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Original Article

Protein is expressed in all major organs after intravenous infusion of mRNA-lipid nanoparticles in swine

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***In vivo* delivery of mRNA is promising for the study of gene expression and the treatment of diseases. Lipid nanoparticles (LNPs) enable efficient delivery of mRNA constructs, but protein expression has been assumed to be limited to the liver. With specialized LNPs, delivery to extrahepatic tissue occurs in small animal models; however, it is unclear if global delivery of mRNA to all major organs is possible in humans because delivery may be affected by differences in innate immune response and relative organ size. Furthermore, limited studies with LNPs have been performed in large animal models, such as swine, due to their sensitivity to complement activation-related pseudoallergy (CARPA). In this study, we found that exogenous protein expression occurred in all major organs when swine were injected intravenously with a relatively low dose of mRNA encapsulated in a clinically relevant LNP formulation. Exogenous protein was detected in the liver, spleen, lung, heart, uterus, colon, stomach, kidney, small intestine, and brain of the swine without inducing CARPA. Furthermore, protein expression was detected in the bone marrow, including megakaryocytes, hematopoietic stem cells, and granulocytes, and in circulating white blood cells and platelets. These results show that nearly all major organs contain exogenous protein expression and are viable targets for mRNA therapies.**

INTRODUCTION

In vivo delivery of mRNA is a versatile strategy that is being harnessed for the development of cancer therapies, gene editing therapies, and vaccines, including those for COVID-19.[1,2] The breadth of potential for RNA therapy lies in the ability to deliver these agents to specific organs and cell types.[3,4] So far, delivery has been mostly limited to hepatic tissue, thus limiting the full perceived potential of RNA therapies.[5] For example, it is not known if exogenous protein expression can be induced in many cell types in the bone marrow or blood after systemic administration, thus limiting RNA therapies as potential treatments for many diseases, such as blood cancers.[6,7]

Lipid nanoparticles (LNPs) are the FDA-approved delivery system used in both of the approved COVID-19 mRNA vaccines.[8] LNPs were first approved in a small interfering RNA (siRNA) therapeutics for hereditary transthyretin amyloidosis.[9] Upon systemic intravenous (i.v.) injection of mRNA-LNPs, protein expression occurs in the liver.[7] However, by altering the lipid composition, delivery to other tissues is detectable in small animal models, specifically the spleen and lung, a single report of expression in the heart, and two reports in bone marrow in mice.[10–12] It is not clear if these findings of extrahepatic expression are applicable to humans, as extrahepatic LNP cargo delivery may be affected by differences in relative organ size and the innate immune response to LNPs.[13–15] Large animal models, such as swine, better resemble human anatomy and immunology but are challenging to use because they also exhibit complement activation-related pseudoallergy (CARPA) that occurs after i.v. administration of nanoparticles in humans.[14–16] It is unknown if protein expression occurs in many tissues after administering mRNA-LNPs, particularly in large animals or with clinically relevant LNPs and dosages. Testing clinically relevant LNP formulations, such as those used in the mRNA COVID-19 vaccine, is important for the development and approval of novel mRNA therapies.

RESULTS

mRNA-LNPs achieve hepatic and extrahepatic expression in swine

We examined whether hepatic and extrahepatic expression occurs following an i.v. injection of mRNA-LNPs in swine (Figure 1A).

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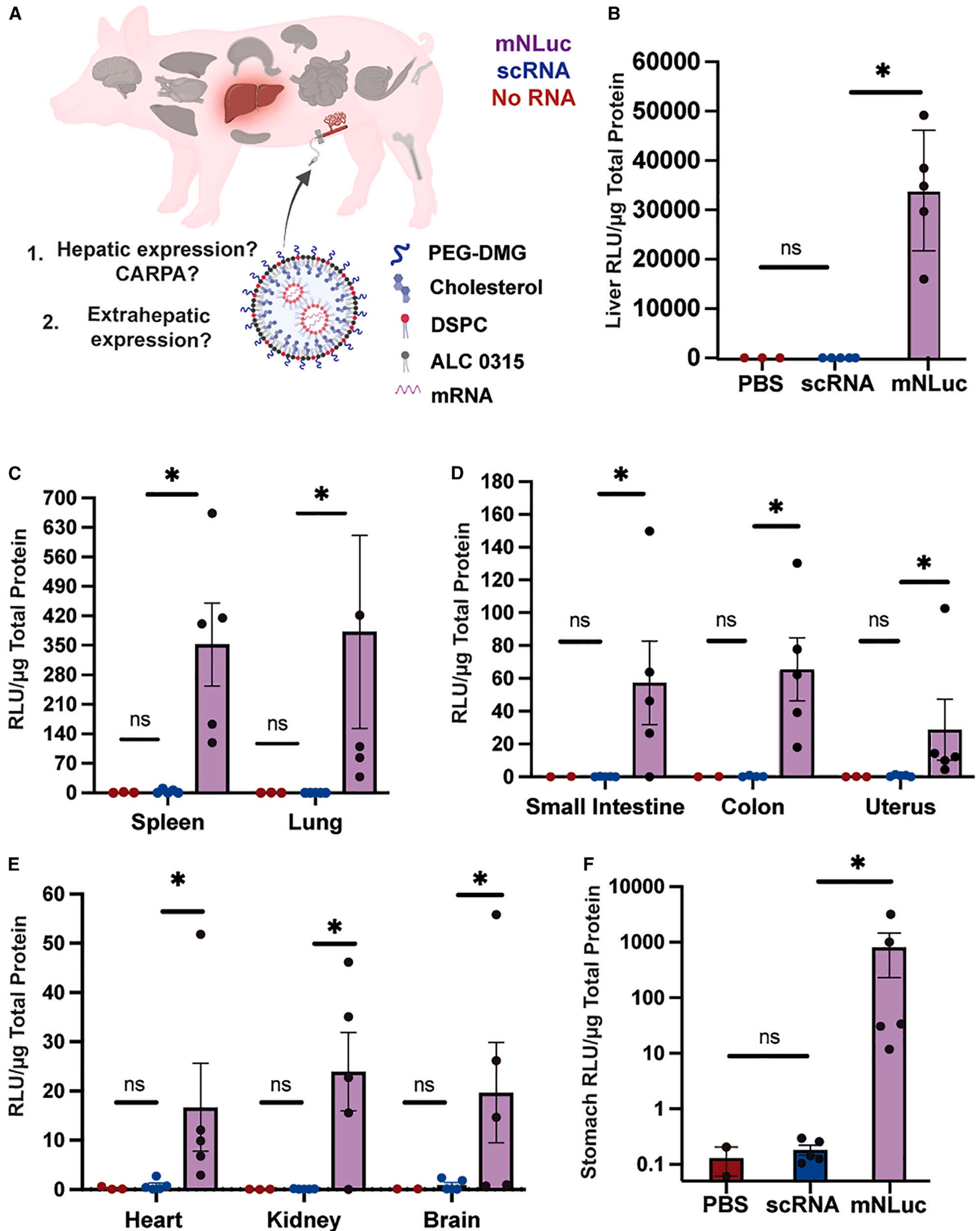

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mRNA encoding the reporter protein NanoLuciferase (NanoLuc, NLuc) was encapsulated in LNPs (mNLuc), with a similar formulation to the COVID-19 mRNA vaccines. Swine were treated with mNLuc at 0.15 mg per kg of body weight (mg/kg). Tissues were analyzed for NanoLuc protein signal at 24 h post-infusion. To mitigate the potential for CARPA, swine were i.v. injected with famotidine, diphenhydramine, and dexamethasone 30 min prior to mRNA-LNP infusion. These immunosuppressive and anti-histamine agents inhibit complement activation and reduce leukocyte trafficking by preventing histamine release from mast cells, preventing CARPA.[17] The mRNA-LNPs were infused over 60 min at a rate of 0.25 mL/min. Swine were closely monitored for signs of CARPA at the time of infusion and for the following 3 h, and there were no significant changes in temperature, heart, or respiratory rates. There was significant exogenous protein expression in the liver (33,703 ± 5,462 relative light units (RLU) /μg total protein, $p < 0.05$, mean ± SEM) compared to control swine treated with scrambled mRNA (scRNA) or PBS (no mRNA) (6 ± 2 and 5 ± 2, respectively) (Figure 1B). NanoLuc protein was also measured in the spleen, lung, small intestine, colon, uterus, heart, kidney, and brain. There was significant NanoLuc signal in the spleen and lungs (352 ± 98 and 382 ± 229 RLU/μg total protein, respectively, $p < 0.05$) compared to PBS- and scRNA-treated pigs (Figure 1C). NanoLuc protein levels were lower but still significant in the small intestine, colon, uterus, heart, kidney, and brain tissues (57 ± 25, 66 ± 19, 29 ± 18, 17 ± 9, 24 ± 8, and 20 ± 10 RLU/μg total protein, respectively, $p < 0.05$) compared to PBS- and scRNA-treated swine (Figures 1D and 1E). While the NanoLuc protein signal was detectable and significant in the stomach tissue of mNLuc-treated swine (837 ± 611 RLU/μg total protein, $p < 0.05$), the levels were variable (Figure 1E). This could have been due to a systemic higher transfection rate of two out of the five pigs, as all the other organs of these subject animals had higher NanoLuc signals.

RNA-LNP can safely deliver mRNA to most tissues

NanoLuc mRNA levels were examined to determine whether the NanoLuc protein signal in the extrahepatic tissues was due to exogenous mRNA delivery or perfusion of these tissues by blood carrying the protein presynthesized in the liver. mNLuc-treated swine had significant levels of NanoLuc mRNA transcripts in the liver compared to scRNA control swine, as measured by quantitative PCR (qPCR) (Figure 2A). The NanoLuc mRNA levels detected in the control swine were likely background noise due to primer dimerization. Furthermore, the lung, small intestine, uterus, spleen, and colon showed significant levels of NanoLuc mRNA compared to scRNA control (Figure 2A). While NanoLuc mRNA transcripts were detectable in the heart and stomach of mNLuc-treated swine, the levels were not significant. NanoLuc mRNA was not detected in the brain or kidney, potentially due to the low level of transfection along with the detection limit of the qPCR assay. The mRNA NanoLuc levels obtained using qPCR cannot be compared between organs because the analysis was performed using a control protein, and the different organs contain different levels of the control protein.

To assess if toxicity contributed to the distribution of LNP, serum from treated mNLuc and PBS control swine were analyzed. Swine treated with mNLuc did not exhibit increased levels of hepatotoxicity enzymes (aspartate aminotransferase [AST], alkaline phosphatase [ALP], and alanine aminotransferase [ALT]) at 4 or 24 h post-infusion compared to baseline samples (Figure 2B). There were also no statistically significant differences in albumin, gamma-glutamyl transferase (GGT), total protein, globulin, or creatinine at any time point compared to baseline (Figures S1A–S1E). To assess potential signs of CARPA and other systemic inflammatory markers, thromboxane B2 (TXB2) was measured.[17] There were no statistically significant differences in TXB2 concentrations between baseline samples, PBS controls, and mNLuc-treated swine 4 h post-injection (Figure S1F).

RNA-LNPs achieve expression in the bone marrow and circulating blood cells

It was not previously known whether exogenous protein expression from i.v.-infused mRNA could occur in hematopoietic stem cells (HSCs) or in the bone marrow and blood cells of large animals. Swine treated with mNLuc exhibited NanoLuc protein signal in both whole blood (4 ± 1 RLU/μL, $p < 0.05$) (Figure 3A) and plasma (14 ± 4 RLU/μg total protein, $p < 0.05$) compared to the control scRNA-treated swine (0.2 ± 0.1 RLU/μL and 0.04 ± 0.02 RLU/μg in whole blood and plasma, respectively) (Figure 3B). Upon blood cell separation, significant NanoLuc protein signal was detected in white blood cells and platelets (133 ± 35 and 113 ± 58 RLU/μg total protein, respectively, $p < 0.05$) but not in red blood cells (1 ± 0.4) compared to the control PBS and scRNA pigs (Figure 3C). Furthermore, the bone marrow was examined for exogenous protein expression. Significant NanoLuc protein signal was detected in the bone marrow of mNLuc swine (431 ± 257 RLU/μg total protein, $p < 0.05$) compared to scRNA swine (1 ± 0.8 RLU/μg) (Figure 4A). Upon cell sorting the bone marrow of swine, resident megakaryocytes and platelets together with HSCs exhibited the highest exogenous protein signal of the cell types (112 ± 41 and 113 ± 40 RLU/μg total protein, respectively, $p < 0.05$) followed by granulocytes (41 ± 7 RLU/μg total protein, $p < 0.05$) and other bone marrow-derived cells (75 ± 13 RLU/μg total protein, $p < 0.05$) (Figure 4B).

DISCUSSION

These results demonstrate that following a low-dose infusion of mRNA-LNPs, exogenous protein and exogenous mRNA transcripts can be detected globally including in the liver, spleen, lung, heart, uterus, colon, stomach, kidney, small intestine, and brain of the swine. Notably, we also detected exogenous protein expression in the bone

Figure 1. Hepatic and extrahepatic protein expression in swine following infusion of NanoLuciferase mRNA-LNPs

(A) Swine were injected with a single dose of mNLuc (purple), scRNA (blue), or PBS (no RNA, red). Hepatic (B) and extrahepatic (C–F) NanoLuciferase protein signals measured *ex vivo* 24 h post-injection. $N = 2–5$. $^*p < 0.05$, ns, not significant. Error bars represent mean ± SEM.

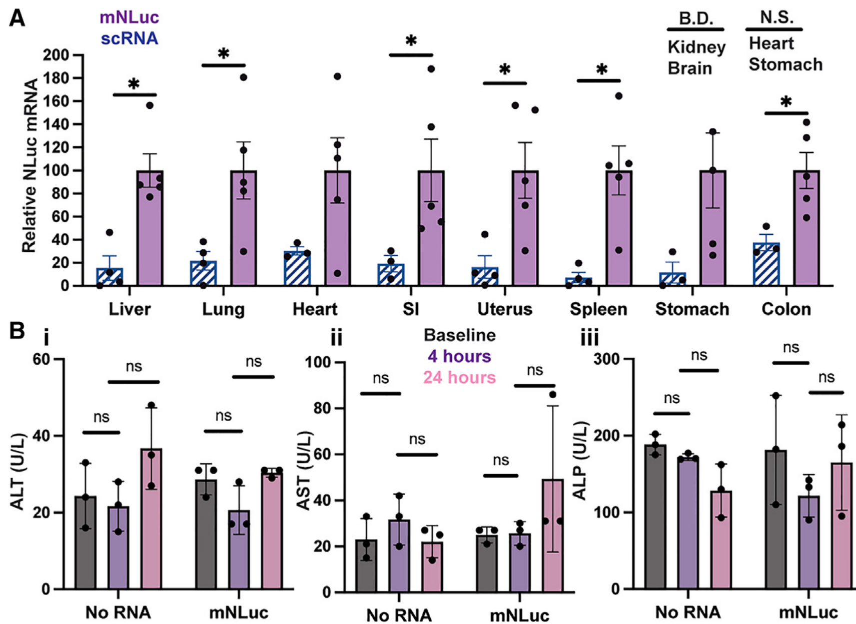

Figure 2. Hepatic and extrahepatic NanoLuciferase mRNA delivery without hepatotoxicity

(A) mRNA NanoLuciferase levels measured by qPCR in mNLuc- (purple) or scRNA (blue)-treated swine. B.D., below detection limit; N.S., additional organs that were not significantly different. (B) Hepatotoxicity enzyme levels of alanine aminotransferase (ALT; i), aspartate aminotransferase (AST; ii), and alkaline phosphatase (ALP; iii) measured prior to injection (black), at 4 h (purple), and at 24 h post-injection (pink) of mRNA-LNPs or PBS. N = 3–5. *$p < 0.05$, ns, not significant. Error bars represent mean ± SEM.

marrow, including megakaryocytes, HSCs, and granulocytes, and in circulating blood cells, such as white blood cells and platelets. These findings are significant, as extrahepatic delivery and expression of RNA using a nanomedicine have previously been limited to small animals and highly specialized nanoparticles decorated with targeting moieties. Here, we tested the hypothesis that administering a potent LNP carrying mRNA i.v. to swine would result in exogenous mRNA expression and protein synthesis in the liver without eliciting overt toxicity. We used a well-characterized FDA-approved LNP formulation similar to the COMIRNATY COVID-19 mRNA vaccine in swine and, surprisingly, observed that it has underexplored tropisms and biodistribution, which could potentially be related to the animal model used for testing. This highlights the importance of testing these agents in large animal models, as the organ-to-body size ratio may affect the relative organ distribution of LNP delivery. This study is also one of the first to deliver this LNP formulation i.v. instead of intramuscularly, which may lead to different pharmacokinetics. The capacity to target extrahepatic tissues could represent a promising template for designing more complex extrahepatic carriers. It is important to note that the dose of mRNA used in this i.v. study is half the dose of Onpattro, an i.v.-administered siRNA-LNP therapy and ∼50-fold higher than the dose of mRNA used in the intramuscularly administered COVID-19 mRNA vaccine.[18,19] The vaccine is not expected to lead to protein expression in organs such as the brain or bone marrow.

Due to their physiological similarities to humans, swine are the preferred animal model to study several systems, such as the cardiovascular system.[20] However, limited studies with LNPs have been performed in swine due to their sensitivity to CARPA.[21] Likely due to the use of premedication with famotidine, diphenhydramine, and dexamethasone and the slow infusion rate, CARPA was not observed in this study. This infusion regimen is clinically relevant, as it is used for Onpattro, a therapy approved by the FDA. Had swine been given a bolus injection of mRNA-LNPs without premedication, we expect that CARPA would have occurred. Hepatotoxicity was also not observed at the dose of mRNA used here, 0.15 mg/kg.[22] In this study, toxicity was evaluated following a single injection of mRNA-LNPs at 4 and 24 h. Similarly to studies performed in non-human primates and mice, we do not expect swine to develop hepato- and immune-toxicity following multiple injections over time; however, this will need to be evaluated in future studies.[23–27]

We observed exogenous mRNA and protein expression across most tissues in swine; however, LNP distribution and uptake were not evaluated. There are several potential mechanisms that may influence delivery of mRNA to extrahepatic sites, including by the direct uptake of mRNA-LNPs or by indirect uptake of mRNA circulating after exocytosis of mRNA-vesicles from the liver. These mechanisms will require further investigation in future studies. Furthermore, localization of exogenous protein within organs was not evaluated except for the bone marrow. We found significant exogenous protein and mRNA transcripts levels in eight non-reticuloendothelial system (RES) organs: the bone marrow, heart, uterus, colon, stomach, kidney, small intestine, and brain. We focused on analyzing expression in cell types in the bone marrow because it has high clinical relevance and interest in the field of gene therapy. Cells in the bone marrow were separated, including megakaryocytes, HSCs, and granulocytes. Each cell type showed significant exogenous protein levels. Localization of exogenous protein within other non-RES organs was not evaluated due to the lack of a NanoLuc antibody compatible with histology and the limited antibody probes available for sorting swine cells. We expect that further analysis would reveal that exogenous protein was localized to the endothelium and immune cells because these cells are often transfected by RNA-LNPs; however, as seen from the bone marrow, other cell types are potentially transfected as well. As expected, the levels of protein expression in non-RES organs are considerably lower than in the liver. The liver had the greatest signal, with a 100-fold higher expression compared to the spleen, lung, and stomach and 1,000-fold higher compared to non-RES organs,

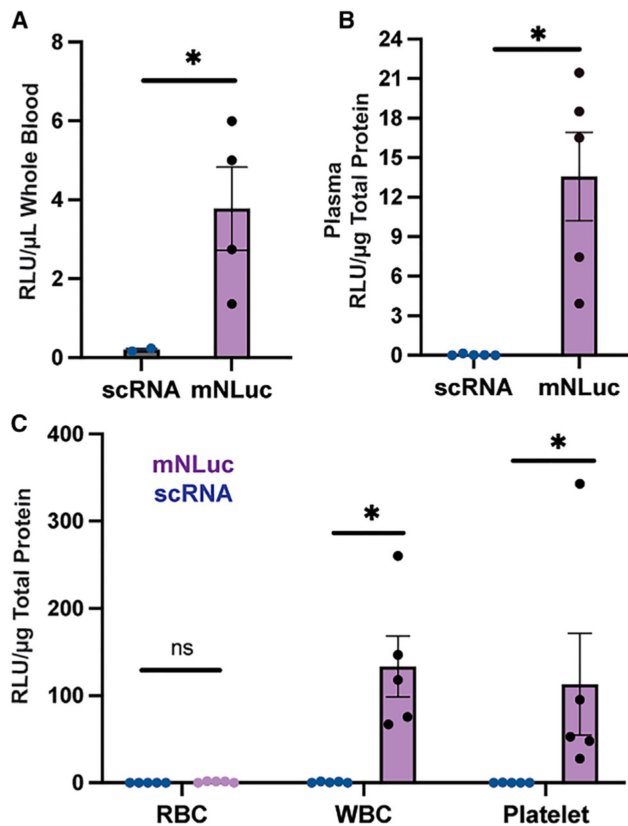

Figure 3. Exogenous protein expression in circulating blood cells

(A and B) NanoLuciferase protein signal in whole blood (A) or plasma (B) of swine treated with mNLuc (purple) or scRNA (blue). (C) NanoLuciferase protein signal in white blood cells (WBCs), red blood cells (RBCs), and platelets. $N = 3–5$. $^*p < 0.05$, ns, not significant Error bars represent mean ± SEM.

such as the small intestine, colon, uterus, heart, kidney, and brain (Figure S1H). These results are similar to previous studies performed in rodents that assessed spleen, liver, lung, heart, and kidney.[11]

An additional limitation to this study is that the swine were young (3–5 weeks old) and not considered to have a fully developed immune system.[28–30] The distribution of protein expression may differ between young and adult swine. While our findings may be more applicable to therapies for children, further studies will have to be conducted with adult swine. Additionally, further investigation is needed to determine if this occurs in other large animal models such as non-human primates or in small animals.

It is important to note that NanoLuc is a sensitive reporter protein that can be detected at lower expression levels than many other reporter proteins.[31] We expect that higher doses of mRNA and further optimization of the LNP is needed to achieve extrahepatic expression of proteins such as GFP at detectable levels in many tissues and cells. In this study, we dosed the swine at 0.15 mg/kg with the goal of analyzing extrahepatic protein expression without causing toxicity. We expect that higher doses to lead to higher levels of protein expression; however, this will likely be accompanied by some measures of toxicity. Future studies will be required to explore the dependence of protein expression on dose. The longevity of exogenous protein expression was not monitored after 24 h post-infusion; however, based on previous work, exogenous protein expression peaks between 7 and 24 h and decreases until it is no longer detectable at 48 h.[32–34] We expect protein expression in swine to behave similarly and reach its peak by 24 h, but this will need to be verified in future studies.

In conclusion, this study highlights the utility of testing LNPs in large animal models, which are more similar to humans than rodents and may reveal new LNP uptake and distribution profiles. It demonstrates the feasibility of developing novel extrahepatic LNP formulations without targeting moieties. We expect that continued advances in mRNA and LNP design will lead to increased levels of protein expression in these tissues and will reach levels necessary for therapeutic efficacy in many diseases.

MATERIALS AND METHODS

mRNA synthesis

mRNA was synthesized in bulk by *in vitro* transcription. Briefly, a DNA template encoding a CleanCap AG bacteriophage T7 promoter site, a NanoLuc coding sequence, and a template-encoded poly(A) tail was linearized with SapI. RNA was produced by *in vitro* transcription reactions containing CleanCap reagent and 5-methoxyuridine-5′-triphosphate (TriLink BioTechnologies, San Diego, CA, USA) and purified using an RNeasy Kit (Qiagen, Toronto, ON, Canada).

mRNA-LNP formulation

5-Methoxyuridine mRNAs encoding NanoLuc or scRNA were encapsulated in LNPs as previously described.[35] Briefly, mRNAs were dissolved in sodium acetate (pH 4) and combined with a lipid solution at an amine-to-phosphate ratio of 6. The lipid solution consisted of ALC-0315, DSPC, cholesterol, and PEG-DMG (Avanti Lipids) at a 50:10:38.5:1.5% molar ratio. The LNPs were dialyzed overnight against Dulbecco's PBS at pH 7.4 in 1,000-fold volume excess. Cholesterol content was measured using the Cholesterol E Assay Kit (Wako Chemicals, Mountain View, CA, USA). To determine mRNA concentration and encapsulation efficiency, RiboGreen assay (Quant-IT RiboGreen RNA Assay Kit, Thermo Scientific) was performed. Encapsulation efficiency was analyzed by comparing signals in LNP samples with or without Triton X-100 detergent, and the size and polydispersity index (PDI) was determined with Malvern Zeta Particle Sizer. The encapsulation efficiency was 88%–94%, the radii were 80–91 nm, and the PDI was 0.12–0.17, each of these varying slightly between each preparation. The LNP stability was analyzed over a 20 h period following formulation by assessing encapsulation efficiency, size, and PDI. The LNPs were stable within the first 8 h post-formulation; however, the size and the PDI increased, and encapsulation efficiency decreased, after 20 h (Figure S1G). LNPs were administered to swine within 3 h after formulation. The LNPs were diluted to a final concentration of 0.15 mg mRNA per mL in PBS (pH 7.4) prior to i.v. injection.

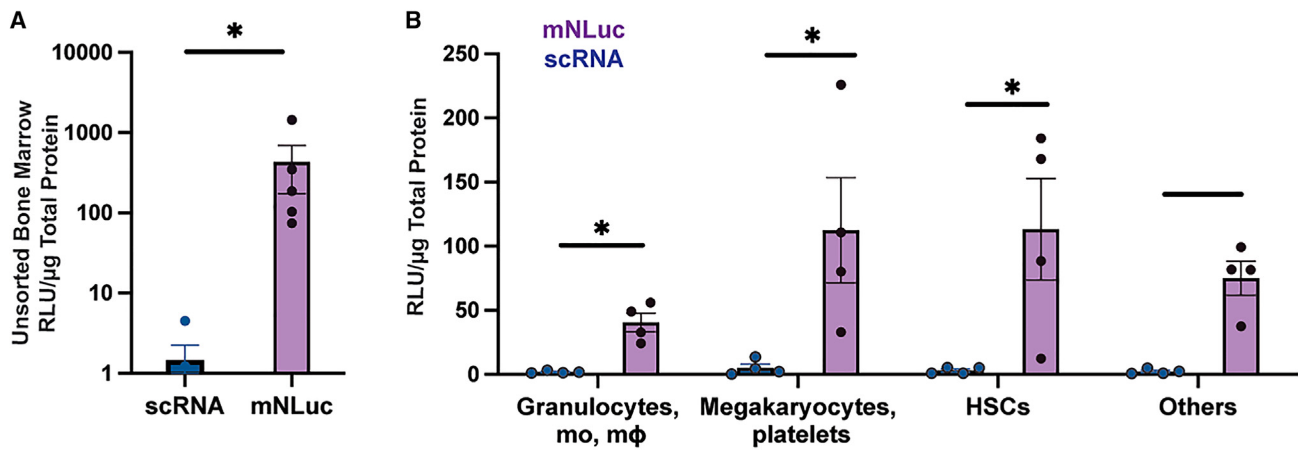

Figure 4. Exogenous protein expression in the bone marrow

(A) NanoLuciferase protein signal in unsorted bone marrow. (B) NanoLuciferase protein signal in sorted bone marrow cells: granulocytes, hematopoietic stem cells (HSCs), megakaryocytes, and platelets. *N* = 3–5. **p* < 0.05, ns, not significant Error bars represent mean ± SEM.

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 were used in the study. Swine weights ranged from 9 to 11 kg (PBS-treated swine: 10, 9, and 11 kg; mNLuc-treated swine: 10, 9, 10, and 9 kg; and scRNA-treated swine: 12, 10, 12, and 11 kg).

mRNA-LNP injections

Swine were anesthetized using an intramuscular injection of tiletamine/zolazepam and xylazine, followed by intubation and maintenance of anesthesia with isoflurane 1%–5% 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 using a modified Seldinger technique, a 4F catheter was placed into the vein, and a baseline blood sample was collected followed by administration of famotidine, diphenhydramine, and dexamethasone. After 30 min, mRNA-LNPs or PBS was infused over 1 h. The dose was chosen based on the animal weight-to-size ratio and the human infusion protocol for Onpattro, an FDA-approved siRNA-LNP therapy; 50% of the RNA dose of Onpattro was used. Following the infusion, the catheter was removed, the silk sutures were tied down, and the groin was closed in two layers using vicryl suture. Swine were awakened from anesthesia and allowed to recover.

Tissue, serum, plasma, and blood cells extraction from swine

At 4 h post-injection, swine were anesthetized, and blood was collected from the femoral vein branch. At 24 h post-injection, blood was collected via cardiac puncture under isoflurane anesthesia, and the various tissues (spleen, lung, small intestine, colon, uterus, heart, kidney, brain, and stomach) were surgically excised. Blood was collected into a syringe containing sodium citrate (0.32% final), and plasma samples were separated by spinning the whole blood twice at 1,500 × *g* for 10 min. Serum was collected in the same way as the plasma; however, the blood was collected without sodium citrate and allowed to clot for 30 min before centrifuging.

Blood cell separation was carried out using differential centrifugation or Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada). For platelet isolation, fresh whole citrated blood was centrifuged at room temperature, 100 × *g*, for 20 min to obtain platelet-rich plasma (PRP) and red blood cells. Platelets were then isolated from PRP by centrifugation at 250 × *g* for 20 min and washed twice by centrifugation in HEPES-buffered Tyrodes (pH 6.5; 134 mM NaCl, 2.9 mM KCl, 0.34 mM NaH_2PO_4, 10 mM HEPES, 5 mM D-glucose). Plasma was prepared by two additional centrifugation steps at 1,500 × *g* for 15 min. Red blood cells were isolated from the lowest fraction of centrifuged citrated blood and washed twice in PBS by centrifugation at 500 × *g* for 5 min. To ensure cell-type purity, white blood cells were isolated by Lymphoprep. One volume fresh whole blood collected in citrate was diluted 1:1 (v/v) in PBS and layered over one volume room temperature Lymphoprep. Density gradient separation was carried out by centrifugation in a room temperature swinging-bucket centrifuge at 1,600 rpm for 20 min with no brake. White blood cells were collected from the Lymphoprep-plasma interface, washed by centrifugation at 2,200× rpm for 2 min with 4 volumes DPBS. All cell fractions were assessed for purity and cell number by Sysmex XN-1000 Hematology Analyzer. Cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% [v/v] NP-40, 0.25% [w/v] deoxycholate, 1 mM EDTA, 2 mM PMSF, cOmplete Protease Inhibitor Cocktail [Roche]).

Bone marrow extraction and cell separation

Following euthanasia, bilateral femurs were circumferentially exposed. A bone saw was used to transect the bone proximally and distally. The bone segment was removed and flushed with 20 mL of sterile saline over a collection vial.

Bone marrow in saline was diluted 2:1 (v/v) with cold red blood cell lysis buffer (Abcam, Cambridge, UK) and rotated at 4°C for 10 min prior to centrifugation at 4°C and 1,200 rpm for 10 min. Bone marrow cell pellets were resuspended in an equivalent volume of red blood cell lysis buffer and centrifuged again before a final resuspension in 1/3 volume DPBS 5% (v/v) FBS. Cells were kept on ice, incubated for 15 min in the dark with antibodies (BD Pharmingen), PE mouse anti-pig monocyte/granulocyte (cat. 561499, BD), mouse anti-pig CD61 antibody (cat. MCA2263647, Bio-Rad, Hercules, CA, USA), and mouse anti-pig CD117 antibody (cat. MCA2598GA488, Bio-Rad), and run through a 30 μm filter immediately prior to sorting on an Accuri BD FACSAria Illu Cell sorter.

Protein extraction and quantification in tissues

Protein was extracted from tissue samples by physical homogenization with a bead mill homogenizer (Bead Rupter Elite, OMNI, Kennesaw, GA, USA). Tissue samples were excised and transferred to preweighed Eppendorf tubes containing 1.4 mm ceramic beads (Thermo Scientific, Waltham, MA, USA). Tissues were lysed by 2 cycles of disruption for 30 s each at 5 m/s in ice-cold RIPA lysis buffer at 20 μL/mg tissue and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. The total protein content in each lysate was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol.

NanoLuc assay

Cell and tissue lysates were transferred to a 96-Flat Well white microplate and mixed with prepared NanoLuc substrate (Promega, Madison, WI, USA) at a 1:1 (v/v) ratio. Luminescence was recorded on the EnVision 2105 Multimode Plate Reader (PerkinElmer). NLuc expression was then reported as the luminescence normalized to the total protein content (RLU/μg total protein). To account for any NLuc signal in organs that might be due to NLuc in blood, we subtracted whole blood NLuc signal from the respective organ NLuc signal by estimating that 6% of the organ volume was blood and using the NLuc values obtained from isolated whole blood. These were the estimated organ volumes: kidney, 132 mL; heart, 33 mL; lung, 66 mL; stomach, 46 mL; small intestine, 46 mL; large intestine, 46 mL; brain, 99 mL; uterus, 20 mL; liver, 86 mL; and spleen, 165 mL. It is important to emphasize that the raw signal in the organs was higher in all 44 organ samples—with the exception of two brain, one kidney, and one small intestine sample—than the raw signal in whole blood. Thus, protein expression in organs was not due simply to expression in whole blood.

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). qPCR was performed using the SYBR Green Master Mix (Thermo Scientific) and DNA primers against NLuc (F: 5′-GTCCGTAACTCCGATCCAAAG-3′; R: 5′-GCCGCT CAGACCTTCATA-3′). NLuc expression was quantified using the ΔΔCt method, relative to the expression of the housekeeping gene (*B-actin*) (F: 5′-GTCCACCTTCCAGCAGAT-3′; R: 5′-CAGTCCG CCTAGAAGCAT-3′). Primers were synthesized by IDT, and the data were collected using the QuantStudio6.

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, blood urea nitrogen, AST, ALP, and ALT levels were analyzed.

Statistical analysis

The statistical analysis was completed using GraphPad Prism (v.9.2.0). To ensure a t test could be used between two groups, the F-test was performed to confirm that 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 analysis of variance (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 $p < 0.05$. The number of replicates varied in some analyses because we continuously collected additional tissues and cells as the experiment progressed and additional swine were enrolled.

DATA AND CODE AVAILABILITY

There is a supplemental figure associated with this article in the supplemental information. Further details of this study are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101314.

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

C.J.K., P.R.C., E.J., and K.B. are directors, shareholders, and/or cofounders of companies developing RNA therapies. C.J.K., P.R.C.,

E.J., F.F., and K.B. have filed intellectual property on RNA-based therapies with the intention of commercializing these inventions.

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