted RNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Analysis of plasminogen concentration in plasma

The plasma plasminogen concentration was analyzed with a Pig Plasminogen ELISA Kit (ab190538, Abcam, Waltham, MA, USA) following the manufacturer's guidelines.

Western blotting

Plasma samples were reduced and heated, and 30 μg of protein was separated on 4%–15% acrylamide gradient gels (Bio-Rad). Following electrophoresis, the samples were transferred to a PVDF membrane and blocked with 5% non-fat dried milk. The membranes were treated with a primary antibody against plasminogen (1:1,500; sheep

anti-human plasminogen, Affinity Biologicals, Ancaster, ON, Canada), washed, and treated with HRP-conjugated secondary antibody (1:2,000, anti-sheep, ab7111, Abcam). Plasminogen protein was detected using ECL substrates (Bio-Rad) and imaged. Protein quantification of the bands was performed using ImageJ software. Relative intensity of plasminogen was determined using immunoglobulin G as the control.

Toxicity analysis

Serum samples were submitted to Idexx BioAnalytics (North Grafton, MA, USA) for a standard toxicology panel. Albumin, creatine kinase, GGT, total bilirubin, total protein, globulin, creatinine, BUN, AST, ALP, and ALT levels were analyzed.

Coagulation profile and ROTEM

Plasma samples were also submitted to Idexx BioAnalytics for a coagulation panel. D-dimer, prothrombin time, partial thromboplastin time, and fibrinogen were analyzed. ROTEM (Rotem Delta, Werfen S.A., Barcelona, Spain) was performed according to the manufacturer's instructions. All reagents were allowed to incubate until they reached 37°C. Swine whole blood or plasma was mixed with 20 μL of 0.2 M $CaCl_2$ and 20 μL EXTEM reagent containing tissue factor. In fibrinolysis experiments, recombinant swine tPA (ab92728, Abcam) was added to the assay mixture at a final concentration of 150 ng/mL for whole blood or 300 ng/mL for plasma. Each test was allowed to proceed for 2 h.

Statistical analysis

To ensure that a t test or analysis of variance (ANOVA) could be used between groups, 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 or with Welch's t test if the SD between groups was significantly different. Two-way ANOVA was used to compare 2 datasets over time or Welch's two-way ANOVA if the SD between groups was significantly different. Significance was designated at p values <0.05. The statistical analysis was completed using GraphPad Prism (v.9.2.0).

DATA AND CODE AVAILABILITY

There are no supplementary materials associated with this publication. All data and details on methodology are included in the main text and are available to journal subscribers. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS

We thank Dr. Kenneth Allen and the Biomedical Resource Center at the Medical College of Wisconsin for their support with conducting experiments. We also thank the animals used in this study. This work was supported by the UBC Centre for Blood Research and by a research grant from the Advancing a Healthier Wisconsin Endowment (AHW) (5520659). The figures were created with BioRender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, M.F.C., F.F., and C.J.K.; methodology, M.F.C., F.F., M.S., K.B., Y.Z., L.M.K., M.R., M.D., and C.J.K.; formal analysis, M.F.C. and F.F.; investigation, M.F.C., F.F., M.S., K.B., Y.Z., L.M.K., G.R., T.C., M.F., A.W., M.R., A.H., and M.D.; resources, M.F.C., F.F., and C.J.K.; writing – original draft, M.F.C., F.F., and C.J.K.; writing – review & editing, M.F.C., F.F., M.S., K.B., Y.Z., L.M.K., M.R., M.D., and C.J.K.; visualization, M.F.C., F.F., and C.J.K.; supervision, M.F.C., F.F., and C.J.K.; project administration, M.F.C., F.F., and C.J.K.; funding acquisition, M.F.C., M.d.M., and C.J.K.; validation, F.F.; data curation, F.F., K.B., L.M.K., and M.F.; supervision, P.R.C., M.d.M., and M.D.

DECLARATION OF INTERESTS

C.J.K., P.R.C., and K.B. are directors, shareholders, and/or co-founders of companies developing RNA therapies. 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.

REFERENCES

1. Robinson, N.B., Krieger, K., Khan, F.M., Huffman, W., Chang, M., Naik, A., Yongle, R., Hameed, I., Krieger, K., Girardi, L.N., and Gaudino, M. (2019). The current state of animal models in research: A review. Int. J. Surg. *72*, 9–13.

2. Kulkarni, J.A., Witzigmann, D., Thomson, S.B., Chen, S., Leavitt, B.R., Cullis, P.R., and van der Meel, R. (2021). The current landscape of nucleic acid therapeutics. Nat. Nanotechnol. *16*, 630–643.

3. Maynard, L.H., Humbert, O., Peterson, C.W., and Kiem, H.-P. (2021). Genome editing in large animal models. Mol. Ther. *29*, 3140–3152.

4. Yang, W., Chen, X., Li, S., and Li, X.-J. (2021). Genetically modified large animal models for investigating neurodegenerative diseases. Cell Biosci. *11*, 218.

5. Akinc, A., Maier, M.A., Manoharan, M., Fitzgerald, K., Jayaraman, M., Barros, S., Ansell, S., Du, X., Hope, M.J., Madden, T.D., et al. (2019). The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. Nat. Nanotechnol. *14*, 1084–1087.

6. Corbett, K.S., Flynn, B., Foulds, K.E., Francica, J.R., Boyoglu-Barnum, S., Werner, A.P., Flach, B., O'Connell, S., Bock, K.W., Minai, M., et al. (2020). Evaluation of the mRNA-1273 Vaccine against SARS-CoV-2 in Nonhuman Primates. N. Engl. J. Med. *383*, 1544–1555.

7. Nguyen, G.N., Everett, J.K., Kafle, S., Roche, A.M., Raymond, H.E., Leiby, J., Wood, C., Assenmacher, C.-A., Merricks, E.P., Long, C.T., et al. (2021). A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. Nat. Biotechnol. *39*, 47–55.

8. Scola, M.R., Nichols, T.C., Zhu, H., Caughey, M.C., Merricks, E.P., Raymer, R.A., Margaritis, P., High, K.A., and Gallippi, C.M. (2011). ARFI ultrasound monitoring of hemorrhage and hemostasis in vivo in canine von Willebrand disease and hemophilia. Ultrasound Med. Biol. *37*, 2126–2132.

9. Strilchuk, A.W., Hur, W.S., Batty, P., Sang, Y., Abrahams, S.R., Yong, A.S.M., Leung, J., Silva, L.M., Schroeder, J.A., Nesbitt, K., et al. (2024). Lipid nanoparticles and siRNA targeting plasminogen provide lasting inhibition of fibrinolysis in mouse and dog models of hemophilia A. Sci. Transl. Med. *16*, eadh0027.

10. Grimm, D. (2023). European researchers face monkey shortage crisis. Science *380*, 567–568.

11. Ziegler, A., Gonzalez, L., and Blikslager, A. (2016). Large Animal Models: The Key to Translational Discovery in Digestive Disease Research. Cell. Mol. Gastroenterol. Hepatol. *2*, 716–724.

12. Walker, R.L., and Eggel, M. (2020). From Mice to Monkeys? Beyond Orthodox Approaches to the Ethics of Animal Model Choice. Animals. *10*, 77.

13. Lunney, J.K., Van Goor, A., Walker, K.E., Hailstock, T., Franklin, J., and Dai, C. (2021). Importance of the pig as a human biomedical model. Sci. Transl. Med. *13*, eabd5758.

14. Käser, T. (2021). Swine as biomedical animal model for T-cell research—Success and potential for transmittable and non-transmittable human diseases. Mol. Immunol. *135*, 95–115.

15. Kulkarni, J.A., Witzigmann, D., Leung, J., Tam, Y.Y.C., and Cullis, P.R. (2019). On the role of helper lipids in lipid nanoparticle formulations of siRNA. Nanoscale *11*, 21733–21739.

16. Juang, L.J., Hur, W.S., Silva, L.M., Strilchuk, A.W., Francisco, B., Leung, J., Robertson, M.K., Groeneveld, D.J., La Prairie, B., Chun, E.M., et al. (2022). Suppression of fibrin(ogen)-driven pathologies in disease models through controlled knockdown by lipid nanoparticle delivery of siRNA. Blood *139*, 1302–1311.

17. Szebeni, J., Fontana, J.L., Wassef, N.M., Mongan, P.D., Morse, D.S., Dobbins, D.E., Stahl, G.L., Bünger, R., and Alving, C.R. (1999). Hemodynamic Changes Induced by Liposomes and Liposome-Encapsulated Hemoglobin in Pigs. Circulation *99*, 2302–2309.

18. Szebeni, J., Bedőcs, P., Rozsnyay, Z., Weiszhár, Z., Urbanics, R., Rosivall, L., Cohen, R., Garbuzenko, O., Báthori, G., Tóth, M., et al. (2012). Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. Nanomedicine *8*, 176–184.

19. Urbanics, R., Bedőcs, P., and Szebeni, J. (2015). Lessons learned from the porcine CARPA model: constant and variable responses to different nanomedicines and administration protocols. Eur. J. Nanomed. *7*.

20. Szebeni, J., Baranyi, L., Savay, S., Lutz, H.U., Jelezarova, E., Bunger, R., and Alving, C.R. (2000). The Role of Complement Activation in Hypersensitivity to Pegylated Liposomal Doxorubicin (Doxil®). J. Liposome Res. *10*, 467–481.

21. Zhou, Y., Liang, Q., Wu, X., Duan, S., Ge, C., Ye, H., Lu, J., Zhu, R., Chen, Y., Meng, F., and Yin, L. (2023). siRNA Delivery against Myocardial Ischemia Reperfusion Injury Mediated by Reversibly Camouflaged Biomimetic Nanocomplexes. Adv. Mater. *35*, e2210691.

22. Yan, S., Zheng, X., Lin, Y., Li, C., Liu, Z., Li, J., Tu, Z., Zhao, Y., Huang, C., Chen, Y., et al. (2023). Cas9-mediated replacement of expanded CAG repeats in a pig model of Huntington's disease. Nat. Biomed. Eng. *7*, 629–646.

23. Moretti, A., Fonteyne, L., Giesert, F., Hoppmann, P., Meier, A.B., Bozoglu, T., Baehr, A., Schneider, C.M., Sinnecker, D., Klett, K., et al. (2020). Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy. Nat. Med. *26*, 207–214.

24. Zhong, C., Jiang, W., Wang, Y., Sun, J., Wu, X., Zhuang, Y., and Xiao, X. (2022). Repeated Systemic Dosing of Adeno-Associated Virus Vectors in Immunocompetent Mice After Blockade of T Cell Costimulatory Pathways. Hum. Gene Ther. *33*, 290–300.

25. Collins, L.T., Ponnazhagan, S., and Curiel, D.T. (2023). Synthetic Biology Design as a Paradigm Shift toward Manufacturing Affordable Adeno-Associated Virus Gene Therapies. ACS Synth. Biol. *12*, 17–26.

26. Dézsi, L., Mészáros, T., Kozma, G., H-Velkei, M., Oláh, C.Z., Szabó, M., Patkó, Z., Fülöp, T., Hennies, M., Szebeni, M., et al. (2022). A naturally hypersensitive porcine model may help understand the mechanism of COVID-19 mRNA vaccine-induced rare (pseudo) allergic reactions: complement activation as a possible contributing factor. GeroScience *44*, 597–618.

27. Saadati, F., Cammarone, S., and Ciufolini, M.A. (2022). A Route to Lipid ALC-0315: a Key Component of a COVID-19 mRNA Vaccine. Chem. Eur J. *28*, e202200906.

28. Hou, X., Zaks, T., Langer, R., and Dong, Y. (2021). Lipid nanoparticles for mRNA delivery. Nat. Rev. Mater. *6*, 1078–1094.

29. Ferraresso, F., Strilchuk, A.W., Juang, L.J., Poole, L.G., Luyendyk, J.P., and Kastrup, C.J. (2022). Comparison of DLin-MC3-DMA and ALC-0315 for siRNA Delivery to Hepatocytes and Hepatic Stellate Cells. Mol. Pharm. *19*, 2175–2182.

30. Suzuki, Y., and Ishihara, H. (2021). Difference in the lipid nanoparticle technology employed in three approved siRNA (Patisiran) and mRNA (COVID-19 vaccine) drugs. Drug Metab. Pharmacokinet. *41*, 100424.

31. Schoenmaker, L., Witzigmann, D., Kulkarni, J.A., Verbeke, R., Kersten, G., Jiskoot, W., and Crommelin, D.J.A. (2021). mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. Int. J. Pharm. *601*, 120586.

32. Shapiro, A.D., Menegatti, M., Palla, R., Boscarino, M., Roberson, C., Lanzi, P., Bowen, J., Nakar, C., Janson, I.A., and Peyvandi, F. (2020). An international registry of patients with plasminogen deficiency (HISTORY). Haematologica *105*, 554–561.

33. Ottawa (ON) (2019). Canadian Agency for Drugs and Technologies in Health; 2019 Aug. Executive Summary. In Clinical Review Report: Patisiran (Onpattro) (Alnylam Netherlands B.V). Indication: Treatment of Polyneuropathy in Adult Patients with Hereditary Transthyretin-Mediated Amyloidosis [Internet].

34. Zhang, S., Yu, B., Liu, Q., Zhang, Y., Zhu, M., Shi, L., and Chen, H. (2022). Assessment of Hematologic and Biochemical Parameters for Healthy Commercial Pigs in China. Animals. *12*, 2464.

35. Li, R., Wang, F., Zhang, Y., Li, C., Xia, C., Chen, H., Lu, X., and Liu, F. (2019). Comparison of hematologic and biochemical reference values in specific-pathogen-free 1-month-old Yorkshire pigs and Yorkshire-Landrace crossbred pigs. Can. J. Vet. Res. *83*, 285–290.

36. Dimitrakakis, N., Waterhouse, A., Lightbown, S., Leslie, D.C., Jiang, A., Bolgen, D.E., Lightbown, K., Cascio, K., Aviles, G., Pollack, E., et al. (2022). Biochemical and Hematologic Reference Intervals for Anesthetized, Female, Juvenile Yorkshire Swine. J. Am. Assoc. Lab. Anim. Sci. *61*, 21–30.

37. Turnbull, I.C., Eltoukhy, A.A., Fish, K.M., Nonnenmacher, M., Ishikawa, K., Chen, J., Hajjar, R.J., Anderson, D.G., and Costa, K.D. (2016). Myocardial Delivery of Lipidoid Nanoparticle Carrying modRNA Induces Rapid and Transient Expression. Mol. Ther. *24*, 66–75.

38. Kamimura, K., Suda, T., Xu, W., Zhang, G., and Liu, D. (2009). Image-guided, Lobe-specific Hydrodynamic Gene Delivery to Swine Liver. Mol. Ther. *17*, 491–499.

39. Abramson, A., Kirtane, A.R., Shi, Y., Zhong, G., Collins, J.E., Tamang, S., Ishida, K., Hayward, A., Wainer, J., Rajesh, N.U., et al. (2022). Oral mRNA delivery using capsule-mediated gastrointestinal tissue injections. Matter *5*, 975–987.

40. Li, J., Kelly, S.C., Ivey, J.R., Thorne, P.K., Yamada, K.P., Aikawa, T., Mazurek, R., Turk, J.R., Silva, K.A.S., Amin, A.R., et al. (2022). Distribution of cardiomyocyte-selective adeno-associated virus serotype 9 vectors in swine following intracoronary and intravenous infusion. Physiol. Genomics *54*, 261–272.

41. Rich, K.A., Wier, C.G., Russo, J., Kong, L., Heilman, P.L., Reynolds, A., Knapp, A., Pino, M.G., Keckley, E., Mattox, L., et al. (2022). Premature delivery in the domestic sow in response to in utero delivery of AAV9 to fetal piglets. Gene Ther. *29*, 513–519.

42. Järveläinen, N., Halonen, P., Nurro, J., Hätinen, O.-P., Korpela, H., Mäkinen, P., Gan, L.-M., Fritsche-Danielson, R., and Ylä-Herttuala, S. (2021). Citrate-Saline-Formulated mRNA Delivery into the Heart Muscle with an Electromechanical Mapping and Injection Catheter Does Not Lead to Therapeutic Effects in a Porcine Chronic Myocardial Ischemia Model. Hum. Gene Ther. *32*, 1295–1307.

43. Liu, S., Li, K., Wagner Florencio, L., Tang, L., Heallen, T.R., Leach, J.P., Wang, Y., Grisanti, F., Willerson, J.T., Perin, E.C., et al. (2021). Gene therapy knockdown of Hippo signaling induces cardiomyocyte renewal in pigs after myocardial infarction. Sci. Transl. Med. *13*, eabd6892.

44. Démoulins, T., Ruggli, N., Gerber, M., Thomann-Harwood, L.J., Ebensen, T., Schulze, K., Guzmán, C.A., and McCullough, K.C. (2020). Self-Amplifying Pestivirus Replicon RNA Encoding Influenza Virus Nucleoprotein and Hemagglutinin Promote Humoral and Cellular Immune Responses in Pigs. Front. Immunol. *11*, 622385.

45. Bravo-Hernandez, M., Tadokoro, T., Navarro, M.R., Platoshyn, O., Kobayashi, Y., Marsala, S., Miyanohara, A., Juhas, S., Juhasova, J., Skalnikova, H., et al. (2020). Spinal subpial delivery of AAV9 enables widespread gene silencing and blocks motoneuron degeneration in ALS. Nat. Med. *26*, 118–130.

46. Gabisonia, K., Prosdocimo, G., Aquaro, G.D., Carlucci, L., Zentilin, L., Secco, I., Ali, H., Braga, L., Gorgodze, N., Bernini, F., et al. (2019). MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. Nature *569*, 418–422.

47. Chan, T., Grisch-Chan, H.M., Schmierer, P., Subotic, U., Rimann, N., Scherer, T., Hetzel, U., Bozza, M., Harbottle, R., Williams, J.A., et al. (2022). Delivery of non-viral naked DNA vectors to liver in small weaned pigs by hydrodynamic retrograde intrabiliary injection. Mol. Ther. Methods Clin. Dev. *24*, 268–279.

48. Aliño, S.F., José Herrero, M., Bodi, V., Noguera, I., Mainar, L., Dasí, F., Sempere, A., Sánchez, M., Díaz, A., Sabater, L., and Lledó, S. (2010). Naked DNA delivery to whole pig cardiac tissue by coronary sinus retrograde injection employing non-invasive catheterization. J. Gene Med. *12*, 920–926.

49. Bikou, O., Tharakan, S., Yamada, K.P., Kariya, T., Aguero, J., Gordon, A., Mazurek, R., Aikawa, T., Kohlbrenner, E., Fish, K.M., et al. (2022). Endobronchial Aerosolized AAV1.SERCA2a Gene Therapy in a Pulmonary Hypertension Pig Model: Addressing the Lung Delivery Bottleneck. Hum. Gene Ther. *33*, 550–559.

50. Li, L., Li, Q., Bao, Y., Li, J., Chen, Z., Yu, X., Zhao, Y., Tian, K., and Li, N. (2014). RNAi-based inhibition of porcine reproductive and respiratory syndrome virus replication in transgenic pigs. J. Biotechnol. *171*, 17–24.

51. Wei, J., Zhang, W., Li, J., Jin, Y., and Qiu, Z. (2022). Application of the transgenic pig model in biomedical research: A review. Front. Cell Dev. Biol. *10*, 1031812.

52. Perleberg, C., Kind, A., and Schnieke, A. (2018). Genetically engineered pigs as models for human disease. Dis. Model. Mech. *11*, dmm030783.

53. Boettcher, A.N., Loving, C.L., Cunnick, J.E., and Tuggle, C.K. (2018). Development of Severe Combined Immunodeficient (SCID) Pig Models for Translational Cancer Modeling: Future Insights on How Humanized SCID Pigs Can Improve Preclinical Cancer Research. Front. Oncol. *8*, 559.

54. Xie, B., Shi, X., Li, Y., Xia, B., Zhou, J., Du, M., Xing, X., Bai, L., Liu, E., Alvarez, F., et al. (2021). Deficiency of ASGR1 in pigs recapitulates reduced risk factor for cardiovascular disease in humans. PLoS Genet. *17*, e1009891.

55. Trefts, E., Gannon, M., and Wasserman, D.H. (2017). The liver. Curr. Biol. *27*, R1147–R1151.

56. Keragala, C.B., and Medcalf, R.L. (2021). Plasminogen: an enigmatic zymogen. Blood *137*, 2881–2889.

57. Drew, A.F., Kaufman, A.H., Kombrinck, K.W., Danton, M.J., Daugherty, C.C., Degen, J.L., and Bugge, T.H. (1998). Ligneous Conjunctivitis in Plasminogen-Deficient Mice. Blood *91*, 1616–1624.

58. Schott, D., Dempfle, C.-E., Beck, P., Liermann, A., Mohr-Pennert, A., Goldner, M., Mehlem, P., Azuma, H., Schuster, V., Mingers, A.-M., et al. (1998). Therapy with a Purified Plasminogen Concentrate in an Infant with Ligneous Conjunctivitis and Homozygous Plasminogen Deficiency. N. Engl. J. Med. *339*, 1679–1686.

59. Kuehn, B.M. (2021). First Treatment for Plasminogen Deficiency Is Approved. JAMA *326*, 379.

60. Ng, W., Jerath, A., and Wąsowicz, M. (2015). Tranexamic acid: a clinical review. Anaesthesiol. Intensive Ther. *47*, 339–350.

61. Cardenas, J.C., Matijevic, N., Baer, L.A., Holcomb, J.B., Cotton, B.A., and Wade, C.E. (2014). Elevated Tissue Plasminogen Activator and Reduced Plasminogen Activator Inhibitor Promote Hyperfibrinolysis in Trauma Patients. Shock *41*, 514–521.

62. Wang, I.-J., Park, S.-W., Bae, B.-K., Lee, S.-H., Choi, H.J., Park, S.J., Ahn, T.Y., Goh, T.S., Lee, M.J., and Yeom, S.R. (2020). FIBTEM Improves the Sensitivity of Hyperfibrinolysis Detection in Severe Trauma Patients: A Retrospective Study Using Thromboelastometry. Sci. Rep. *10*, 6980.

63. Swaisgood, C.M., Aronica, M.A., Swaidani, S., and Plow, E.F. (2007). Plasminogen Is an Important Regulator in the Pathogenesis of a Murine Model of Asthma. Am. J. Respir. Crit. Care Med. *176*, 333–342.

64. Shaw, M.A., Gao, Z., McElhinney, K.E., Thornton, S., Flick, M.J., Lane, A., Degen, J.L., Ryu, J.K., Akassoglou, K., and Mullins, E.S. (2017). Plasminogen Deficiency Delays the Onset and Protects from Demyelination and Paralysis in Autoimmune Neuroinflammatory Disease. J. Neurosci. *37*, 3776–3788.

65. Berri, F., Rimmelzwaan, G.F., Hanss, M., Albina, E., Foucault-Grunenwald, M.-L., Lê, V.B., Vogelzang-van Trierum, S.E., Gil, P., Camerer, E., Martinez, D., et al. (2013). Plasminogen Controls Inflammation and Pathogenesis of Influenza Virus Infections via Fibrinolysis. PLoS Pathog. *9*, e1003229.

66. Cralley, A.L., Moore, E.E., Coleman, J.R., Vigneshwar, N., Bartley, M., Kissau, D., Eitel, A., Hom, P., Mitra, S., Ghasabyan, A., et al. (2023). Hemorrhagic shock and tissue injury provoke distinct components of trauma-induced coagulopathy in a swine model. Eur. J. Trauma Emerg. Surg. *49*, 1079–1089.

67. Sukhanov, S., Higashi, Y., Yoshida, T., Danchuk, S., Alfortish, M., Goodchild, T., Scarborough, A., Sharp, T., Jenkins, J.S., Garcia, D., et al. (2023). Insulin-like growth factor 1 reduces coronary atherosclerosis in pigs with familial hypercholesterolemia. JCI Insight *8*, e165713.

68. Teramoto, N., Koshino, K., Yokoyama, I., Miyagawa, S., Zeniya, T., Hirano, Y., Fukuda, H., Enmi, J., Sawa, Y., Knuuti, J., and Iida, H. (2011). Experimental pig model of old myocardial infarction with long survival leading to chronic left ventricular dysfunction and remodeling as evaluated by PET. J. Nucl. Med. *52*, 761–768.

69. Ishikawa, K., Aguero, J., Oh, J.G., Hammoudi, N., Fish, L.A., Leonardson, L., Picatoste, B., Santos-Gallego, C.G., Fish, K.M., and Hajjar, R.J. (2015). Increased Stiffness Is the Major Early Abnormality in a Pig Model of Severe Aortic Stenosis and Predisposes to Congestive Heart Failure in the Absence of Systolic Dysfunction. J. Am. Heart Assoc. *4*, e001925.

70. Li, K., Wagner, L., Moctezuma-Ramirez, A., Vela, D., and Perin, E. (2021). A Robust Percutaneous Myocardial Infarction Model in Pigs and Its Effect on Left Ventricular Function. J. Cardiovasc. Transl. Res. *14*, 1075–1084.

71. Camacho, P., Fan, H., Liu, Z., and He, J.-Q. (2016). Large Mammalian Animal Models of Heart Disease. J. Cardiovasc. Dev. Dis. *3*, 30.

72. Tan, W., Li, X., Zheng, S., Li, X., Zhang, X., Pyle, W.G., Chen, H., Wu, J., Sun, H., Zou, Y., et al. (2021). A Porcine Model of Heart Failure With Preserved Ejection Fraction Induced by Chronic Pressure Overload Characterized by Cardiac Fibrosis and Remodeling. Front. Cardiovasc. Med. *8*, 677727.

73. Crisostomo, V., Baez, C., Abad, J.L., Sanchez, B., Alvarez, V., Rosado, R., Gómez-Mauricio, G., Gheysens, O., Blanco-Blazquez, V., Blazquez, R., et al. (2019). Dose-dependent improvement of cardiac function in a swine model of acute myocardial infarction after intracoronary administration of allogeneic heart-derived cells. Stem Cell Res. Ther. *10*, 152.

74. Velayutham, N., and Yutzey, K.E. (2022). Porcine Models of Heart Regeneration. J. Cardiovasc. Dev. Dis. *9*, 93.

75. (2018). European Medicines Agency Committee for Medicinal Products for Human Use (CHMP). In Assessment Report OnpattroAssessment Report Onpattro.

76. Moeser, A.J., Roney, A., Fardisi, M., and Thelen, K. (2022). Biological sex: an understudied factor driving disease susceptibility in pigs. J. Anim. Sci. *100*, skac146.

77. Semenas, E., Nozari, A., and Wiklund, L. (2010). Sex differences in cardiac injury after severe haemorrhage and ventricular fibrillation in pigs. Resuscitation *81*, 1718–1722.

78. Bartlett, D.W., and Davis, M.E. (2006). Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. Nucleic Acids Res. *34*, 322–333.

79. Szebeni, J. (2014). Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals. Mol. Immunol. *61*, 163–173.

80. Doessegger, L., and Banholzer, M.L. (2015). Clinical development methodology for infusion-related reactions with monoclonal antibodies. Clin. Transl. Immunol. *4*, e39.

81. Sudowe, S., Rademaekers, A., and Kölsch, E. (1997). Antigen dose-dependent predominance of either direct or sequential switch in IgE antibody responses. Immunology *91*, 464–472.

82. Arastu, A.H., Elstrott, B.K., Martens, K.L., Cohen, J.L., Oakes, M.H., Rub, Z.T., Aslan, J.J., DeLoughery, T.G., and Shatzel, J. (2022). Analysis of Adverse Events and Intravenous Iron Infusion Formulations in Adults With and Without Prior Infusion Reactions. JAMA Netw. Open *5*, e224488.

83. Fülöp, T., Kozma, G.T., Vashegyi, I., Mészáros, T., Rosivall, L., Urbanics, R., Storm, G., Metselaar, J.M., and Szebeni, J. (2019). Liposome-induced hypersensitivity reactions: Risk reduction by design of safe infusion protocols in pigs. J. Control. Release *309*, 333–338.

84. Cáceres, M.C., Guerrero-Martín, J., Pérez-Civantos, D., Palomo-López, P., Delgado-Mingorance, J.I., and Durán-Gómez, N. (2019). The importance of early identification of infusion-related reactions to monoclonal antibodies. Ther. Clin. Risk Manag. *15*, 965–977.

85. Őrfi, E., Mészáros, T., Hennies, M., Fülöp, T., Dézsi, L., Nardocci, A., Rosivall, L., Hamar, P., Neun, B.W., Dobrovolskaia, M.A., et al. (2019). Acute physiological changes caused by complement activators and amphotericin B-containing liposomes in mice. Int. J. Nanomed. *14*, 1563–1573.

86. Wang, H., Lin, S., Wu, X., Jiang, K., Lu, H., and Zhan, C. (2023). Interplay between Liposomes and IgM: Principles, Challenges, and Opportunities. Adv. Sci. *10*, e2301777.

87. Barthel, D., Schindler, S., and Zipfel, P.F. (2012). Plasminogen is a complement inhibitor. J. Biol. Chem. *287*, 18831–18842.

88. Motayagheni, N., Phan, S., Eshraghi, C., Nozari, A., and Atala, A. (2017). A Review of Anesthetic Effects on Renal Function: Potential Organ Protection. Am. J. Nephrol. *46*, 380–389.

89. Phornphutkul, K.S., Anuras, S., Koff, R.S., Seeff, L.B., Mahler, D.L., and Zimmerman, H.J. (1974). Causes of Increased Plasma Creatine Kinase Activity after Surgery. Clin. Chem. *20*, 340–342.

90. Kim, J.W., Kim, J.D., Yu, S.B., and Ryu, S.J. (2013). Comparison of hepatic and renal function between inhalation anesthesia with sevoflurane and remifentanil and total intravenous anesthesia with propofol and remifentanil for thyroidectomy. Korean J. Anesthesiol. *64*, 112–116.

91. Alstrup, A.K., Afzelius, P.M., Jensen, S.B., Leifsson, P.S., Wegener, K.M., and Nielsen, O.L. (2020). Effects of Long-term Anesthesia, Blood Sampling, Transportation, and Infection Status on Hearts and Brains in Pigs Inoculated with Staphylococcus aureus and Used for Imaging Studies. J. Am. Assoc. Lab. Anim. Sci. *59*, 74–84.

92. Hotchkiss, C.E., and Young, M.A. (2020). Comparative Risk of Human Injury/Exposure While Collecting Blood from Sedated and Unsedated Nonhuman Primates. J Am Assoc Lab Anim Sci *59*, 371–376.

93. Raoufinia, R., Mota, A., Keyhanvar, N., Safari, F., Shamekhi, S., and Abdolalizadeh, J. (2016). Overview of Albumin and Its Purification Methods. Adv. Pharm. Bull. *6*, 495–507.

94. Wiedermann, C.J. (2022). Moderator Effect of Hypoalbuminemia in Volume Resuscitation and Plasma Expansion with Intravenous Albumin Solution. Int. J. Mol. Sci. *23*, 14175.

95. Tao, W., Mao, X., Davide, J.P., Ng, B., Cai, M., Burke, P.A., Sachs, A.B., and Sepp-Lorenzino, L. (2011). Mechanistically Probing Lipid-siRNA Nanoparticle-associated Toxicities Identifies Jak Inhibitors Effective in Mitigating Multifaceted Toxic Responses. Mol. Ther. *19*, 567–575.

96. Sato, Y., Matsui, H., Sato, R., and Harashima, H. (2018). Neutralization of negative charges of siRNA results in improved safety and efficient gene silencing activity of lipid nanoparticles loaded with high levels of siRNA. J. Control. Release *284*, 179–187.

97. Zimmermann, T.S., Karsten, V., Chan, A., Chiesa, J., Boyce, M., Bettencourt, B.R., Hutabarat, R., Nochur, S., Vaishnaw, A., and Gollob, J. (2017). Clinical Proof of Concept for a Novel Hepatocyte-Targeting GalNAc-siRNA Conjugate. Mol. Ther. *25*, 71–78.

98. Breda, L., Papp, T.E., Triebwasser, M.P., Yadegari, A., Fedorky, M.T., Tanaka, N., Abdulmalik, O., Pavani, G., Wang, Y., Grupp, S.A., et al. (2023). In vivo hematopoietic stem cell modification by mRNA delivery. Science *381*, 436–443.

99. Dilliard, S.A., Cheng, Q., and Siegwart, D.J. (2021). On the mechanism of tissue-specific mRNA delivery by selective organ targeting nanoparticles. Proc. Natl. Acad. Sci. USA *118*, e2109256118.

100. Chen, Y., Tan, W., and Cheng, J. (2006). Sus scrofa plasminogen mRNA, complete cds. https://www.ncbi.nlm.nih.gov/nuccore/DQ530369.