CrossMark click for updates

PNAS PLUS

Systemic delivery of factor IX messenger RNA for protein replacement therapy

Suvasini Ramaswamy[a], Nina Tonnu[a], Kiyoshi Tachikawa[b], Pattraranee Limphong[b], Jerel B. Vega[b], Priya P. Karmali[b], Pad Chivukula[b], and Inder M. Verma[a,1]

[a]Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, CA 92037; and [b]Arcturus Therapeutics, San Diego, CA 92121

Contributed by Inder M. Verma, January 17, 2017 (sent for review November 30, 2016; reviewed by Paul E. Monahan and James M. Wilson)

Safe and efficient delivery of messenger RNAs for protein replacement therapies offers great promise but remains challenging. In this report, we demonstrate systemic, in vivo, nonviral mRNA delivery through lipid nanoparticles (LNPs) to treat a Factor IX (*FIX*)-deficient mouse model of hemophilia B. Delivery of human FIX (hFIX) mRNA encapsulated in our LUNAR LNPs results in a rapid pulse of FIX protein (within 4–6 h) that remains stable for up to 4–6 d and is therapeutically effective, like the recombinant human factor IX protein (rhFIX) that is the current standard of care. Extensive cytokine and liver enzyme profiling showed that repeated administration of the mRNA–LUNAR complex does not cause any adverse innate or adaptive immune responses in immune-competent, hemophilic mice. The levels of hFIX protein that were produced also remained consistent during repeated administrations. These results suggest that delivery of long mRNAs is a viable therapeutic alternative for many clotting disorders and for other hepatic diseases where recombinant proteins may be unaffordable or unsuitable.

lipid nanoparticles | nonviral mRNA delivery | hemophilia B therapy | systemic delivery | hepatic diseases

Aberrant gene expression is the underlying cause for many pathologies and restoring the normal state by targeting genes through expression or knockdown is conceptually a simple solution (1). RNA-based therapeutics have some inherent advantages over DNA and viral vectors but their therapeutic use has been plagued by problems of poor translatability, lack of stability, inefficient delivery, and adverse immune reactions. Incremental improvements (5′ caps, codon optimization, use of optimized 5′ and 3′ UTRs, poly(A) modifications, modified nucleosides like 5-methyl cytosine (5MC), pseudouridine and 2 thio-UTP, etc.) have substantially improved the stability and translatability of RNAs while also making them immunologically silent. Furthermore, lipid nanoparticles (LNPs) have been developed as a nonviral option to encapsulate and deliver nucleic acids in vivo.

Efficient in vivo delivery, however, has long been a major challenge because currently available LNPs can induce liver damage and stimulate an immune response (2). Lipid nanoparticles typically comprise four different lipids—an ionizable lipid, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG) lipid. When formulated into LNPs, these amine-containing ionizable lipids electrostatically complex with the negatively charged RNA to facilitate cellular uptake. These improvements have resulted in increasing use of small interfering RNA (siRNAs) as a potential therapeutic for systemic in vivo delivery to treat diseases like transthyretin amyloidosis, hepatitis B virus, hypercholesterolemia, cancer, and so forth (Arbutus, Alnylam Pharmaceuticals, Quark Pharmaceuticals, Allergan, Calando Pharmaceuticals, and others) (3). However, obvious differences between mRNAs and siRNAs in terms of length, stability, charge density, and so forth, make the synthesis, packaging, and delivery of mRNAs more challenging. Currently available LNPs also induce liver damage and elicit an immune response. Thus, despite many technological advancements, the development of mRNA as drugs for the purpose of protein replacement is still fraught with technical challenges (4–6).

In this study, we demonstrate the successful use of lipid-enabled and unlocked nucleic acid modified RNA (LUNAR), a safe, reproducible and effective LNP mRNA delivery platform that can be used to treat diseases requiring protein replacement, such as hemophilia. LUNAR is composed of four lipid components: Proprietary Arcturus Therapeutics's lipid (ATX), cholesterol, a phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and a pegylated lipid. The ATX lipid has been designed to contain an ionizable amino head group and a biodegradable lipid backbone. The ionizable amino head group renders the lipid with a pK_a of <7. At acidic pH (e.g., pH 3.5), the amino group is protonated and interacts with the negatively charged RNA, thus forming nanoparticles and encapsulating the RNA. However, at physiological pH (e.g., pH 7.4), which is above the pK_a of the amino head group, LUNAR nanoparticles bear neutral charge, thereby mitigating the toxicity commonly observed with positively charged cationic transfection vectors. The pH sensitivity of the amino head group also enables protonation of the lipid once inside the endosomes, thereby promoting their interaction with the oppositely charged anionic endosomal lipids, causing destabilization of the endosomal membrane and release of RNA payload into the cytosol. Furthermore, ester groups have been incorporated into the lipidic backbone of ATX lipids by design. Ester bonds possess good chemical stability at physiological pH but can be readily cleaved by esterases inside tissue and intracellular compartments once the cargo has been delivered. Such cleavage will result in formation of hydrophilic cleavage

Significance

Abnormal gene expression is the underlying cause for many pathological states, and restoring normalcy through overexpression or knockdown is a conceptually simple solution. Despite the advantages over DNA and viral vectors, RNA-based therapeutics have been plagued by problems of poor translatability, stability, and adverse immune reactions. Efficient in vivo delivery has also been challenging because currently available lipid nanoparticles (LNPs) can induce liver damage and elicit a strong immune response. In this study, we demonstrate the successful use of LUNAR—a safe, reproducible, and effective LNP mRNA delivery platform that can be used to treat diseases requiring protein replacement. We achieve therapeutic delivery of mRNAs in a preclinical model of hemophilia and demonstrate alleviation of disease symptoms.

Author contributions: S.R., P.C., and I.M.V. designed research; S.R. and N.T. performed research; K.T., P.L., J.B.V., and P.P.K. contributed new reagents/analytic tools; P.L. generated the F9 and Luc mRNAs; J.B.V. and P.P.K. generated and formulated mRNAs into LUNAR LNPs; P.C. developed LUNAR technology; S.R. analyzed data; and S.R. and I.M.V. wrote the paper.

Reviewers: P.E.M., Shire; and J.M.W., University of Pennsylvania Perelman School of Medicine.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. Email: verma@salk.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1619653114/-/DCSupplemental.

MEDICAL SCIENCES

products that can be rapidly metabolized. Taken together, these design features make LUNAR nanoparticles a safe and potent RNA delivery system.

Hemophilia B is a genetic bleeding disorder caused by mutations in the gene that encodes coagulation factor IX (*FIX*) (7). Patients with dysfunctional FIX protein are unable to form normal clots and are susceptible to life-threatening bleeds that occur either spontaneously or as a result of minor injuries (8). They also suffer from recurrent bleeding into joints and muscles, which leads to significant joint pain, deformities, and loss of mobility (7). Currently, patients with hemophilia B are treated prophylactically or after a bleed with i.v. dosing of plasma-derived or recombinant human FIX protein (rhFIX) (7). Both these products are 90% effective in stopping hemorrhages (8). However, both have a short half-life of 18–24 h and must be given two to three times a week to prevent hemorrhages and secondary symptoms, such as hemophilic arthropathy (9) (8–10). This means that patients on prophylactic treatment plans require indwelling venous ports and are at risk for developing infection, sepsis, and thrombosis. Patients may also experience an anaphylactic reaction to recombinant or plasma-derived FIX and can commonly develop antibodies that prevent the injected FIX proteins from forming clots (7, 11).

Hemophilia B is an ideal candidate for protein replacement therapy via mRNA treatment because it is caused by a single defective protein that is normally produced by the liver and secreted into the bloodstream (8). Furthermore, very small amounts of WT hFIX protein (5–10% of normal levels) are needed to prevent symptoms in human hemophilia B patients (12). Functional Factor IX protein also undergoes O- and N-linked glycosylation, β-hydroxylation, and γ-carboxylation and all these posttranslational modifications best occur in the host hepatocytes (the natural site of FIX production and secretion). In this study, we achieve delivery of mRNAs to the liver via LUNAR LNPs at therapeutic levels in a preclinical model of hemophilia.

Results

A Lipid Nanoparticle Containing Modified RNA Is Safe and Effectively Delivers RNA to the Liver. To harness the potential of mRNA-based therapy, we developed a lipid nanoparticle delivery system, LUNAR. We tested the in vivo efficiency of this system by using LUNAR to deliver siRNA against factor VII to mice and achieved up to 97% down-regulation of the target protein as detected by an ELISA (Fig. 1*A*). Compared with other lipid formulations from industry leaders like DLin-MC3-DMA or MC3 [heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate], LUNAR is five times more efficient. MC3 is the ionizable lipid component of Patisiran, which is currently in clinical trials for treatment of transthyretin-mediated familial amyloid polyneuropathy (FAP) (13, 14). Additionally, doses of up to 10 mg/kg did not cause any increase in circulating aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels (markers associated with acute liver toxicity), thus establishing the safety of this technology (Fig. 1*B*).

We next delivered LUNAR:GFP mRNA in a single dose through an i.v. injection in male, (6- to 8-wk-old) C57BL/6 mice and saw increased GFP expression after 24 h in a dose-dependent manner in the hepatocytes (Fig. 1*C*). This result shows that unlike viral vectors, which require an additional transcriptional step, LUNAR:GFP mRNA is rapidly and efficiently translated in hepatocytes into a functional protein.

We also benchmarked mRNA delivery with our LUNAR technology against the well-known MC3 LNP formulation. As shown in Fig. 1*D*, when administered to 7-wk-old female balb/c mice, at 6 h postdosing, LUNAR:mRNA formulation resulted in up to twofold more hFIX protein compared with the MC3–mRNA LNP formulation (2 mg/kg dose).

We then examined the biodistribution of LUNAR–LNPs in vivo using LUNAR encapsulated luciferase at a final dose of 0.5 mg/kg. Imaging with Xenogen in vivo imaging systems (IVIS) at 5-h postinjection showed most of the Luc mRNA was delivered to the liver where robust expression was seen (Fig. 1*E*). The spleen, as part of the reticuloendothelial system, gave a weak luciferase signal but the other organs, namely the heart, lungs, and kidney, showed undetectable levels of luciferase expression. These data suggest that the LUNAR–mRNA LNPs are more selectively delivered to the liver.

LUNAR-Delivered Human FIX mRNA Can Restore Normal Clotting Activity in Hemophilic Mice. We went on to test whether LUNAR-delivered mRNA could treat hemophilia in FIX knockout ($FIX^{-/-}$) mice. These mice lack circulating hFIX protein, exhibit prolonged clotting times, and show less than 5% WT FIX activity in the one-stage FIX activity test (a physiologically relevant assay to measure clotting activity) assay (15) (Fig. 2*A*). Analysis of the mouse plasma from our $FIX^{-/-}$ deficient strain by a Western blot further confirms the lack of hFIX protein, thus confirming that the delayed clotting in a one-stage FIX activity test assay is indeed due to lack of circulating FIX (Fig. 2*A* and the panel below). We i.v. administered synthetic human FIX mRNA encapsulated in LUNAR LNPs (LUNAR:hFIX) at a dose of 2 mg/kg in 8- to 12-wk-old $FIX^{-/-}$ males. ELISA results show that animals treated with LUNAR:hFIX had serum levels of over 2,500 ng/mL of hFIX protein at 6, 12, and 24 h postinjection (Fig. 2*B*). These levels are within normal physiological range (4–5 μg/mL in human plasma and 2–3 μg/mL in mouse plasma) and well above the clinically acceptable level of 1% (equivalent to 50 ng/mL). We further confirmed that the circulating FIX protein was also functional and alleviated the clotting defect as measured by a one-stage FIX activity test (Fig. 2*C*). These increased levels corresponded with a therapeutic rescue of the clotting defect, because it represents about 45% of WT FIX activity levels at 6 and 12 h postinjection. Clotting activity stayed around 30% at 48 h postinjection, whereas the circulating hFIX protein levels had dropped to 780 ng/mL. Symptoms in patients with hemophilia B can be managed and severe joint damage avoided with prophylactic doses that maintain a little over 1% of WT clotting activity (16). We further confirmed the hFIX protein levels as reported by our ELISA using a Western blot that detected a ∼55-kDa band, as expected (Fig. 2*D*).

After a 2-wk wash-out period, we examined whether we could achieve a longer therapeutic effect by increasing the treatment dose to 4 mg/kg in the same cohort of $FIX^{-/-}$ animals. We detected a significant increase in circulating hFIX protein levels that corresponded with a sixfold increase in FIX protein activity at 48 h postinjection, and clotting activity remained higher than the therapeutically relevant levels of 10% even at 144 h postinjection (*SI Appendix*, Fig. S1 *A* and *B*). It thus appears that repeated administration of LUNAR:hFIX can induce consistent and therapeutic increases in clotting ability for up to 6 d after one administration.

Hyperfunctional Variants of FIX Can Further Extend the Therapeutic Effect. Two catalytically enhanced FIX variants, R338A-hFIX and R338L-hFIX, have been shown to induce a 3-fold and 6- to 10-fold increase in clotting activity, respectively, compared with WT hFIX (12, 17). We explored whether these hyperfunctional variants could further extend and improve the therapeutic efficiency of our LUNAR–mRNA formulation. We formulated LUNAR LNPs with WT, R338A, or R338L-hFIX mRNA and delivered 4 mg/kg of each i.v. into 12- to 16-wk-old $FIX^{-/-}$ males. We measured clotting activity at 48 h postinjection and, as previously reported, clotting function was dramatically enhanced for both the R338A (115%) and R338L (88%) variants compared with the WT protein (20%) (Fig. 3*B*). ELISA (Fig. 3*A*) and Western blot analysis (Fig. 3*B*) found that although the WT protein was present in greater amounts (both in the liver and in systemic circulation) compared with the R338A and R338L

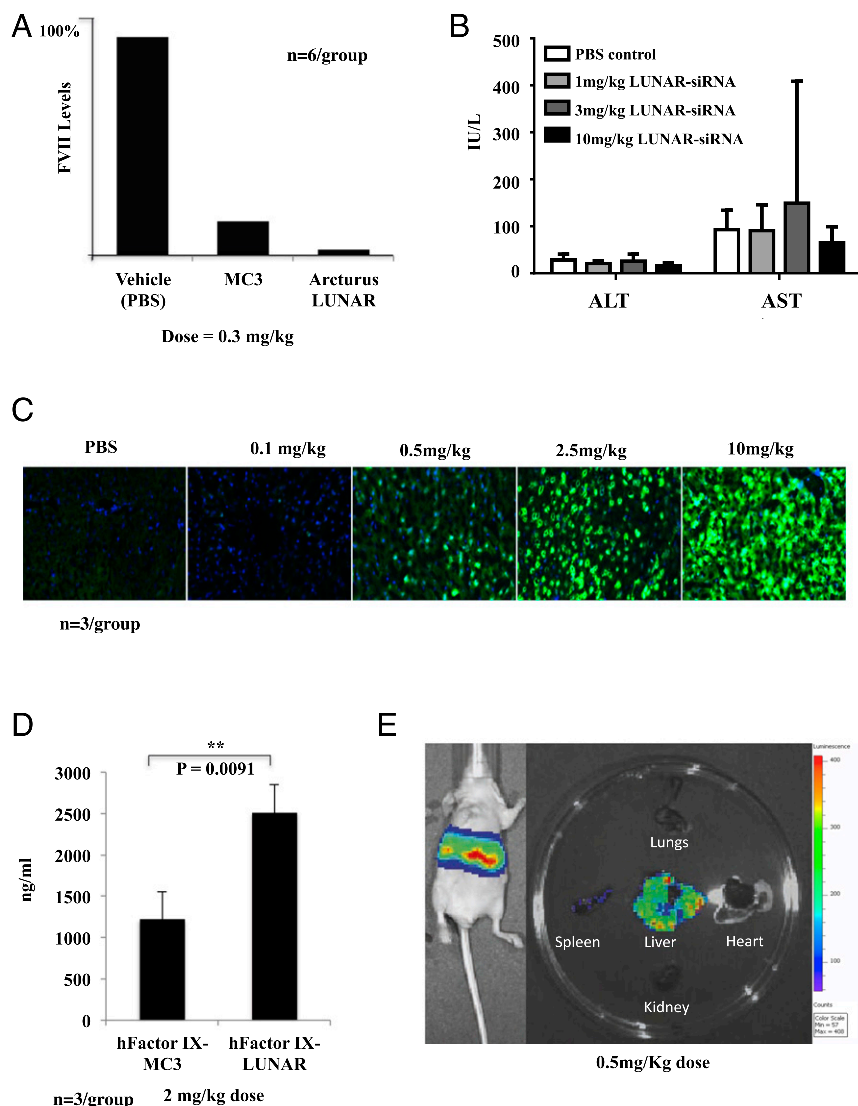

Fig. 1. Lipid-enabled and unlocked nucleic acid modified RNA (LUNAR) is safe and effectively delivers RNA to the liver. (*A*) Comparing the knockdown efficiencies of LUNAR:FVII siRNA formulation with MC3 (a lipid formulation currently approved for clinical use) and vehicle (PBS). At a dose of 0.3 mg/kg, LUNAR:FVII siRNA gave up to 97% knockdown of FVII protein levels in mouse serum, which is up to five times more than the levels achieved by MC3 ($n = 6$). (*B*) The LUNAR:FVII siRNA treatment of doses up to 10 mg/kg in five male and five female CD-1 mice did not cause significant elevations in AST/ALT levels, compared with saline controls. Serum was collected 48 h postdose for clinical chemistry (performed on a clinical chemistry analyzer at Contract Research Lab BTS Research, Inc.). (*C*) LUNAR:GFP mRNA complexes were i.v. (tail vein) administered to 6- to 8-wk-old male C57Bl6 mice at doses of 0.1, 0.5, 2.5, and 10 mg/kg ($n = 3$ per group). At 24 h posttreatment, the animals were killed and livers were flash frozen for immunofluorescence analysis. Tissue sections imaged at 4× magnification are shown. (*D*) In vitro transcribed hFIX mRNA, packaged in the LUNAR and MC3 formulations, was administered once to 7-wk-old female balb/c mice at 2 mg/kg ($n = 3$ per group). The animals were bled at 6-h postdosing and the serum FIX levels were assessed by an ELISA (Assay Pro: EF1009-1) at 1:200 dilution as per the manufacturer's instructions. As can be seen, the LUNAR formulation was up to two times more efficient than the clinically approved MC3 lipid formulation. (*E*) Luc mRNA encapsulated in LUNAR LNPs was administered to mice at a dose of 0.5 mg/kg ($n = 3$). Mice were imaged on an IVIS system 5-h postinjection following which they were killed and tissues were also imaged ex vivo. LUNAR-delivered mRNA was concentrated in the liver.

variants (Fig. 3 *A* and *C*), its therapeutic efficacy was lower. Thus, it seems that despite lower levels of functional protein in circulation, the R338A mRNA has a higher therapeutic efficacy in vivo.

LUNAR:R338A FIX Is More Effective Than Recombinant Human FIX Protein Over Multiple Administrations. To compare the therapeutic efficacy of repeated doses of LUNAR:R338A hFIX mRNA with the current standard of care, we treated FIX$^{-/-}$ male and female mice with either recombinant human hFIX protein (Benefix, Pfizer) at the recommended dose of 200 IU/kg or LUNAR:R338A at 4 mg/kg. This treatment was repeated three times at 10-d intervals. LUNAR:R338A hFIX mRNA treatment induced 8–10 times more circulating hFIX protein levels and clotting activity than Benefix treatment at 24 h postinjection (Fig. 4 *A* and *B*). By 96 h postinjection, both circulating FIX protein levels and clotting activity had returned to baseline in Benefix-treated animals. In contrast, LUNAR:hFIX mRNA-treated animals still showed 20% clotting activity at this time point (Fig. 4*C*). Such levels are enough to maintain asymptomatic physiological status under normal conditions and to provide the benefits of prophylactic therapy (18, 19). By 240-h or 10-d posttreatment, both treatment groups had returned to the baseline hFIX protein levels and clotting activity. The treated animals were redosed twice again and the results were consistent across the three treatment cycles (Fig. 4 *A*–*C*). We thus find that,

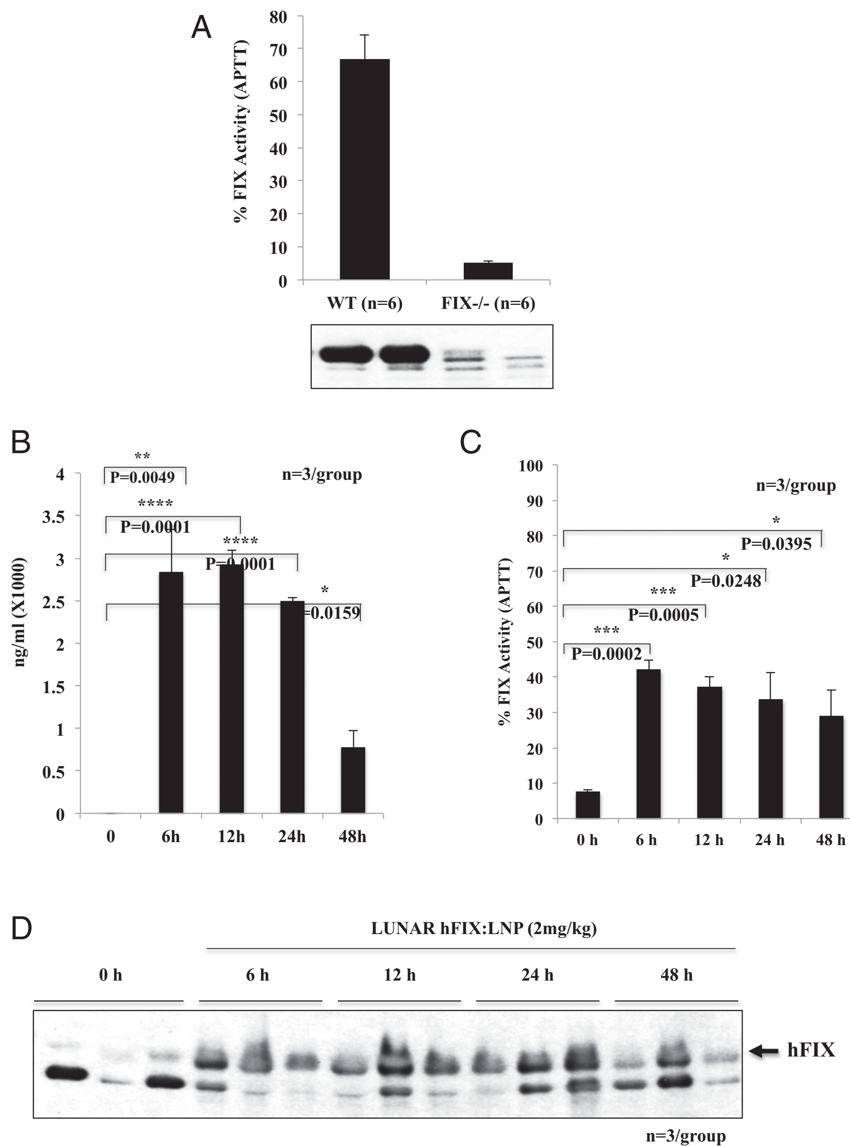

Fig. 2. LUNAR-delivered human FIX-mRNA can restore normal clotting activity in hemophilic mice. (*A*) FIX activity for the WT and hemophilic mice measured by clotting time in an APTT assay (percentage of activity calculated based on a standard curve generated from serial dilutions of pooled normal serum). Panel below shows the Western blot for FIX protein in the WT and hemophilic mouse serum. $FIX^{-/-}$ mice had less than 5% of WT FIX activity. (*B*) ELISA for circulating hFIX in $FIX^{-/-}$ animals after i.v. delivery of hFIX mRNA encapsulated in LUNAR LNPs (dose = 2 mg/kg; n = 3). Significance was tested using a two-tailed Student's t test. (*C*) FIX activity upon injection of hFIX mRNA:LUNAR LNPs as measured by clotting time in an APTT assay. Significance was tested using a two-tailed Student's t test (dose = 2 mg/kg; n = 3). (*D*) Circulating hFIX levels in the serum of hemophilic mice over a 24-h time course after i.v. delivery of hFIX mRNA:LUNAR LNPs at a dose of 2 mg/kg.

whereas rhFIX maintains therapeutic efficacy for anywhere from 24 to 72 h, the mRNA:LNP treatment is more effective. Because LUNAR:hFIX mRNA provides therapeutic efficacy for 6 d (*SI Appendix*, Fig. S1 *A* and *B*), these data suggest that LUNAR-mediated delivery of R338A hFIX mRNA can provide longer and higher therapeutic efficacies than the current standard of care without eliciting a dampening immune response.

Moreover, neither treatment caused any overt adverse events or weight loss over the 30-d treatment period (Fig. 4*D*) and repeated LUNAR:R338A treatment did not cause liver damage as measured by circulating AST, ALT, and ALP levels (20) (*SI Appendix*, Fig. S2). These preliminary data suggest that our LUNAR mRNA delivery platform is a safe and effective approach for long-term protein replacement therapies. Whereas there is no evidence of overt toxicity, more studies need to be done to comprehensively evaluate the safety of this technology in larger mammals with a different hepatic metabolism.

LUNAR-Delivered FIX mRNA Is Safely Targeted to the Liver and Does Not Elicit Adverse Immune Reactions. Restoration of FIX activity following administration of the FIX mRNA:LNPs prompted us to examine the kinetics, biodistribution, and immune toxicity of these LUNAR-LNPs. To evaluate both the acute and chronic effects of LUNAR-LNPs, a single cohort of Factor IX-deficient mice was treated with WT hFIX:LUNAR LNPs at 6 and 8 wk of age, respectively. At 20 wk of age, we i.v. administered this same cohort with FIX and Luc mRNAs encapsulated at a 1:1 ratio in the LUNAR LNPs at a final dose of 4 mg/kg (2 mg/kg each) and assessed tissue distribution based on a luciferase read-out. The luciferase signal, acting as a proxy for localization of the hFIX mRNA, was concentrated in the liver at 7 h postinjection (Fig. 5*A*)

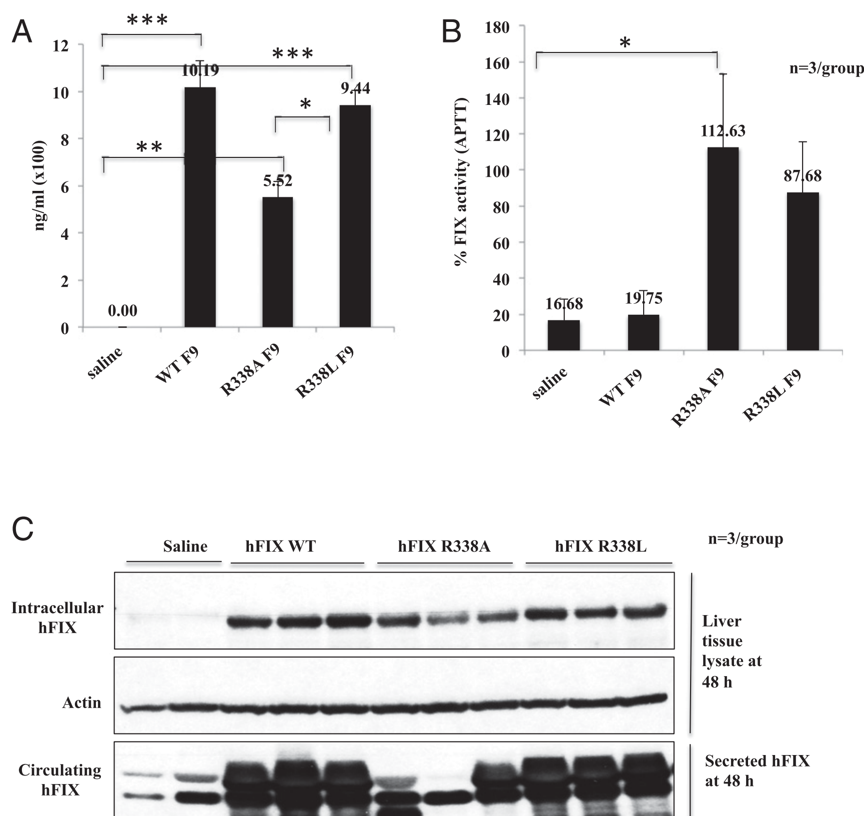

Fig. 3. Hyperfunctional variants of mRNA extend the therapeutic efficacy. (*A*) ELISA for circulating hFIX, hFIX R338A, and hFIX R338L in $FIX^{-/-}$ animals after i.v. delivery of the three different mRNAs encapsulated in LUNAR LNPs (dose = 4 mg/kg; n = 3). An ANOVA and post hoc Tukey's were used to test for significant differences between groups. (*B*) Clotting efficiency of the three variant hFIX mRNAs (WT, R338A, and R338L) in mouse serum were determined by an APTT assay at 48 h after i.v. delivery of the mRNA-LUNAR LNP formulation. The hyperfunctional variants R338A and R338L exhibited greater therapeutic efficiency than the WT protein, i.e., they restored clotting efficiency to 100% with lower amounts of circulating protein (based on Western, *C* and ELISA, *A*). An ANOVA and post hoc Tukey's were used to test for significant differences between groups. (*C*) Western blot to examine hFIX levels both in the liver (where they are synthesized) and in the serum (where they are functional). Upon i.v. administration of the WT and variant hFIX mRNAs, hFIX protein can be detected in protein lysates from the liver (*Uppermost* vs. *Middle*) and from the serum (*Lowermost*). The WT variant was produced and secreted into circulation at amounts significantly higher than that of the R338A variant.

and consistent with our previous experience, the signal gradually declined by 48 h. Levels of circulating hFIX protein were measured by a Western blot (Fig. 5*B*) and an ELISA (Fig. 5*C*) on mouse serum samples. As observed before (Fig. 2), clotting activity was also significantly increased at times as early as 4 and 7 h postinjection (Fig. 5*D*).

Because patients with hemophilia B can develop antibodies to recombinant or plasma-derived FIX protein, which renders the treatment less effective over time (7), we also tested the safety and efficiency of repeated LUNAR:hFIX treatment over this 4-mo period, long enough for any adaptive immune response to occur. We monitored the levels of circulating FIX protein through an ELISA and a Western blot and found no significant reduction following repeat administrations (Fig. 5 *B* and *C*). The one-stage FIX activity test assay, as a measure of clotting efficiency also remained comparable to the early administrations, thus suggesting no dampening in protein levels or function due to adverse adaptive immune responses.

We did not note any gross adverse effects or weight loss in any of the treated animals over the 4-mo period as they gained weight and grew normally (Fig. 5*E*). Unlike Fig. 4*D*, increase in weight is due to the fact that the injected animals are younger (6 wk vs. 5 mo). At 7, 24, and 48 h after this third administration, animals were killed and their livers were histopathologically examined for any gross pathological changes. Barring minor necrotic lesions in two animals, we did not find any additional abnormalities, thus lending support to the safety of LUNAR LNPs with repeated administration (Fig. 5*F*). We also analyzed the cytokine profile of these animals as markers of strong adaptive or innate immune responses. Using a multiplexed cytokine assay (Bio-Plex, Bio-Rad) we found that administration of LUNAR:hFIX mRNA did not elicit any strong innate or adaptive immune responses. As can be seen in *SI Appendix*, Fig. S3, levels of proinflammatory cytokines like TNFα and IFNγ did not rise sharply over a 48-h period following administration. Cytokines like IL-6, MIP-1β (macrophage inflammatory protein-1β), RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemotactic protein-1), G-CSF (granulocyte-colony stimulating factor), KC [chemokine (C-X-C motif) ligand 1], and GM-CSF (granulocyte-macrophage colony-stimulating factor) showed increases at 4–7 h after administration but returned to the baseline within 24 h, suggesting absence of any long-term repercussions. These preliminary results confirm that repeated LUNAR:hFIX treatment is safe, does not elicit a dampening immune response, and is thus a suitable alternative for protein therapy.

Discussion

Messenger RNA-based therapies are attractive because, unlike DNA, mRNA does not need to enter the nucleus, and so does not carry any risk of random integration or mutagenesis and can be translated into a functional protein once it has breached the cell membrane. The delivery of messenger RNA to host cells also

Fig. 4. R338A FIX mRNA:LUNAR is therapeutically more effective than recombinant human FIX protein, which is the current standard of care. (*A*) Animals were administered the rhFIX or LUNAR:hFIX mRNA every 10 d for a total of three repetitions (with two males and two females in each group). Animals showed higher levels of FIX protein in the serum, as measured by an ELISA; when administered, the LUNAR:R338A hFIX mRNA LNPs compared with recombinant human FIX protein. The exogenously administered FIX was cleared from the system in 10 d (or 240 h) at which point the next dose was administered. (*B*) Administration of LUNAR R338A hFIX mRNA LNPs gave higher and longer therapeutic levels of functional FIX protein in the serum. Over the three rounds of injections, the LUNAR:R338A hFIX mRNA LNPs formulation gave higher therapeutic efficiency at both 24 and 96 h. The clotting activity was restored to baseline levels at 10 d postadministration, suggesting that the LUNAR LNP formulation remained therapeutically efficient for 4–9 d in vivo, whereas the recombinant protein remained effective for 1–3 d. (*C*) We also analyzed the cumulative APTT activity over the three rounds of repeat administrations and compared the therapeutic efficacy of the rhFIX protein with our LUNAR-mRNA LNP formulation. At 24 h, the LUNAR-mRNA administration resulted in dramatically high levels of therapeutic clotting activity compared with the rhFIX protein (130% vs. 20%). Even at 96 h, while the rhFIX protein was no longer therapeutically efficient, the hFIX mRNA:LUNAR LNP formulation gave significantly higher clotting efficiency (~20%), enough to rescue the phenotypic defect. (*D*) Additionally, over the course of the 30 d, as animals were given repeated doses of the rhFIX or the LUNAR:R338A hFIX, the animals remained healthy and their body weights remained stable.

ensures accurate translation and posttranslational modifications of the target therapeutic protein. The short-term stability of RNAs also limits potential side effects as the normal cellular metabolic pathways can restore the system to baseline fairly quickly. Production of the in vitro transcribed mRNAs is also relatively simple, inexpensive, scalable, and reproducible once the variables are standardized.

Whereas systemic delivery of siRNAs for therapy has been achieved many times and is in clinical use (Alnylam and Arbutus), the delivery of longer messenger RNAs remains an attractive, yet challenging therapeutic prospect. It has been limited due to the strong immunogenicity and limited stability of naked RNA in vivo, both of which are critical for the long-term, systemic administration that is needed for most protein replacement therapies.

LNPs are the most common nonviral vectors because they are easy to prepare, versatile, biocompatible, and do not cause insertional mutagenesis (2). Despite their promise, older LNP formulations have lower transfection rates, induced cytokine release, stimulated the toll-like receptor-mediated immune response, and damaged liver tissue (2). In fact, most studies avoid repeat administration of LNPs and tend to ignore a rigorous study of liver function after acute and chronic administration. Previous reports (5, 6) have tested the use of mRNA therapeutics in vivo through

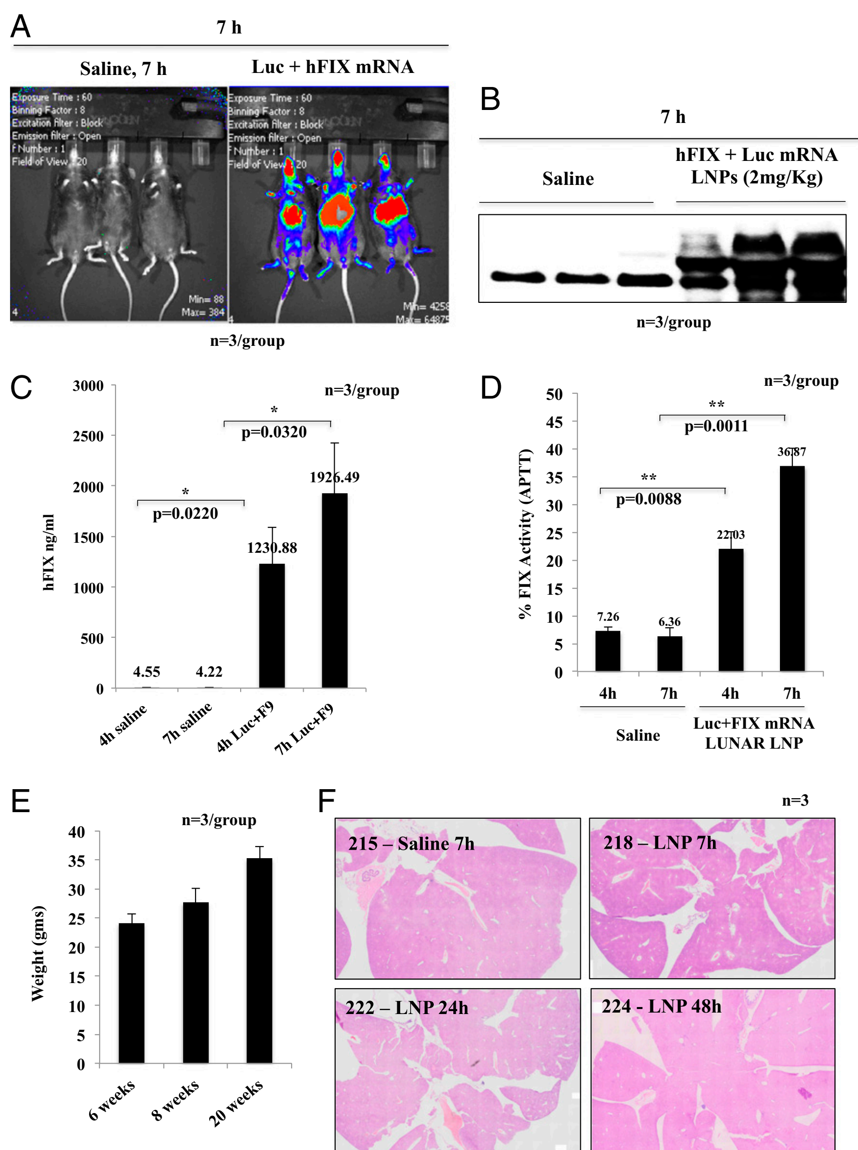

Fig. 5. Repeated dosing over a 3- to 4-mo period does not elicit adverse immune reactions. A small cohort of $FIX^{-/-}$ animals ($n = 3$ per group) was dosed three times with the mRNA:LUNAR LNP formulation over a period of 20 wk. The first two injections were 2 wk apart (WT hFIX mRNA:LUNAR LNP), whereas the last injection was after a 3-mo interval (and a 1:1 mix of FIX and luciferase mRNAs at a final dose of 4 mg/kg). At the third injection, the animals were examined for biodistribution and kinetics of the hFIX mRNA using the luciferase signal as a proxy for the localization of the LUNAR-hFIX. (*A*) Intravital imaging system (IVIS) from Xenogen was used to image animals at 7 h after LUNAR-hFIX + Luc mRNA administration. Animals were imaged at 15 min after administration of 15 mg/mL luciferin. As can be seen, most of the delivered LNPs enter the liver and are expressed there at this time point. (*B*) Hemophilic animals injected with the Luc + hFIX mRNA:LUNAR LNP complexes were bled at the indicated time points and the serum was assayed for the presence of human FIX protein by a Western blot. Circulating levels of FIX protein peak early by 4–7 h postinjection. (*C*) Levels of circulating hFIX in the mouse serum were measured by an ELISA. Levels are reported in nanograms per milliliter based on a standard curve generated from serial dilution of a known standard. (*D*) The serum from these animals was assayed for clotting efficiency by an APTT assay. The level of FIX protein produced at 4 h is enough to achieve therapeutic efficacy at up to 20% of normal levels. Despite repeat dosing, therapeutic levels of FIX are attained reproducibly and consistently, suggesting no antibody or cell-mediated neutralization of the LUNAR-hFIX mRNA LNPs or the FIX protein. (*E*) As a proxy for any toxicity, body weights were tracked during this period and the animals, being young, demonstrated normal weight gain. (*F*) At the end of the third administration, mouse livers were fixed, sectioned, stained with H&E, and examined for any histopathological abnormalities. Normal tissue architecture was seen in most cases, suggesting no adverse events.

direct injection into the heart (VEGF mRNA for myocardial infraction) and aersolization into the lung (surface protein B). These studies, however, use naked, chemically modified mRNA and this poses significant hurdles for long-term, systemic administration that is needed for most protein replacement therapies. Another study has also reported the delivery of longer mRNAs through their LNP delivery platform (21); however, the therapeutic effect lasted for only 2 d and safety with repeat administration was not tested.

To address these problems and improve safety and tolerance in vivo, we incorporated biodegradable ionizable lipids (ATX) in our LUNAR technology. Here we show that the LUNAR-delivery of mRNAs is safe and well tolerated in immunocompetent mice even after repeat dosing over a period of 4 mo. Animals that received multiple doses showed no adverse events, hepatotoxicity, weight loss, or innate or adaptive immune reactions in response to treatment. LUNAR-encapsulated mRNAs are preferentially targeted to the liver, which is the site for many physiological

functions, and is also a relevant target for many genetic diseases such as hemophilia, Niemann-Pick type C2 (NPC2), factor VII deficiency, α-1-antitrypsin deficiency, and familial tyrosinemia (22, 23). Expression in the native environment is also expected to confer accurate posttranslational modifications and a greater systemic immune tolerance (23). Furthermore, we demonstrate proof of concept for the therapeutic use of this LUNAR-mRNA technology by using it to treat hemophilia B in our FIX-deficient mouse model.

Hemophilia B is a debilitating disease for which there is no cure. Current standard of care requires frequent (two to three times per week) i.v. dosing with plasma-derived or recombinant FIX protein and carries significant risk of side effects, including allergic and anaphylactic responses, infection, and sepsis (7, 9, 11). Whereas the recombinant purified protein is safer than the plasma-derived concentrates or cryoprecipitates in terms of the risk of blood-borne infections, and so forth, it is expensive and often in limited supply. The problem is even more acute in emerging economies where financial constraints place a significant burden. Patients can also develop antibodies against the exogenous FIX, which can lower the therapeutic efficiency and prevent the protein from initiating clot formation (9). A number of new-generation FIX products, with longer half-lives through PEG-ylation or fusion to IgG or albumin, are in clinical trial or have been recently approved (7). However, they too, cannot be predicted to completely prevent the formation of neutralizing antibodies. Agents that promote thrombin formation without requiring factors VIII and IX are also in clinical trials, but these have an increased risk of thrombosis (7).

Hemophilia B is an ideal candidate for mRNA therapy, because, the disease is caused by a single malfunctioning protein and low levels of protein replacement (1–5% of WT FIX protein levels) can prevent the majority of symptoms in hemophilic patients (12). Delivery of hFIX mRNA through viral vectors [adenovirus and adeno-associated virus (AAV)] can increase circulating levels of FIX protein, increase FIX activity, and decrease bleeding in both mice and humans (12, 24). Six early-stage clinical trials are currently underway, testing viral vector gene therapy in patients with hemophilia (7). However, AAV vectors can cause hepatitis and immune-mediated hepatocyte destruction in human patients (17, 24). Viral vectors also carry a small, albeit definite risk of insertional mutagenesis (25). The preexistence of antibodies against these viral vectors in the patients further complicates the problem and imposes significant restrictions on the treated population.

We show here that LUNAR-encapsulated hFIX mRNA is effectively delivered to the liver, translated into functional FIX protein by hepatocytes, and released into the circulation where it restores clotting activity to therapeutic levels. We found that this mRNA therapy could rapidly alleviate the clotting defect seen in $FIX^{-/-}$ mice (within 4 h of administration), and this therapeutic effect maintains the animals in an asymptomatic state for up to 6 d postinjection. More importantly, the animals maintained similar and sustained levels of circulating hFIX protein and clotting activity in response to repeat administrations, suggesting an absence of inhibitory antibodies against the hFIX protein.

We also found that we could increase the therapeutic effect by using the hyperactive FIX mRNA variants R338A and R338L hFIX. Previous reports showed that adenovirus-encapsulated R338A-FIX or R338L-FIX mRNA could increase clotting activity in a one-stage FIX activity test by 3-fold and 6- to 10-fold, respectively, compared with WT FIX, without causing thromboses in major organs or eliciting an immune response (12, 17). We found similar results suggesting that lower doses of a hyperactive FIX mRNA variant could be used to achieve the same therapeutic response. A lower dose would further lower the risk of side effects and the cost of drug production. We then compared the R338A mRNA:LUNAR LNPs against the recombinant human FIX protein (which is the current standard of care) and found it to be 8–10 times more therapeutically effective.

We believe this mRNA therapeutics-based approach to treating hemophilia B could be translated to other hepatic diseases where purification and delivery of accurately modified recombinant proteins may be expensive or technically challenging. Additionally, in vitro transcription (IVT) mRNA can be manufactured at relatively low costs and the production and purification processes are scalable and robust once standardized (1). The production costs for GMP (good manufacturing practice) batches are also 5- to 10-fold lower for IVT mRNA than for recombinant protein therapeutics produced in eukaryotic cells. Scale up of the LUNAR nanoparticle delivery system has currently been established up to multigram scale that can support early clinical evaluation. Based on these results, we also feel that our ability to deliver long mRNAs to the liver will allow us to explore many new mRNA-based therapeutic options, not only for protein replacement, but also for allergy tolerization, infectious disease vaccines, and cancer immunotherapy (1).

Materials and Methods

Synthesis of ATX. Di((Z)-non-2-en-1-yl) 8,8′((tertbutoxycarbonyl)azanediyl) dioctanoate (13.85 mmol, 9 g) was dissolved in dry dichloromethane (DCM) (150 mL). Trifluoroacetic acid (TFA) was added at 0 °C to initiate a reaction. The reaction temperature was slowly allowed to warm to room temperature for 30 min with stirring. TLC showed that the reaction was completed. The reaction product was concentrated under vacuum at 40 °C and the crude residue was diluted with DCM and washed with a 10% (wt/vol) $NaHCO_3$ solution. The aqueous layer was reextracted with DCM, and the combined organic layers were washed with brine solution, dried over Na_2SO_4, and concentrated. The collected crude product was dissolved in dry DCM (85 mL) under nitrogen gas. Triphosgene was added and the reaction mixture was cooled to 0 °C, and Et_3N was added dropwise. The reaction mixture was stirred overnight at room temperature. TLC showed that the reaction was completed. DCM solvent was removed from the reaction mass by distillation under N_2. The reaction product was cooled to 0 °C, diluted with DCM (50 mL), and 2-(dimethylamino)ethanethiol HCl (0.063 mol, 8.3 g) was added, followed by Et_3N (dry). The reaction mixture was then stirred overnight at room temperature. TLC showed that the reaction was completed. The reaction product was diluted with 0.3 M HCl solution (75 mL), and the organic layer was separated. The aqueous layer was reextracted with DCM, and the combined organic layers were washed with 10% (wt/vol) K_2CO_3 aqueous solution (75 mL) and dried over anhydrous Na_2SO_4. Concentration of the solvent gave a crude mass of 10 g. The crude compound was purified by silica gel column (100–200 mesh) using 3% MeOH/DCM. The yield was 3.1 g.

Generation and Maintenance of FIX-Deficient Mice. The FIX-deficient hemophilic mice were previously generated as described in ref. 15. The targeting vector was constructed by inserting the 7.2-kb XhoI–BstBI fragment into the NotI site of the pPNT (mammalian expression plasmid with a PGK promoter) upstream of the phosphoglycerate kinase (PGK)-neomycin (Neo) cassette, and the 5.5-kb BamHI fragment that is downstream of the coding region into the BamHI site of the pPNT. The targeting vector was linearized with NotI and introduced into the 129Sv embryonic stem (ES) cell line by electroporation, and stable transfectants were selected. Individual ES clones were screened for homologous recombination by Southern blot analysis with an 800-bp fragment as a probe. Positive ES clones were injected into C57BL6 blastocysts as described, and the resulting chimeric males were bred to C57BL6 and 129Sv to establish an inbred line of mutant mice. Genotypes of mice were established by Southern blot hybridization using tail biopsy DNA. An inbred line of hemophilic mice was established through repeated crossing for more than seven generations. Hemophilic offspring were identified by genotyping using real-time PCR. Hemophilia B mice used in this study were 8–20 wk old (16–35 g). Purified human FIX (Benefix, Pfizer) diluted in PBS was injected i.v. into mice in the event of any bleeding or hemophilic complications. Lipid nanoparticles were i.v. delivered by a retroorbital injection under isofluorane anesthesia at a dose of either 2 or 4 mg/kg (as specified). Animals were retroorbitally bled under isofluorane and liver and spleen were collected when needed after killing at the designated time points.

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Salk Institute for Biological Studies.

Intravital IVIS Imaging. Twenty minutes before imaging, animals were intraperitoneally administered 150 μL of luciferin in PBS (15 mg/mL, PerkinElmer). Animals were then anesthetized by isofluorane and imaged in the Xenogen IVIS instrument.

Bioplex Chemokine Analysis. To assess immune reactivity to these lipid nanoparticle formulations, hemophilic animals were administered LNPs encapsulating FIX mRNA at 2 mg/kg and at 4 mg/kg 2 wk apart. After two rounds of mRNA:LNP injections, animals were maintained normally without any additional interventions for an additional 90 d, at which point they were administered Luc + FIX mRNAs at 4 mg/kg encapsulated in the same LNP formulation at 1:1 ratio. The animals were then imaged for luciferase expression and retroorbitally bled at defined time points to look for any changes in the systemic cytokine profile. Blood was collected into 1/10th volume 3.2% sodium citrate and plasma was collected after two centrifugation steps (4,900 × *g* and 14,900 × *g*). The resultant plasma from multiple time points in the experiment was stored at −80 °C until assayed using the Mouse Group I Cytokine kit (Bio-Rad) as per the manufacturer's instructions. Briefly, serum samples were diluted four times and incubated with a mixture of spectrally color-coded, magnetic beads wherein each color corresponded to antibodies against a single cytokine (23 cytokines in all). After antigen capture and multiple washes using a magnetic wash station, the samples were incubated with a PE-conjugated streptavidin–biotin complex at the end of which the level of each cytokine was determined by the Bioplex MAGPIX reader as per the manufacturer's instructions. Absolute concentrations of each cytokine were then determined based on a serially diluted standard curve generated from the manufacturer-provided standard.

Generating Tail PCR. Human F9 plasmid DNA (10 ng) was used to generate the poly(A) tail 120 PCR products in a 50-μL PCR with 2X KAPA HiFi PCR mix (KR0370) as per the manufacturer's instructions. The product was then checked on a 2% gel from Life Technologies and approximately quantified based on the intensity of the low molecular weight ladder (Life Technologies, 10068-013), and it was cleaned with the Qiagen PCR purification kit and resuspended in 50 μL water.

IVT for Synthesis. The following protocol is for a 200 μL IVT reaction using the NEB HiScribe T7 reagents that should yield around 1 mg of RNA: 2.5× NTP mix was prepared as required by thawing individual 100-mM NTP stocks [ATP, GTP, CTP, and N1 methyl pseudouridine (N1MPU) nucleotides] and pooling them together. For the IVT reaction, around 2–4 μg of the template was used for a 200-μL reaction. The 10× IVT reaction buffer, the 2.5 × dNTP mix, the template DNA, and the T7 RNA polymerase were mixed well by pipetting and incubated at 37 °C for 4 h. To degrade the DNA template, the IVT reaction was diluted with 700 μL of nuclease-free water, and then 10× DNase I buffer and 20 μL of the RNase-free DNase I were added to the IVT mix and incubated at 37 °C for 15 min. The diluted (to 1 mL) and DNase-treated reaction was then purified by Qiagen RNeasy Maxi columns as per the manufacturer's instructions with a final elution in RNase-free water. The purified RNA was then quantified by UV absorbance where the A260/A280 should be around 1.8–2.2, depending on the resuspension buffer used.

Enzymatic Capping of IVT mRNA. For enzymatic capping, we used a 50× scaled-up version of NEB's standard one-step capping and 2′O-methylation reaction that is suitable for treating up to 1 mg of IVT transcripts. Whereas NEB recommends the use of only 10 μg RNA in a 20-μL reaction, this is based on the assumption that transcript length is as short as 100 nt. A higher substrate-to-reaction volume should be acceptable for mRNA transcripts, which are generally longer (∼300–600 nt) in length. The ratio used here is a compromise between economy and the risk of overloading the enzyme and is subject to further optimization. Before initiating the capping reaction, the RNA was denatured at 65 °C for 5 min and then snap chilled to relieve any secondary conformations. Each mg of mRNA was capped using the following components: 1 mg of mRNA in 700 μL of nuclease-free water, 100 μL capping buffer, 50 μL (10 mM) GTP, 50 μL (4 mM) SAM, 50 μL (10 units/μL) of Vaccinia capping enzyme, and 50 μL of mRNA 2′O-methyltransferase (50 units/μL). The reaction was incubated at 37 °C for 1 h. The resulting capped mRNA was eluted using RNase-free water, repurified on an RNeasy column, and quantified by nanodrop. The mRNA was also visualized on the gel by running 500 ng of the purified product per lane in a denaturing gel after denaturation and snap chilled to remove secondary structures.

Preparation of LUNAR-mRNA and MC3-mRNA Nanoparticles. Using LUNAR technology, a proprietary lipid delivery technology platform, Arcturus Therapeutics produced lipid nanoparticles containing FIX mRNA by mixing appropriate volumes of lipids in ethanol with an aqueous buffer containing mRNA, using a Nanossemblr microfluidic device, followed by downstream processing. For the encapsulation of mRNA, the desired amount of mRNA was dissolved in 5 mM citric acid buffer, pH 3.5, whereas lipids at the desired molar ratio were dissolved in ethanol. The molar percentage ratio for the constituent lipids is 50% ionizable lipid (ATX, Arcturus proprietary ionizable amino lipid or MC3), 7% DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids), 40% cholesterol (Avanti Polar Lipids), and 3% DMG-PEG (1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene glycol, PEG chain molecular weight: 2000) (NOF America Corporation). The lipid and mRNA solutions were then combined in the microfluidic device (Precision NanoSystems) at a flow ratio of 1:3 (ethanol:aqueous phase). The total combined flow rate was 12 mL/min. Lipid nanoparticles thus formed were purified by dialysis against phosphate buffer overnight using Spectra/Por Flot-a-lyzer ready to use dialysis device (Spectrum Labs) followed by concentration using Amicon Ultra-15 centrifugal filters (Merck Millipore). Particle size was determined by dynamic light scattering (ZEN3600, Malvern Instruments). Encapsulation efficiency was calculated by determining unencapsulated RNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes) to the LNP slurry (Fi) and comparing this value to the total siRNA content that is obtained upon lysis of the LNPs by 1% Triton X-100 (Ft), where percentage of encapsulation = (Ft − Fi)/Ft × 100.

Preparation of LUNAR-siRNA and MC3-siRNA Nanoparticles. Using a proprietary lipid delivery technology platform, LUNAR technology, Arcturus Therapeutics created the lipid nanoparticles containing siRNA. The LNPs were prepared by mixing appropriate volumes of lipids in ethanol with an aqueous phase containing siRNA duplexes, using a Nanoassemblr microfluidic device, followed by downstream processing. For the encapsulation of RNA, the desired amount of RNA was dissolved in 5 mM citric acid buffer, pH 3.5. Lipids at the desired molar ratio were dissolved in ethanol. The molar percentage ratio for the constituent lipids is 58% ATX (proprietary ionizable amino lipids), 7% DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids), 33.5% cholesterol (Avanti Polar Lipids), and 1.5% DMG-PEG (1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene glycol, PEG chain molecular weight: 2000) (NOF America Corporation). At a flow ratio of 1:3 ethanol:aqueous phases, the solutions were combined in the microfluidic device (Precision NanoSystems) using two HPLC prep pumps (AZURA P 2.1L, Knauer). The total combined flow rate was 12 mL/min, per microfluidics chip. Anywhere from one to four microfluidics chips were used, in a custom unit for parallelization (Precision NanoSystems), allowing a variable throughput for different batch sizes. The microfluidics chips use a herringbone micromixer for extremely quick mixing times, yielding high encapsulation and narrow particle size distribution. The mixed material was then diluted three times with deionized water after leaving the micromixer outlet, reducing the ethanol content to 6.25%. The diluted LNP slurry was concentrated by tangential flow filtration with hollow fiber membranes (mPES Kros membranes, Spectrum Laboratories), and then diafiltration was performed with modified DPBS, without magnesium or calcium (HyClone). A total of 10 diavolumes were exchanged, effectively removing the ethanol. MC3 siRNA nanoparticles were formulated as described previously (26). Particle size was determined by dynamic light scattering (ZEN3600, Malvern Instruments). Encapsulation efficiency was calculated by determining unencapsulated siRNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes) to the LNP slurry (Fi) and comparing this value to the total RNA content that is obtained upon lysis of the LNPs by 1% Triton X-100 (Ft), where % encapsulation = (Ft − Fi)/Ft × 100.

Mouse Plasma. Blood samples were collected from the retroorbital plexus into 0.1 volume of 3.2% sodium citrate. After two sequential centrifugation steps (4,500 × *g* and 14,500 × *g*), the plasma was stored at −70 °C for all future analyses.

One-Stage FIX Activity Test. Factor IX activity was determined by a one-stage FIX activity test assays as follows: Fifty microliters of APTT reagent (Pacific Hemostasis, Thermo Fisher), 50 μL of factor IX-deficient human plasma (George King Biomedical), and 50 μL of a 1:5 (or 1:10) dilution of mouse test plasma in Hepes buffer (50 mM Hepes), were incubated at 37 °C in an ST4 coagulometer (Stago). After 3 min, clotting was initiated by the addition of 50 μL of 33 mM $CaCl_2$ (Pacific Hemostasis, Thermo Fisher) in Hepes buffer (Life Technologies). Factor IX activity of duplicate samples was determined from a log–log standard curve that was constructed from the clotting time results for dilution (1:5–1:1,280) of pooled normal mouse plasma.

The authors clarify that, whereas this assay relies on the principle of activated partial thromboplastin time (APTT), it is actually a modification of the original method developed by Langdell, Wagner, and Brinkhous. The

one-stage factor IX activity assay performs the APTT test in the presence of standardized factor IX-deficient plasma, so that the degree of correction of clotting of the factor IX-deficient plasma is directly related to the amount of factor IX activity that is supplemented by the addition of animal plasma sample. We also see some FIX activity in our hemophilic animals when measured by the one-stage FIX activity assay, even though there is little to no FIX protein in circulation. This is a technical artifact due to the lower sensitivity of the one-stage FIX activity assay at lower ranges (<15%).

Western Blot Analysis. Mouse plasma was subjected to barium citrate adsorption twice (19). Briefly, 4 μL of 1 M BaCl2 was added to 50 μL of mouse plasma, incubated at room temperature for 5 min, and centrifuged at 3,800 × *g* for 10 min. The precipitated proteins were dissolved in 25 μL of citrate–saline buffer and precipitated again by $BaCl_2$. The pellets were dissolved in 75 μL of citrate–saline buffer, and 10–15 μL samples were electrophoresed through a SDS 4–12% gradient polyacrylamide gel. The gel was blotted on a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore), blocked with casein, and sequentially incubated with goat anti-human factor IX antibody (GA-FIX-AP, Affinity Biologicals), and horseradish peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology). ECL chemiluminescence reagent (Amersham) was used as a substrate to detect antibody-bound protein bands.

ELISA. Levels of hFIX protein in the mouse serum were measured by a sandwich ELISA using capture and detection antibodies from Affinity Biologicals (Anti-FIX GA FIX paired antibodies ELISA kit) as per the manufacturer's instructions. The ELISA was performed in duplicate for at least two or more biological replicates per experimental group.

AST/ALT/ALP Assays by Statvet and Arcturus. Liver function was monitored through AST, ALT, and ALP level test activity were done as an indicator of liver function. The tests were conducted by Statvet Diagnostics as per their optimized protocol. A Beckman Coulter AU480 analyzer was used to do a hepatic analysis on the mouse serum samples.

ELISA for Benchmarking LUNAR and MC3 Formulations for mRNA Delivery. In vitro transcribed FIX mRNA, packaged in the LUNAR and MC3 formulations, was administered once to 7-wk-old female balb/c mice at 0.25, 0.5, and 2 mg/kg. The animals were bled at 6-h postdosing and the serum FIX levels were assessed by an ELISA (Assay Pro: EF1009-1) at 1:200 dilution as per the manufacturer's instructions.

Statistical Analysis. Cohort size in all animal experiments is stated in the figures (n = 3 or 4). The data reported are the average of biological and technical replicates along with the SE in each case. Statistical analyses were conducted with GraphPad Prism software version 4.0 (GraphPad). Experimental differences were evaluated by Student's two-tailed *t* test, assuming equal variance or using an ANOVA as stated in the legend. P values of <0.05 were considered statistically significant.

ACKNOWLEDGMENTS. The authors thank Angel I. Leu, Arisa I. Cale, and Bijan Godarzi for their help and support toward vector construction, in vitro translation, and mRNA synthesis; Mathias Leblanc, the chief veterinarian at Salk Institute for Biological Studies, for all his help with the evaluation of liver toxicity upon LUNAR treatment; and the Sanford Burnham Prebys Histology Core for tissue processing and histology services. This work was supported by the Waitt Advanced Biophotonics Core Facility of the Salk Institute for Biological Studies with funding from the NIH National Cancer Institute Cancer Center Support Grant P30 014195, National Institute of Neurological Disorders and Stroke Neuroscience Core Grant, and the Waitt Foundation. I.M.V. is an American Cancer Society Professor of Molecular Biology and holds the Irwin and Joan Jacobs Chair in Exemplary Life Science. This work was also supported in part by NIH Cancer Center Core Grant P30 CA014195-38, Ipsen, the H. N. and Frances C. Berger Foundation, the Glenn Center for Aging Research, the Leona M. and Harry B. Helmsley Charitable Trust Grant 2012-PG-MED002, and the California Institute for Regenerative Medicine (CIRM-TR4-06809).

1. Sahin U, Karikó K, Türeci Ö (2014) mRNA-based therapeutics: Developing a new class of drugs. *Nat Rev Drug Discov* 13(10):759–780.
2. Zatsepin TS, Kotelevtsev YV, Koteliansky V (2016) Lipid nanoparticles for targeted siRNA delivery: Going from bench to bedside. *Int J Nanomedicine* 11:3077–3086.
3. Yin H, et al. (2014) Non-viral vectors for gene-based therapy. *Nat Rev Genet* 15(8): 541–555.
4. Jirikowski GF, Sanna PP, Maciejewski-Lenoir D, Bloom FE (1992) Reversal of diabetes insipidus in Brattleboro rats: Intrahypothalamic injection of vasopressin mRNA. *Science* 255(5047):996–998.
5. Zangi L, et al. (2013) Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat Biotechnol* 31(10): 898–907.
6. Kormann MS, et al. (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat Biotechnol* 29(2):154–157.
7. Peyvandi F, Garagiola I, Young G (2016) The past and future of haemophilia: Diagnosis, treatments, and its complications. *Lancet* 388(10040):187–197.
8. Nazeef M, Sheehan JP (2016) New developments in the management of moderate-to-severe hemophilia B. *J Blood Med* 7:27–38.
9. Srivastava A, et al.; Treatment Guidelines Working Group on Behalf of The World Federation Of Hemophilia (2013) Guidelines for the management of hemophilia. *Haemophilia* 19(1):e1–e47.
10. Lozier JN, et al. (1990) Factor IX New London: Substitution of proline for glutamine at position 50 causes severe hemophilia B. *Blood* 75(5):1097–1104.
11. Mancuso ME, et al. (2009) Improved treatment feasibility in children with hemophilia using arteriovenous fistulae: The results after seven years of follow-up. *Haematologica* 94(5):687–692.
12. Brunetti-Pierri N, et al. (2009) Bioengineered factor IX molecules with increased catalytic activity improve the therapeutic index of gene therapy vectors for hemophilia B. *Hum Gene Ther* 20(5):479–485.
13. Suhr OB, et al. (2015) Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: A phase II multi-dose study. *Orphanet J Rare Dis* 10:109.
14. Butler JS, et al. (2016) Preclinical evaluation of RNAi as a treatment for transthyretin-mediated amyloidosis. *Amyloid* 23(2):109–118.
15. Wang L, et al. (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. *Proc Natl Acad Sci USA* 94(21):11563–11566.
16. Petrini P (2001) What factors should influence the dosage and interval of prophylactic treatment in patients with severe haemophilia A and B? *Haemophilia* 7(1):99–102.
17. Monahan PE, et al. (2015) Employing a gain-of-function factor IX variant R338L to advance the efficacy and safety of hemophilia B human gene therapy: Preclinical evaluation supporting an ongoing adeno-associated virus clinical trial. *Hum Gene Ther* 26(2):69–81.
18. Peters RT, et al. (2010) Prolonged activity of factor IX as a monomeric Fc fusion protein. *Blood* 115(10):2057–2064.
19. Björkman S, Berntorp E (2001) Pharmacokinetics of coagulation factors: Clinical relevance for patients with haemophilia. *Clin Pharmacokinet* 40(11):815–832.
20. Manno CS, et al. (2003) AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 101(8):2963–2972.
21. DeRosa F, et al. (2016) Therapeutic efficacy in a hemophilia B model using a biosynthetic mRNA liver depot system. *Gene Ther* 23(10):699–707.
22. Gorczynski RM (1992) Immunosuppression induced by hepatic portal venous immunization spares reactivity in IL-4 producing T lymphocytes. *Immunol Lett* 33(1):67–77.
23. Knolle PA, Gerken G (2000) Local control of the immune response in the liver. *Immunol Rev* 174:21–34.
24. Nathwani AC, et al. (2014) Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 371(21):1994–2004.
25. Keles E, Song Y, Du D, Dong WJ, Lin Y (2016) Recent progress in nanomaterials for gene delivery applications. *Biomater Sci* 4(9):1291–1309.
26. Jayaraman M, et al. (2012) Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angewandte Chemie* 51(34):8529–8533.