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Transient yet Robust Expression of Proteins in the Mouse Liver via Intravenous Injection of Lipid Nanoparticle-encapsulated Nucleoside-modified mRNA

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[Abstract] With the recent availability of the SARS-CoV-2 mRNA-based vaccines, public attention has been drawn to this new technology and how it may be applied to other indications. Temporal activation of key hepatic regenerative pathways can induce liver regeneration, overcoming the lack of donor organs for liver transplantation and ineffectiveness of alternative treatments. Recombinant protein therapies and genetic therapies that target these pathways require frequent and repeated injections or, when integrated into the genome, may lead to deleterious effects. In contrast, nucleoside-modified mRNA encapsulated in lipid nanoparticles (mRNA-LNP) are non-integrative and induce transient yet robust expression of proteins that could serve as an ideal therapeutic tool to treat specific liver diseases. For instance, our recent publication in *Nature Communications* used mRNA-LNP to express hepatic mitogens, hepatocyte growth factor, and epidermal growth factor to induce liver regeneration following both acute and chronic liver injuries. Initial testing with firefly luciferase mRNA-LNP transfection and *in vivo* imaging confirmed specific hepatotropic delivery. In this protocol, we describe in detail the necessary steps to deliver mRNA-LNP to the murine liver and, following intravenous injection of eGFP mRNA-LNP, verify transfection efficiency using flow cytometry and liver cell specificity using immunofluorescence analyses. This procedure presents an unprecedented tool that can be customized with mRNA-LNP encoding any protein of interest to be expressed by virtually all hepatocytes, ~70% endothelial cells, and ~40% Kupffer cells for promoting liver function and/or regeneration.

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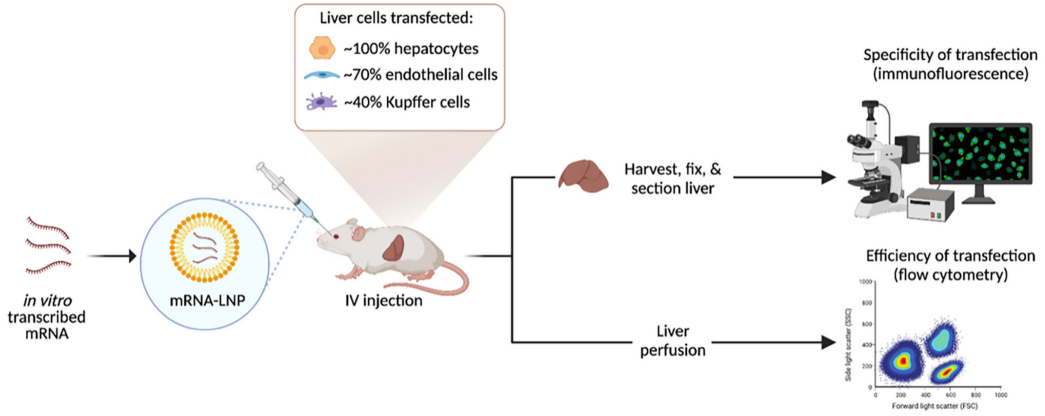
Graphic abstract:



Experimental Design of mRNA-LNP IV Injection and Analysis of Liver Cell Specificity and Efficiency of Transfection (Created with BioRender.com)

Keyword: Nucleoside-modified mRNA, Lipid nanoparticle, Liver regeneration, Protein expression, Retro-orbital injection

[Background] Recently, interest has been garnered for the use of nucleoside-modified mRNA-LNP (mRNA-LNP) due to their safe, non-integrative nature, as well as for their fame in the new publicly available SARS-CoV-2 vaccines produced by Pfizer-BioNTech and Moderna. Unlike viral gene delivery systems that come with a risk of mutagenesis and systemic adverse effects, intravenously administered mRNA-LNP are directed to the liver, with robust yet transient protein expression induced for only about 5 days (Rizvi *et al.*, 2021). mRNA-LNPs also provide an advantage over recombinant protein replacement therapy, which has limitations due to short protein half-life, requiring repetitive dosing to achieve benefit (Chan *et al.*, 1991; Appasamy *et al.*, 1993; Ido *et al.*, 2004; Hardwicke *et al.*, 2008). Other methods for delivering protein expression to the liver, such as ASGPR-mediated hepatocyte targeting, also come with limitations such as off-target effects and reduced effectiveness in diseased conditions (D'Souza and Devarajan, 2015).

Previous studies have shown that mRNA-LNPs are effective in treating several hepatic diseases that have a single gene mutation, such as alpha-1 antitrypsin deficiency and Crigler-Najjar syndrome type 1. In these cases, mRNA-LNP treatment is more potent than traditional protein replacement therapy, as it can be delivered in smaller volumes and allows the native cell machinery to translate the proteins and apply the appropriate post-translational modifications, and mRNA-LNPs are easier and cheaper to produce than recombinant proteins (Trepotec *et al.*, 2018). mRNA-LNP-encoded Cas9 can also be used as a tool for *in vivo* gene editing to correct these single-gene mutations (Yin *et al.*, 2016). Clinical trials are currently underway testing the therapeutic benefit of mRNA-LNP for applications such as vaccines, cancer immunotherapy, cancer treatment, and as an alternative to the many diseases currently being treated with protein replacement therapy (Sahin *et al.*, 2014; Pardi *et al.*, 2018; Magadum *et al.*, 2019).

In this application, our LNP formulation targets the liver, as shown with the restricted presence of luciferase activity in the liver after luciferase mRNA-LNP injection (Rizvi *et al.*, 2021), by binding circulating apolipoprotein E (ApoE) that in turn targets ApoE receptors on the surface of hepatocytes (Akinc *et al.*, 2010). Using injection of eGFP mRNA-LNP, we specifically demonstrated that only parenchymal cells of the liver were transfected among other organs tested. We also detected expression of eGFP in endothelial cells of the liver, spleen, and intestine, and in $CD45^+$ blood cells, most likely monocytes/macrophages, in the liver, spleen, intestine, and lungs following eGFP mRNA-LNP injection. Nevertheless, liver tropism can be altered by modifying the LNP formulation, route of administration, or by conjugating with specific antibodies for other cell types to redirect the mRNA-LNP away from the liver (Parhiz *et al*., 2018). This protocol provides a step-by-step description for the utilization of mRNA-LNP to induce rapid, robust, and transient expression of any genes directly and specifically to the liver. As a proof-of-principle, we demonstrated that mRNA-derived protein expression is stable for 3-4 days after administration of luciferase mRNA-LNP as luciferase activity was the highest in the liver 5 hours after injection and lasted 3-4 days (Rizvi *et al*., 2021). We have validated the efficiency and clinical benefit of this protocol to deliver hepatic mitogens to accelerate the regeneration of the liver from acetaminophen-induced acute liver injury and CDE diet-induced NAFLD-like chronic liver injury (Rizvi *et al.*, 2021). This protocol can be tailored to express any protein of interest in virtually all hepatocytes, most endothelial cells, and a subpopulation of Kupffer cells of the liver. Overall, this protocol provides a fully validated protein expression strategy to the liver that can be broadly utilized for therapeutic intervention of liver disorders via transient, robust, and cell-specific expression via nucleoside-modified mRNA-LNP.

Materials and Reagents

mRNA-LNP production:

1. T7 RNA polymerase (Megascript, Ambion, catalog number: AMB13345)
2. One-methylpseudouridine (m1Ψ)-5’-triphosphate (TriLink, catalog number: N-1081)
3. m7G capping kit with 2’-O-methyltransferase (ScriptCap, CellScript, catalog number: C-MSC11610)
4. Fast Protein Liquid Chromatography (FPLC) (Akta Purifier, GE Healthcare)
5. Agarose (Denville Scientific, catalog number: CA3510-8)
6. TAE buffer (Promega, catalog number: V4271)
7. mRNA-LNP to be stored at -80°C
   Note: All nucleoside-modified mRNAs are available from the company RNAx created by Dr. Drew Weissman.

eGFP DNA sequence used for mRNA synthesis:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC
GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC

TCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAG
CACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAA
CCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTG
GAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCA
GCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACC
CAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCG
TGACCGCCGCCGGGATCACTCTCGGCATGGACG AGCTGTACAAG

***In vivo* administration of mRNA-LNP:**

1. Dulbecco's Phosphate Buffered Saline (DPBS 1×), Sterile, (-) Calcium Chloride, (-) Magnesium Chloride (Gibco, catalog number: 14190-144)
2. Isothesia Isoflurane, USP (Henry Schein Animal Health, catalog number: 029405)
3. 1/2cc Lo-Dose Insulin Syringe, 0.5 ml, 29 G, sterile (Exel International, catalog number: 26028)

Tissue fixation, cryopreservation, sectioning, and immunofluorescence on sections:

1. FluorSave reagent (EMD Millipore Corp, MilliporeSigma™, catalog number: 345789-20ML)
2. Triton-X 100 (Sigma Life Science, catalog number: T9284-100ML)
3. 16% Paraformaldehyde Aqueous Solution, EM Grade (Electron Microscopy Sciences, catalog number: 15710)
4. Tissue Plus 4585 O.C.T. Compound, Clear (Fisher Healthcare, catalog number: 23-730-571)
5. Sucrose (MP Biomedicals, LLC, catalog number: 0219401891)
6. Normal Donkey Serum (Jackson ImmunoResearch Laboratories, Inc., catalog number: 017-000-121)
   Note: Storage of freeze-dried powder at 2-8°C. After rehydration, aliquot undiluted serum, and store at -20°C or below.
7. DAPI, 4',6-Diamidino-2-Phenylindole, Dilactate (Invitrogen, Molecular Probes, catalog number: D3571)
   Note: Storage of powder at room temperature. After rehydration, aliquot undiluted solution, and store at -20°C or below.

Liver perfusion and flow cytometry:

1. Intravenous (I.V.) administration set (Med Vet International, IV10, Fisher Scientific, catalog number: NC0196893)
2. Catheter, IV, 24 G × 3/4", Sterile, SURFLO®, (Terumo™, catalog number: SROX2419CA)
3. Pump Tubings, 3P Set of 12 PVC Flow Tubes of 1.52 mm (Gilson Inc, catalog number: 3P F117942)
4. 40 µm Sterile Cell Strainer (Fisher Scientific, Fisherbrand, catalog number: 22-363-547)

5. 100 µm Sterile Cell Strainer (Fisher Scientific, Corning, catalog number: 07-201-432)
6. 60 mm Petri Dish (Fisher Scientific, Fisherbrand, catalog number: FB0875713)
7. 1cc Insulin Syringe, 1.0 ml, 28 G, sterile (Exel International, catalog number: 26027)
8. Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap (Falcon, catalog number: 352235)
9. Liver Perfusion Medium 1×, called "*Solution 1*" in procedure, ~30 ml per mouse (ThermoFisher Scientific, Gibco, catalog number: 17701038)
   Note: Store at 2-8°C protected from light.
10. Earl's Balanced Salt Solution (EBSS), Ca^{2+}, Mg^{2+}, phenol red, pH 7.4, called "*Solution 2*" in procedure, ~20 ml per mouse (ThermoFisher Scientific, Gibco, catalog number: 24010043)
    Note: Store at 2-8°C.
11. Liver Digest Medium, called "*Solution 3*" in procedure, ~50 ml per mouse (ThermoFisher Scientific, Gibco, catalog number: 17703034)
    Note: Store from -20°C to -5°C, protected from light.
12. Hepatocyte Wash Medium (ThermoFisher Scientific, Gibco, catalog number: 17704024)
    Note: Store at 2-8°C protected from light.
13. Collagenase Type IV (Worthington Biochemical Corporation, catalog number: LS004188)
    Note: Store at 2-8°C.
14. DNase I, Deoxyribonuclease I, from bovine pancreas (Sigma-Aldrich, catalog number: DN25)
    Note: Store at -20°C.
15. Trypsin-EDTA (1×), 0.05%, phenol red (ThermoFisher Scientific, Gibco, catalog number: 25300-054)
    Note: Store at -5°C to -20°C.
16. Phosphate Buffered Saline (PBS), 10× solution (Fisher Scientific, Fisher BioReagents, catalog number: BP39920)
    Note: Dilute to 1× working solution prior to use for most applications.
17. Small Cotton-Tipped Applicators, 3 inches, Wooden Shaft, Sterilized (Fisher Scientific, Fisherbrand, catalog number: 23-400-118)
    Note: Autoclave to sterilize.
18. Ketamine Hydrochloride (Covetrus, catalog number: 056344)
    Note: Controlled substance.
19. Xylazine Hydrochloride (MP Biomedicals, catalog number: 0215830701)
20. Dulbecco's Phosphate Buffered Saline (DPBS 1×), Sterile, (-) Calcium Chloride, (-) Magnesium Chloride (Gibco, catalog number: 14190-144)
21. Fc Block, CD16/CD32 Purified Rat anti-Mouse (BD Biosciences, BD Pharmingen, catalog number: 553141)
    Note: Store undiluted at 4°C.
22. Ethylenediaminetetraacetic acid (EDTA), 0.5M, UltraPure™, pH 8.0 (Life Technologies, Invitrogen, catalog number: 15575-038)

23. Fetal Bovine Serum (FBS) Characterized (GE Life Sciences, HyCLone, catalog number: SH30071.03)
    Note: Store at -10°C or below.
24. Zombie NIR Fixable Viability Dye (Biolegend, catalog number: 423105)
    Note: Store at -20°C in a dry place protected from light.
25. Red Blood Cell Lysing Buffer Hybri-Max (Millipore Sigma, Sigma Aldrich, catalog number: R7757-100ML)
26. Antibodies (Table 1)

Table 1. List of antibodies used for immunofluorescence on frozen fixed tissues (IF-Fr) and flow cytometry (FC)

Antibody	Cat. No	Species	Application	Dilution
GFP	A10262 (Invitrogen)	Chicken	IF-Fr	1:300
HNF4α	sc-6556 (Santa Cruz)	Goat	IF-Fr	1:50
CK19	602-670 (Abbomax)	Rabbit	IF-Fr	1:100
CD31	557355 (BD Pharmingen)	Rat	IF-Fr	1:100
CD45	NB100-77417 (Novus)	Rat	IF-Fr	1:100
α-SMA	19245S (Cell Signaling)	Rabbit	IF-Fr	1:300
F4/80	sc25830 (Santa Cruz)	Rabbit	IF-Fr	1:100
CD31-PE/Dazzle 594	102429 (BioLegend)	Rat	FC	1:40
CD11b-PE efluor 610	61-0112-80 (Invitrogen)	Rat	FC	1:80
CD45-PE efluor 610	61-0451-80 (Invitrogen)	Rat	FC	1:40

27. DNase I stock solution (10 mg/ml) (see Recipes)
28. NPC Digest Solution (see Recipes)
29. Wash Medium (see Recipes)
30. Ketamine/Xylazine Anesthetic Solution (see Recipes)
31. FACs Buffer (see Recipes)
32. 4% Paraformaldehyde (see Recipes)
33. 3% Normal Donkey Serum (see Recipes)
34. DAPI stock solution (see Recipes)

Equipment

1. Magnetic Stirring Bars (Fisherbrand, catalog number: 14512132)
2. HotHands Hand Warmers, used as heating pads for anesthetized mice (Kobayashi Consumer Products, LLC, hothands.com)
3. Flow cytometer (Stratedigm, S1000 Exi 3 laser, 10 detector)
4. Peristaltic perfusion pump (Gilson, model: Minipuls 3, catalog number: F155005)
5. High-speed refrigerated centrifuge (Eppendorf, model: 5804R, catalog number: 022623508)

6. Fluorescence microscope (Nikon, model: Eclipse Ni-E)
7. Cryostat (Leica, catalog number: CM1950)
8. Magnetic stirrer (Research Productions Inc., Biomega)
9. Zetasizer Nano ZS dynamic light scattering instrument (Malvern Instruments Ltd, Malvern, UK)
10. Electrophoresis machine (Bio-Rad)

Software

1. FlowJo™ Software for Mac, Version 10.7.1. (Becton, Dickinson and Company, Ashland, OR. 2019. https://www.flowjo.com/)
2. Fiji – ImageJ image processing software (Schindelin *et al*., 2012)

Procedure

A. mRNA production
   1. Produce mRNAs using T7 RNA polymerase on a linearized plasmid encoding eGFP.
   2. Transcribe mRNAs to contain 101 nucleotide-long poly(A) tails. Use one-methylpseudouridine (m1Ψ)-5'-triphosphate instead of UTP to generate modified nucleoside-containing mRNA.
   3. Cap RNAs using the m7G capping kit with 2'-O-methyltransferase to obtain cap1.
   4. Purify mRNA by Fast Protein Liquid Chromatography (FPLC).
   5. Analyze all mRNAs by agarose gel electrophoresis and store frozen at -20°C.
      Note: All nucleoside-modified mRNAs are available from the company RNAx created by Dr. Katalin Kariko and Dr. Drew Weissman. The sequence of nucleoside-modified mRNA is listed in Materials and Reagents.

B. LNP encapsulation of the mRNA
   1. Encapsulate FPLC-purified m1Ψ-containing mRNAs in LNP using a self-assembly process in which an aqueous solution of mRNA at pH = 4.0 is rapidly mixed with a solution of lipids dissolved in ethanol.
      Note: LNPs used in this study contain an ionizable cationic lipid (pKa in the range of 6.0-6.5, proprietary to Acuitas Therapeutics)/phosphatidylcholine/cholesterol/PEG-lipid. The proprietary lipid and LNP composition are described in US patent US10,221,127 entitled "Lipids and lipid nanoparticle formulations for delivery of nucleic acids" (https://www.lens.org/lens/patent/183-348-727-217-109). They had a diameter of ~80 nm as measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) instrument. Acuitas is willing to work with academic investigators who would like to test the LNP used in this work.

C. *In vivo* administration of mRNA-LNP
   1. Thaw mRNA-LNP on ice immediately before use.

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2. Dilute mRNA-LNP fresh on ice in sterile Dulbecco's Phosphate Buffered Saline (PBS) prior to each experiment.
   Note: Dilute mRNA-LNP to a working concentration so that no more than 50 µl will be injected into each mouse. Make extra to account for the volume lost in the syringe tip. We used 5-10 µg of mRNA-LNP in each ~20 g body weight mouse.
3. Anesthetize mice using an isoflurane chamber (Figure 1).
4. Remove mice from the anesthesia chamber and quickly inject mRNA-LNP into the retro-orbital sinus using 1/2cc insulin syringes.
   Note: Tail vein injection is also an efficient method of intravenous delivery for this application.

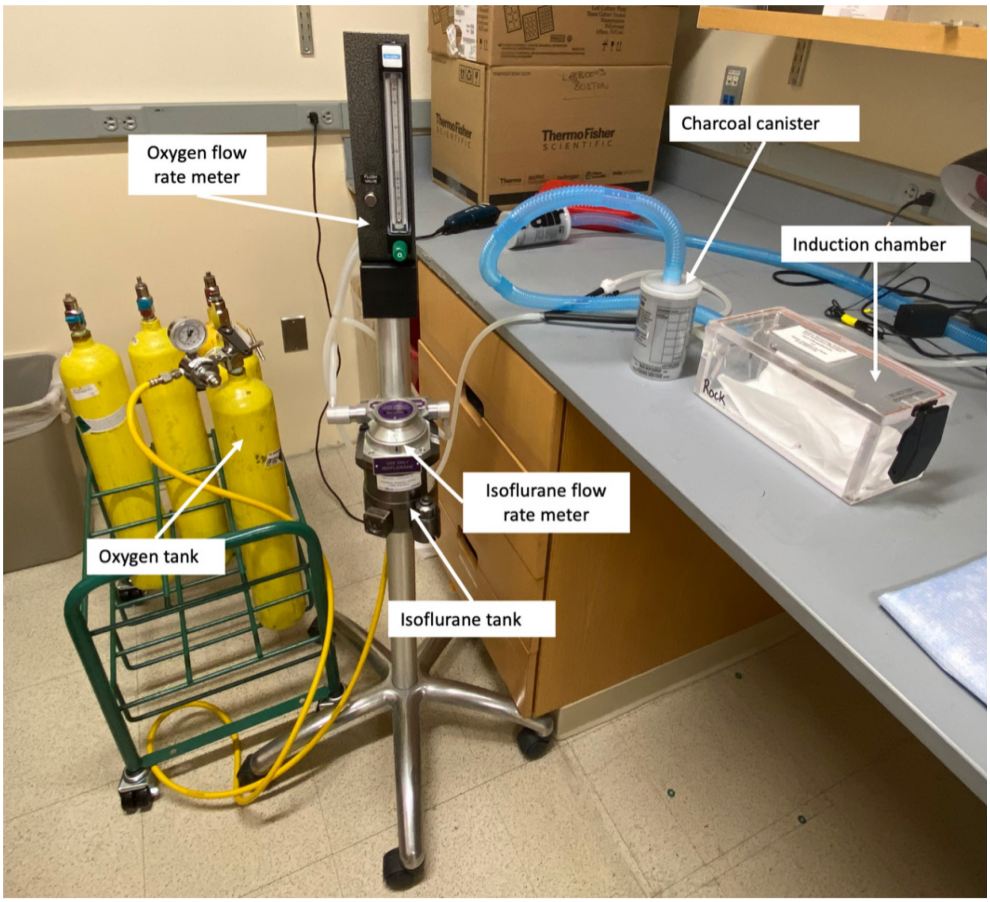

Figure 1. Isoflurane setup used to anesthetize mice

D. Tissue fixation, cryopreservation, sectioning, and immunofluorescence on sections (Data in Figure 3A)
   1. Collect intact liver and other organs including spleen, intestine, and lung directly in 4% paraformaldehyde (PFA) diluted in 1× PBS.
      Note: Expression of protein from mRNA-LNP can be seen between 5 h and 7 days after mRNA-LNP injection. Tissue can therefore be collected at any point in that time frame depending on the stability of the protein produced and the time of the readout desired. mRNA-LNPs are

equally well-expressed in male and female liver cells.

2. Fix for at least 2 h at room temperature.
   Note: Overnight at 4°C preferred.
3. For cryopreservation, wash tissues three times with 1× PBS and dip in 15% sucrose solution for 15 min.
4. Transfer tissues to 30% sucrose solution and keep until they sink to the bottom.
   Note: If tissues are very fatty, they may not sink; waiting overnight is sufficient in these cases.
5. Embed the tissues in O.C.T. compound. The blocks can be stored at -80°C until further required.
6. Cut 5 µm liver sections with cryostat and store the slides at -20°C until required for immunostaining.
7. Defrost the frozen slides at room temperature for 30 min and dry.
8. Dip the slides in 1× PBS for 10 min.
9. Permeabilize the tissues using 0.3% Triton-X in 1× PBS for 10 min.
10. Rinse the slides three times in 1× PBS, 10 min each.
11. Block the tissue with 3% normal donkey serum diluted in 1× PBS for 30 min.
12. Incubate the sections overnight at 4°C with chicken anti-mouse GFP antibody diluted in 1× PBS at 1:300 concentration as indicated in Table 1. Other markers can be used to identify specific cell types transfected, such as those outlined in Figure 3A.
13. Wash the slides three times with 1× PBS, 10 min each.
14. Incubate with fluorescently labeled anti-chicken secondary antibody for 1 h at room temperature protected from light at a concentration of 1:500 diluted in 1× PBS.
15. Wash the slides three times with 1× PBS for 10 min each.
16. Incubate with DAPI (1:3,000 in 1× PBS) for 3-5 min protected from light.
17. Rinse the slides two times with 1× PBS for 5 min each.
18. Mount using FluorSave reagent.
19. Image the slides using a fluorescent microscope.

E. Liver perfusion

1. Warm all solutions to 40°C in water bath.
   Note: Solutions 1 and 2 are kept at 4°C. Solution 3 is thawed fresh prior to use. To make sure the whole surgery is at 37°C, set the water bath to 40°C. Maintain the temperature of the solutions while the perfusion is being done.
2. Sterilize pump tubing with 70% ethanol, then wash with Solution 1.
3. Attach I.V. administration set to catheter at one end and to pump tubing at the other end and fill with Solution 1.
   Note: Fill with solution once the animal is prepared to be dissected to avoid a temperature drop in solution.
4. Anesthetize the mouse using Ketamine/Xylazine Anesthetic Solution at 100 µl/20 g body weight of mice, and place mice on heating pads to maintain their body temperature.

Note: The anesthetic solution is made with a concentration of ketamine:concentration of xylazine ratio; see recipe below. Check the responsiveness of the mouse by pinching its foot.

5. Immobilize all four limbs of the mouse with tape once under deep anesthesia on a moisture-absorbing surface. Open the abdomen and the chest area and push intestines to the side with a sterile cotton-tipped applicator, thereby exposing the vessels. Locate the inferior vena cava (IVC) in the chest area and portal vein under the liver (Figure 2).
   Note: The IVC is a large blood vessel carrying blood from the lower extremities to the right atrium of the heart and is easily distinguished in the chest cavity extending between the diaphragm and heart.

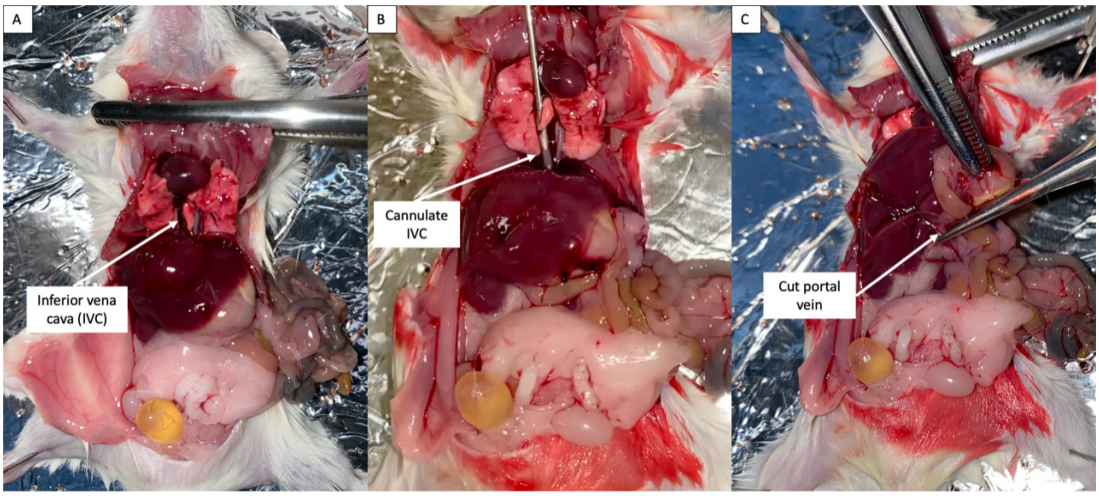

Figure 2. Identification (A) and cannulation (B) of IVC and cutting of portal vein (C)

6. As shown in Figure 2, carefully cannulate the IVC using the catheter and start the flow of the pump at 4 ml/min (pump setting 6.5-6.8). The liver will fill up with the solution turning brown within a few seconds. Quickly locate and cut the portal vein. Tape down the catheter and tubing securely.
7. After about 30 ml of Solution 1, run approximately 10 ml of Solution 2, and finally run Solution 3 for 10-15 min.
   Note: To gauge the progress of liver digestion, poke the liver with a cotton-tipped applicator after 10 min of perfusion with Solution 3. Under-digested liver will remain elastic, whereas a completely digested liver will retain an indentation.
8. When the liver is digested, remove the catheter, excise the gallbladder, and then carefully remove the liver without disrupting the liver capsule if possible.
9. Transfer the liver to a 60 mm Petri dish containing 10 ml of Solution 3. Disrupt the liver capsule with forceps and wash out the hepatocytes by swirling the dish. Collect hepatocytes and filter through a 100 µm filter unit into a sterile 50 ml conical tube that contains 10 ml of cold Wash Medium (see Recipes below).
10. For studies of non-parenchymal cells (NPC), collect the tissue retained on the filter and digested

further as directed in Step E12.

11. Pellet hepatocytes by centrifuging at 50 × *g* for 2 min at 4°C. Collect the supernatant and centrifuge it at 300 × *g* for 5 min and resuspend in cold Wash Medium. This is NPC Fraction 1. Resuspend the hepatocyte pellet in 50 ml cold Wash Medium.
12. Transfer tissue fragments collected in Step E10 to a 60 mm Petri dish. Add 5 ml of NPC Digest Solution (see Recipes below). Add a magnetic stirring bar and stir at 37°C for 10 min. After 10 min, pipette the solution up and down to assist the breakup of remaining tissue fragments.
    Note: Use a 1 ml pipette and cut the end of the 1 ml pipette tip a little to assist in picking up tissue fragments. Pipette up and down a couple of times to dissociate the tissue and release NPCs into the solution.
13. Collect dissociated cells by passage through a 40 µm strainer. Spin at 300 × *g* and resuspend the pellet in cold Wash Medium. This is NPC Fraction 2.
14. Transfer remaining tissue from Step E13 to a new dish with 5 ml of 0.05% trypsin, and incubate at 37°C with stirring for 10-15 min. After stirring, pipette the solution up and down to assist the breakup of remaining tissue fragments.
15. Collect newly dissociated cells by passage through a 40 µm strainer. Spin at 300 × *g* and resuspend in cold Wash Medium. This is NPC Fraction 3.
16. Pool all NPC Fractions collected in Steps E11, E13, and E15, pellet the cells by centrifugation, and resuspend in 5 ml cold Wash Medium. This gives the total NPC fraction.

F. Flow Cytometry (Data in Figure 3B)

1. Centrifuge hepatocytes at 50 × *g* and NPCs at 300 × *g* and resuspend in 1-2 ml RBC lysis buffer.
2. Keep in the lysis buffer for 2 min at room temperature, then add 10 ml to 20 ml Wash Medium.
3. Wash cells by centrifuging hepatocytes at 50 × *g* and NPCs at 300 × *g*. Resuspend hepatocytes and NPCs in 1-2 ml PBS depending upon the number of wells/samples required for analysis.
4. Incubate fractions in Zombie NIR dye for 30 min at room temperature. Wash with Wash Media, centrifuge appropriately, then resuspend in FACS Buffer (see Recipes below).
5. Dispense 100 µl cell suspension in each well of a 96 well plate, as necessary.
6. Add 2 µl Fc block to each well and wait for 10 min.
7. Add respective flow cytometry antibodies and incubate for 20 min at room temperature. Figure 3B outlines markers for endothelial cells, leukocytes, and pan-immune cells, for example.
8. Centrifuge plate at 700 × *g* for 4 min.
9. Remove supernatant, and add 100 µl FACS Buffer to each well.
10. Centrifuge plate at 700 × *g* for 4 min.
11. Remove supernatant, and add 100 µl FACS Buffer to each well.
12. Resuspend thoroughly, then transfer cells into the filter top of FACS tubes prepared with 300 µl FACS buffer.
13. Run cells on a flow cytometer.

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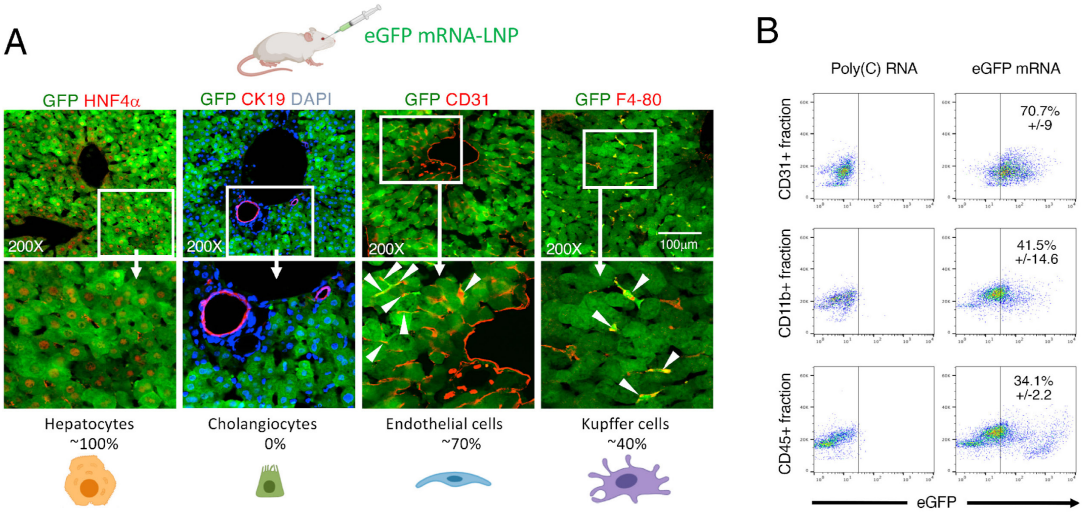

Figure 3. Identification of transfected liver cell types 5 h after IV eGFP mRNA-LNP injection. A. Co-immunostaining for eGFP (green) and liver cell type-specific markers (red, HNF4a for hepatocytes, CK19 for cholangiocytes, CD31 for endothelial cells, and F4-80 for KC); 200× magnification pictures and close-ups are shown. White arrowheads indicate co-stained cells. B. Representative flow cytometry analyses showing percent of eGFP$^+$ cells in CD11b$^+$ (most likely identifying KCs/macrophages as inflammation is minimal, therefore the presence of CD11b$^+$ neutrophils and CD11b$^+$ NK cells unlikely), CD45$^+$ (for blood cells), and CD31$^+$ (for endothelial cells) NPC fractions from mice injected with Poly(C) RNA-LNP or eGFP mRNA-LNP. All NPC fractions were gated for live cells present in Zombie NIR-negative populations. Data are presented as mean values ± SEM for n = 3 mice per group. The scale bar represents 100 μm for all 200× magnification images. Data were originally published in Nature Communications (Rizvi *et al.*, 2021).

Recipes

1. DNase I stock solution (10 mg/ml)
   Suspend 100 mg of DNase I in 0.15 M NaCl.
   Aliquot the DNase I in appropriate volumes to prevent frequent freeze and thaw.
   Store the aliquots at -20°C.
2. NPC Digest Solution
   2.5 mg/ml Collagenase IV and 0.1 mg/ml DNase I in EBSS (~5 ml per mouse); add 50 μl DNase I (10 mg/ml) and 12.5 mg Collagenase IV to 5 ml EBSS.
3. Wash Medium
   10% FBS and 0.1 mg/ml DNase I in Hepatocyte Wash Medium; add 5 ml FBS and 500 μl DNase I (10 mg/ml) in 50 ml Hepatocyte Wash medium.
4. Ketamine/Xylazine Anesthetic Solution
   100 μl/20g body weight of mouse cocktail; for a 10 ml of cocktail, add 1.75 ml Ketamine (100 mg/ml), 0.25 ml Xylazine (100 mg/ml) to 8 ml saline or sterile water.

5. FACs Buffer
   2 mM EDTA + 2% FBS in 1× PBS; add 1 ml FBS and 200 µl of 0.5M EDTA to 50 ml of 1× DPBS
6. 4% Paraformaldehyde
   Dilute 16% paraformaldehyde with PBS.
7. 3% Normal Donkey Serum
   Reconstitute freeze-dried normal donkey serum to a 100% stock concentration by adding 10.0 ml dH_2O. Aliquot and store at -80°C. Dilute stock to 3% working concentration using 1× PBS.
8. DAPI stock solution
   To make a 5 mg/ml DAPI stock solution (10.9 mM for the dilactate), dissolve the contents of DAPI (10 mg) in 2 ml of deionized water (dH_2O). Aliquot and store at -80°C. Use 1:3,000 diluted solution for staining nuclei.

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Competing interests

In accordance with the University of Pennsylvania policies and procedures and our ethical obligations as researchers, we report that Drew Weissman is named on patents that describe the use of nucleoside-modified mRNA as a platform to deliver therapeutic proteins. Relevant to this study, Drew Weissman and Norbert Pardi are also named on a patent describing the use of modified mRNA in lipid nanoparticles US patent US8,278,036 entitled “RNA containing modified nucleosides and methods of use thereof.” Mitchell Beattie and Ying Tam are employees of Acuitas Therapeutics, a company focused on the development of lipid nanoparticulate nucleic acid delivery systems for therapeutic applications. All other authors declare no competing interests.

Ethics

All animal studies were approved by the Boston University IACUC (PROTO201800461 approved until 10/25/2023) and were consistent with local, state, and federal regulations as applicable.

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