Delivery of non-viral naked DNA vectors to liver in small weaned pigs by hydrodynamic retrograde intrabiliary injection

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ORIGINAL ARTICLE

Hepatic gene therapy by delivering non-integrating therapeutic vectors in newborns remains challenging due to the risk of dilution and loss of efficacy in the growing liver. Previously we reported on hepatocyte transfection in piglets by intraportal injection of naked DNA vectors. Here, we established delivery of naked DNA vectors to target periportal hepatocytes in weaned pigs by hydrodynamic retrograde intrabiliary injection (HRII). The surgical procedure involved laparotomy and transient isolation of the liver. For vector delivery, a catheter was placed within the common bile duct by enterotomy. Under optimal conditions, no histological abnormalities were observed in liver tissue upon pressurized injections. The transfection of hepatocytes in all tested liver samples was observed with vectors expressing luciferase from a liver-specific promoter. However, vector copy number and luciferase expression were low compared to hydrodynamic intraportal injection. A 10-fold higher number of vector genomes and luciferase expression was observed in pigs using a non-integrating naked DNA vector with the potential for replication. In summary, the HRII application was less efficient (i.e., lower luciferase activity and vector copy numbers) than the intraportal delivery method but was significantly less distressful for the piglets and has the potential for injection (or re-injection) of vector DNA by endoscopic retrograde cholangiopancreatography.

INTRODUCTION

Delivery of genetic information remains a limiting factor for developing clinical effective drugs for the treatment of inherited or acquired genetic defects. Regarding liver gene therapy, all ongoing human trials and approved applications for liver gene therapy are based on gene addition using non-integrating viral vectors, predominantly adeno-associated viral vectors, which is challenging because of capsid immunogenicity, and toxicity in particular, when repeated delivery due to the growing liver in newborn or children is necessary (for overviews, see Dunbar et al.,1 Baruteau et al.,2 Colella et al.,3 Mendell,4 and Dave and Cornetta5). Non-viral-based delivery of naked DNA is less likely to elicit an immune response, but it has its drawbacks, including poor delivery concomitant with transient gene expression. While sustained gene expression from naked DNA vectors is achievable by using non-plasmid-based vectors such as minicircles (MCs)6,7 or nanovectors8 (see below), the delivery of non-viral vectors remains a hurdle as it needs physicochemical approaches for the DNA to penetrate through tissue and cell membranes. Various delivery systems for non-viral vectors are in development.9,10 A classic experimental approach for targeting nucleic acids to rodent liver is hydrodynamic gene transfer, also called hydroporation.11–13 This method is based on infusing via the rodent tail vein a large volume of a solution containing naked DNA vectors in a short time, typically a volume equal to 10% of mouse body weight in <10 s. Only transient elevation of liver transaminases due to some liver damage but no systemic inflammation or toxicity was reported in these approaches.

The observation that hydrodynamic tail vein infusion for liver targeting is safe in rodents led to the development of hydroporation methods in larger animals for future human gene therapy. While hydrodynamic (tail) vein infusion is feasible and efficient in mice, this method is not available for large animal models and direct liver targeting is required.14,15 In vivo delivery of gene vectors to the liver was established via the jugular vein (anterograde) and through different methods, see Dunbar et al.1 Baruteau et al.2 Colella et al.3 Mendell,4 and Dave and Cornetta5). Non-viral-based delivery of naked DNA is less likely to elicit an immune response, but it has its drawbacks, including poor delivery concomitant with transient gene expression. While sustained gene expression from naked DNA vectors is achievable by using non-plasmid-based vectors such as minicircles (MCs)6,7 or nanovectors8 (see below), the delivery of non-viral vectors remains a hurdle as it needs physicochemical approaches for the DNA to penetrate through tissue and cell membranes. Various delivery systems for non-viral vectors are in development.9,10 A classic experimental approach for targeting nucleic acids to rodent liver is hydrodynamic gene transfer, also called hydroporation.11–13 This method is based on infusing via the rodent tail vein a large volume of a solution containing naked DNA vectors in a short time, typically a volume equal to 10% of mouse body weight in <10 s. Only transient elevation of liver transaminases due to some liver damage but no systemic inflammation or toxicity was reported in these approaches.

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afferent and efferent vessels (e.g., portal vein [anterograde] and the biliary tract [retrograde] in dogs and pigs). Furthermore, catheter-based and/or balloon-assisted applications to target the liver, assistant with computer-controlled injection system were developed (for an overview see Yokoo et al.1). The injection of DNA through the biliary system may be a less invasive intervention compared to, for example, portal vein infusion, because transient obstruction of the portal vein flow can become life threatening in a short time. Furthermore, it was hypothesized that retrograde intrabiliary entry permits the circumvention of the sinusoidal endothelial cells, an anatomical barrier that may be a limiting factor for DNA delivery to hepatocytes through the portal vein.2,22 In addition, bile acid was reported to contain fewer nucleosomes compared to blood serum.24 Thus, the infusion of gene transfer vectors retrograde via the bile duct has been used extensively with various viral25–26 and non-viral vectors, the latter including naked DNA21,31–36 or vector DNA complexes (liposomes or nanoparticles32,34–36). Bile duct injection studies were conducted with mice,25,26,31,33 rats,25,26,32–34,37–39,41,42,44 dogs,31,32 and non-human primates.27

As mentioned above, the administration of non-integrating, non-viral, and non-plasmid naked DNA vectors have the potential for long-term expression and repeated delivery.26 MGs are devoid of any bacterial plasmid DNA backbone, allow sustained transgene expression in quiescent cells and tissues, and remain in the genome as episomal elements. MC-DNA vectors are propagated in genetically modified Escherichia coli strains and can be purified thereafter by conventional plasmid DNA extraction methods.5,41,42 A comparable antibiotic-free selection system called “nanoplastid” for the generation and delivery of naked DNA vectors with improvements regarding scale-up production and purification quality was developed by Nature Technology (Lincoln, NE, USA).43 Hydrodynamic injection through the tail vein in combination with therapeutic MC vectors for gene addition using a liver-specific expression cassette was successfully applied for the long-term correction of genetic mouse models for phenyleketonuria and cystathionine β-synthase deficiency.41,42,44

As a prerequisite to treating newborn or infant patients with inherited metabolic disorders of the liver, we established hydrodynamic portal vein injection in small pigs after weaning.45 While pigs suffered substantially from surgical distress-induced portal vein catheterization, we reported on stable hepatocyte transfection for the luciferase promoter of MC.P3Luc3 as a DNA vector45 and subsequently included an improved nanovector-based vector. Both vectors expressed luciferase as a reporter gene from the liver-specific promoter P3.46 This application route resulted in low but detectable luciferase activity and low vector copy numbers using vector MC.P3Luc3. Expression and copy number both could be significantly improved by replacing the MC-DNA vector with a nanovector-based vector. In addition, we observed that our setting for intrabiliary injection was less stressful for the piglets than for portal vein infusion. Moreover, we speculate that the retrograde intrabiliary injection route may eventually be amenable for human liver gene therapy by either endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC), which are established methods allowing physical access to liver cells via the bile duct.47–49

RESULTS
Surgical procedure and injection parameters for hydrodynamic retrograde intrabiliary infusion (HRII)

The setup for HRII of a solution containing vector DNA involved surgical intervention under deep anesthesia by placing a catheter in the common bile duct and transient isolation of the liver by clamping the portal vein, hepatic artery, and vena cava caudalis. This procedure is described in detail in the Materials and methods section and depicted in Figure 1A. The infusion parameters for HRII were established basically with two piglets, A1 and A2 (Table 1). Outflow was examined by contrast medium injection and monitored via real-time X-ray (Figures 1B–1E), and liver biopsies for analyses were collected before and after HRII for histological and transmission electron microscopy (TEM) analyses (Figure 2). The conditions tested were injections of either 30 or 100 mL of saline solution in 10 s (i.e., flow rates of 3 or 10 mL/s, respectively). The difference in flow rate is equivalent to an ~11-fold pressure increase based on Bernoulli’s principle of pressure ratio change, which is quadratic to the volume flow increase (i.e. a (3.3)²-fold increase from 30 to 100 mL/s). Our injection parameters, including flow rates of 3 and 10 mL/s, were based on 2 other reports of retrograde biliary injections into pigs (10–50 kg body weight [b.w.]),21,22 and on previous experience with portal vein injections into small pigs (3–7 kg b.w.).45 Kumbari and coworkers27 reported hydrodynamic injections of a volume of 40 mL at a flow rate of 2 mL/s retrograde via the bile duct into pigs (40–50 kg b.w.), with no liver rupture, while we did not see any liver damage by injecting via the portal vein a total volume of 30 mL with a flow rate of 10–20 mL/s and a maximal pressure of 120 mm Hg.45 Based on this, when we tested consecutive infusions of 30 mL of saline solution at 3 mL/s under liver isolation by transient obstruction of all 3 vessels, we observed no outflow (using contrast solution), no transient swelling of the liver, and no signs for lesions or rupture (see Figures 1B–1E). These conditions were chosen as “sham” infusion and performed (for flushing) before all injections with DNA vector solutions thereafter to test for the isolation (or clamping) of the liver by...
transient swelling. For maximal pressure, we increased the flow rate 3-fold–10 mL/s. As can be seen in Table 1, we used (except for pig C4) a volume of 100 mL solution containing vector DNA with a flow rate of 10 mL/s, where we observed rupture or liver damage in only 1 (pig C1) of the 12 pigs. After injection, clamping of all 3 vessels was continued for 1 min for liver isolation and optimal transduction with naked DNA vectors (S.F. Aliño, personal communication).

HRII infusion of naked DNA vectors is not toxic and does not lead to any histological abnormalities

During liver isolation and hydrodynamic infusion, all vital parameters (not shown), including standard circulating (enzyme) markers of liver function did not change or showed minor and/or transient elevation (see Table S1 for aspartate transaminase [AST], alanine transaminase [ALT], and lactate dehydrogenase [LDH]). For instance, AST and even more LDH were slightly elevated 6 h post-injection in only 1 (pig C1) of the 12 pigs. After injection, clamping of all 3 vessels was continued for 1 min for liver isolation and optimal transduction with naked DNA vectors (S.F. Aliño, personal communication).

We found that piglets recovered after surgery with no manifestations of morbidity or discomfort but also no indication for elevated bilirubin levels or jaundice, indicating that HRII leads to less suffering and was thus less stressful than the intraportal delivery route. As a means to quantitatively assess the surgical distress besides the inflammatory response (see above) to newborns, we compared the total time under anesthesia and for the duration of the surgical intervention between intraportal delivery compared to HRII infusion (see Tables S4 and S5). Here, we found a much shorter duration for both sedation and surgery for the HRII method compared to the intraportal injection (199 ± 49 min for the duration of sedation and 94 ± 32 min for the duration of surgical intervention for HRII, compared to 254 ± 45 min and 160 ± 42 min, respectively, for intraportal injection). We thus concluded that the procedure for the intrabiliary infusion route is not only less stressful than for portal vein injection but altogether safer, as we found no indication for toxicity.

Transfection of pig liver cells 10 days after naked DNA vector infusion via the bile duct

Using the setup and conditions described above, we tested liver cell transfection with DNA MC-vector MC.P3Luc3, which expressed firefly luciferase from the liver-specific promoter P3. The same vector was used successfully in our previous studies for hydrodynamic tail vein injections into mice and in small pigs after hydrodynamic portal

**Figure 1. Scheme of the common bile duct access for hydrodynamic retrograde intrabiliary infusion (HRII) and outflow examination via real-time X-ray (pig A1)**

(A) Scheme of a pig liver with vena portae, Arteria hepatica, vena cava caudalis, and the gallbladder, including biliary tract. Laparotomy and transient clamping (flaps) of vena portae, Arteria hepatica, vena cava caudalis, and the cystic duct (asterisk) was performed to target periportal hepatocytes. Access of the HRII procedure was performed by enterotomy and moving the catheter forward through the papilla duodeni major (not shown) into the common hepatic duct (black arrow in B). The position of the catheter was ensured and tightly fixed with clamps. (B) Pre-injection to check catheter position (the circle with a black arrow indicates the tip of the catheter). (C) Post-contrast medium injection while obstructing vena cava caudalis (“clamping of one”) with no outflow visible (circle). (D) Post-injection of contrast medium (22 mL 0.9% NaCl plus 8 mL contrast medium) without clamping; outflow in direction of the heart visible (circle). (E) Post-injection of contrast medium with clamping of all 3 vessels with no outflow visible (circle).
vein infusions. Here, we tested the efficacy of two vectors doses, 2 mg and 12 mg, and analyzed for vector presence in pig livers after liver resection 6, 3 days, or 10 days after infusion (pigs B1, B2, B4 and 12 mg, and analyzed for vector presence in pig livers after the injection of 2 mg vector DNA resulted 10 days later in low luciferase expression in pig B3 (Figures 3A and 3D). As can be seen from Table 2, activities in all of the samples in pig B1) and dropped to very low expression at day 3 (i.e., only 1 in 75 samples remained positive in pig B3 [1.33%]). Ten days after injection, luciferase expression was very low but detectable in 7 of 75 samples in pig B1 (176-37) 5.0 6 h 5.0 300 – 10; 10 (100) H&E staining, TEM (Figure 2) Figure 3A

B1 (176-37) 5.0 6 h 5.0 300 – 12 (2.40) 10; 10 (100) – Figure 3A

B2 (176-27) 5.3 3 d 6.9 300 12 (2.18) 10; 10 (100) Figure 3A

B3 (176-31) 5.7 3 d 6.9 300 12 (2.11) 10; 10 (100) Figure 3C

B4 (176-26) 5.4 10 d 6.5 n.d. 2 (0.37) 10; 10 (100) Figure 3C

B5 (176-28) 6.3 10 d 9.6 400 12 (1.88) 10; 10 (100) Figure 3E

B6 (176-29) 6.4 10 d 9.1 300 12 (1.88) 10; 10 (100) Figure 3E

B7 (176-30) 5.3 10 d 7.2 300 12 (1.26) 10; 10 (100) – Figure 3F

C1 (176-34) 4.3 6 h 4.7 n.d. 12 (2.79) 10; 10 (100) – Figure 4A

C2 (176-36) 6.7 6 h 6.7 n.d. 12 (1.79) 10; 10 (100) Figure 4B

C3 (176-32) 5.4 10 d 7.2 300 12 (2.22) 10; 10 (100) Figure 4B

C4 (176-33) 5.2 10 d 7.0 380 12 (2.31) 3; 10 (30) Figure 4C

At the time of surgical intervention, all piglets were 4 to maximally 6 weeks of age. Note that a sham infusion was performed before all of the vector injections (30 mL saline solution in 10 s). n.d., no data available.

“10 days” is between 9 and 11 days after injection.

1Group B pigs were injected with vector MC.P3Luc3, while group C pigs were injected with vector nSMARter.P3Luc1.

2Euthanized on day 3 due to poor health conditions.

3Not analyzed due to liver rupture.

Saturating PCR was positive in all 75 liver samples (representing the 5 liver lobes) after 10 days with 12 mg vector (100% transfection). In contrast, injection of 2 mg vector was less efficient as only between 36% (pig B5) and 85% (pig B4) of all liver samples tested were positive 10 days after injection. The determination of vector genomes (per diploid liver genome) revealed for the transfection with the 12 mg vector between 54 and 523 vector genomes 6 h after infusion (average of 162 vector genomes in pig B1). This number dropped several hundredfold over the next 3 days to 0.2–1.0 vector genomes (average of 0.4 in pig B3) and remained stable at day 10 (0.05–0.7 vector genomes; average of 0.3 in pigs B6 and B7).

A somewhat similar picture was found for luciferase activity, in which the injection of 2 mg vector DNA resulted 10 days later in low luciferase expression—only 6 of the total 75 samples were positive (8%) in pig B4, while luciferase expression was undetectable in pig B5 (Figures 3C and 3D). As can be seen from Table 2, activities in all of the samples of pigs B4 and B5 were very low and close to or below the detection limit. Six hours after injecting 12 mg vector DNA, luciferase expression was found in 45% of all of the tested samples (34 of 75 in pig B1) and dropped to very low expression at day 3 (i.e., only 1 in 75 samples remained positive in pig B3 [1.33%]). Ten days after injection, luciferase expression was very low but detectable in 7 of 75 samples in pig B1 (176-37) 5.0 6 h 5.0 300 – 10; 10 (100) H&E staining, TEM (Figure 2) Figure 3A

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weight increase from 250 to 345 g during the 10-day incubation period, which is equivalent to a 38% increase (see Table 1). Ideally, autonomous replication should overcome the episomal loss of DNA vector during cell growth and division. Naked DNA vectors harboring scaffold/matrix attachment region (S/MAR) elements with the potential for autonomous replication53 are in development and investigation in mice in our laboratory. We, therefore, included in this study with young pigs an infusion of nanovector nSMARter.P3Luc1, harboring the same luciferase expression cassette as we used for MC.P3Luc3 (see Figure S1). Based on the observations with vector MC.P3Luc3, we tested only a 12-mg vector dose and analyzed for vector presence in pig livers 6 h and 10 days after HRII (pigs C1–C4; Table 1). As described below, the lower flow rate of 3 mL/s resulted in a similar vector transduction efficacy as observed for the higher pressure in pig C3.

The results for nanovector-DNA transfection and luciferase activity are depicted in Table 2 and Figure 4. Saturating PCR performed 6 h and 10 days after HRII was again positive in almost all 75 liver samples representing all of the liver lobes confirming ~100% transfection. Vector quantification revealed 6 h after HRII an average of 288 nanovector genomes (pig C2) and 10 days after between 7 and 8 vector genomes in all of the samples tested from pigs C3 and C4. This is equivalent to a 38-fold decrease in 10 days. Thus, 25× more nanovector genomes were present in the liver 10 days after infusion compared to vector MC.P3Luc3 (average of 0.3 in pigs B6 and B7; see above). Luciferase expression was detected in almost all of the samples tested 6 h after the infusion of nanovector nSMARter.P3Luc1 (Figure 4A), although with low activity (0.3 relative light unit [RLU]/μg), which was comparable to what was found for MC.P3Luc3 (Table 2). This activity decreased to low detection 10 days after HRII but remained detectable in some liver lobes.

In summary, using the nanovector nSMARter.P3Luc1 compared to MC.P3Luc3, we observed in pigs a much higher transfection and luciferase expression after 6 h and a 10-fold higher number of vector genomes with more significant luciferase expression 10 days after infusion by HRII.

**Localization of naked DNA vectors in hepatocytes after HRII**

In an attempt to localize the transfected target in liver cells, we prepared cryosections and formalin-fixed paraffin-embedded pig liver biopsies of all five liver lobes for luciferase detection. As can be seen in Figure 5, 3,3′-diaminobenzidine (DAB)-chromogen staining revealed a luciferase-positive reaction in hepatocytes. Additional positive signals can be seen for hepatocellular bile acid and hemosiderin, which was indirectly confirmed by the 3-amino-9-ethylcarbazole (AEC)-chromogen staining.

**DISCUSSION**

Non-viral gene delivery is an attractive approach for gene therapy because of the favorable safety profile over, for example, adeno-associated virus (AAV), which is the leading vector system for clinical applications for *in vivo* gene therapy for various organs,54 including the liver, despite accumulating evidence for genotoxicity and hepatocellular carcinoma (for a recent discussion on the long-term safety issues of AAV vectors, see Dave and Cornetta5). Non-viral delivery of naked DNA vectors to the liver through the tail vein using hydrodynamic forces is an established procedure for rodents to target (primarily perivenous) hepatocytes; however, this approach is not translatable
to larger animal models or human subjects. Various metabolic activities are specifically and exclusively located in the periportal hepatocytes, which require direct naked DNA vector targeting via, for example, the portal vein or the bile duct. We have previously reported on hydrodynamic intraportal injection of naked DNA MC vectors into small pigs immediately after weaning. We aimed here to reduce the invasiveness of intraportal injections with sustained or improved therapeutic efficacy for vector delivery by targeting periportal hepatocytes in weaned pigs via the bile duct by HRII.

Hydrodynamic injection via the bile system has been described in rats and by ERCP guidance in adult pigs (by infusing plasmid DNA), but not in small pigs after weaning. We aimed here to reduce the invasiveness of intraportal injections with sustained or improved therapeutic efficacy for vector delivery by targeting periportal hepatocytes in weaned pigs via the bile duct by HRII.

Optimal conditions were found with up to 12 mg naked DNA vector in a volume of 100 mL and an infusion time of 10 s, followed by 1 min of clamping. Transfection of hepatocytes in all of the tested liver samples (75 of 75) was observed with 12 mg vectors expressing luciferase from a liver-specific promoter up to day 10. Vector copy number and luciferase expression were thus higher compared to hydrodynamic intraportal injection, even when up to 12 mg vector MC.P3Luc3 was tested (unpublished data).

Table 2. Vector transfection rate, luciferase activity, and copy number (per diploid liver genome) in liver cells of pigs infused with either vector MC.P3Luc3 (B1–B7) or nSMARTer.P3Luc1 (C2–C4).

| Pig   | Infusion | Transfected | Liver lobe |
|-------|----------|-------------|------------|
| Pig B1 (12 mg; 6 h) | 75/75 (100%) | Right middle | Vector genomes: 54.3, 0.17 |
| Pig B3 (12 mg; 3 d) | 75/75 (100%) | Right middle | Vector genomes: 59.8, 0.191 |
| Pig B4 (2 mg; 10 d) | 64/75 (85%) | Right lower | Vector genomes: 57.7, 0.335 |
| Pig B5 (2 mg; 10 d) | 27/75 (36%) | Right lower | Vector genomes: 523, 0.266 |
| Pig B6 (12 mg; 10 d) | 75/75 (100%) | Left middle | Vector genomes: 55.8, 0.48 |
| Pig B7 (12 mg; 10 d) | 75/75 (100%) | Left lower | Vector genomes: 114, 0.29 |
| Pig C2 (12 mg; 10 d) | 74/75 (99%) | Caudal | Vector genomes: 114, 0.29 |
| Pig C3 (12 mg; 10 d) | 75/75 (100%) | Caudal | Vector genomes: 114, 0.29 |
| Pig C4 (12 mg; 10 d) | 75/75 (100%) | Caudal | Vector genomes: 114, 0.29 |

n.d., no detectable luciferase activity (below the threshold of 0.08 RLU/μg).

Fraction of transfection liver samples from a total of 75 tested samples (i.e., PCR+ for vector DNA [in %]). The 75 samples were collected from different parts and represent all 5 liver lobes.

Luciferase activity (RLU/μg of protein lysate) averaged in all of the positive samples per lobe (threshold 0.08 RLU/μg).
particular, for experimentation with large animals, the effort and quality aspects in the production of sufficient highly pure vector DNA cannot be underestimated. For these reasons, we switched during our experiments with young pigs from MC DNA to the nanovector platform, which offers a simpler way for vector generation and purification, with significantly higher yields than for MC vector preparation concomitant with high purity and quality, including exclusively the monomeric form of circular nanovector. Here, we used the naked DNA vector nSMARter.P3Luc1, which harbored the identical transgene expression cassette for luciferase-like vector MC.P3Luc3. In addition, this nanovector contains an S/MAR element with the potential for autonomous replication to overcome the episomal loss of vector DNA during cell growth and division. Here, we used the naked DNA vector nSMARter.P3Luc1, which harbored the identical transgene expression cassette for luciferase-like vector MC.P3Luc3. In addition, this nanovector contains an S/MAR element with the potential for autonomous replication to overcome the episomal loss of vector DNA during cell growth and division.

HRII infusion leads to no detectable histological damage of liver tissue (Figure 2). Furthermore, we thought to investigate the cellular localization of luciferase expression by the immunohistological staining of cryosections. Here, AEC-chromogen was used to identify luciferase-positive reaction and to distinguish from hepatocellular bile and hemosiderin (Figure 5). The latter two exhibit a more crystal-like, shiny brown appearance compared to hepatocytes, which are more matte brown. Although it is difficult to distinguish positive reactions from hepatocellular bile and hemosiderin because both stainings are brown, the positive reaction could be observed as focal and nuclear.

Figure 3. Sketches of pig liver lobes and efficacy of MC.P3Luc3 naked DNA vector delivery and luciferase expression upon HRII

| Pig | Injection | Transfection Rate | Luciferase Expression |
|-----|-----------|-------------------|-----------------------|
| B1  | 12 mg     | 100%              | 45%                   |
| B3  | 12 mg     | 100%              | 1.33%                 |
| B4  | 2 mg      | 85%               | 8%                    |
| B5  | 2 mg      | 36%               | undetectable          |
| B6  | 12 mg     | 100%              | 6.7%                  |

Note that “10 days” means between 9 and 11 days post-injection.
in hepatocytes, which indicates that the transfected cells are hepatocytes.

In summary, we found that the HRII application route was feasible and safe in piglets, with a reduced impact on animal welfare but lower efficacy (i.e., lower luciferase activity and vector copy numbers when compared to intraportal injections). In addition, the intrabiliary injection approach has the potential for injection (or re-injection) of vector DNA by ERCP-guided hydrodynamic delivery (alternatively, percutaneous transhepatic cholangiography) in combination with sequential multilobular injections, which opens new avenues for direct gene delivery to the liver.

MATERIALS AND METHODS

Handling of naked DNA/MC vector MC.P3Luc3 and nanovector nSMARter.P3Luc1

For pigs B1–B7, purified MC vector MC.P3Luc3 (2,324 bp) that expressed the firefly luciferase gene from the liver-specific promoter P3 was used.6,41,56 For vector DNA preparation, the parental plasmid was transformed into the bacterial strain ZYCY10P3S2T, expressing integrase and endonuclease, both under the control of the arabinose-inducible promoter pBAD.6 MC-DNA was generated via intramolecular recombination after adding arabinose. The excised bacterial backbone was degraded in vivo, and the corresponding MC contained the liver-specific P3 promotor for hepatocyte expression of luciferase as a reporter gene, plus a bovine growth hormone polyadenylation signal element BGHpA. Vector DNA was purified by using a commercial kit from Macherey-Nagel NucleoBondXtra Maxi EF.

For pigs C1–C4, purified nSMARter.P3Luc1 (4,644 bp) vector that contained the firefly luciferase gene under the control of the P3 promoter, a replicating element scaffold/matrix attachment region (S/MAR), and an isolator Ele40 was used.57 The vector was generated in the laboratory of Dr. Richard Harbottle (German Cancer Research Center, Heidelberg, Germany). Purified DNA was provided by Nature Technology. Both vector constructs were validated for luciferase expression under cell culturing conditions and in mouse liver upon hydrodynamic tail vein infusion (see Viecelli et al.,41 Grisch-Chan et al.,42 Stoller et al.,45 and data not shown).

Animal handling

Animal experiments were performed according to the guidelines and policies of the Veterinary Office of the state of Zurich, Switzerland, and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Animal studies were approved by the Veterinary Office of the state of Zurich and the Cantonal Committee for Animal Experiments, Zurich (license to perform animal experiments ZH176-2016/28163). Three-week-old female domestic pigs (between 4.3 and 6.7 kg b.w.; Table 1) were separated from their mothers after weaning and brought to a loose barn with porcine mates for adaptation 5 days before surgery.

Anesthesia and postoperative monitoring

The medications for pre-anesthetic medication were azaperone (2 mg/kg intramuscularly [i.m.]), ketamine (5 mg/kg i.m.), and atropine (0.03 mg/kg i.m.); for induction, an isoflurane mask was used, and for maintenance, isoflurane and oxygen. Animals were kept for 6 h, 3 days, or 10 days (i.e., 9–11 days) after surgical intervention. Liver transaminases (AST, ALT, and LDH) were measured in the Unit for Clinical Chemistry and Biochemistry at University Children’s Hospital Zurich by automated analyzer.
UniCel DXC600 (Beckman Coulter, Nyon, Switzerland) at on the day of surgery and the day of sacrifice. Plasma and urine were stored shock-frozen for any later analyses. The following medications were used for postoperative care: buprenorphine 0.01 mg/kg intravenously (i.v.) (3×/day [t.i.d.]) and enrofloxacin 5% 2.5 mg/kg i.m. once daily for 3 days. In case of fever, metamizole 25 mg/kg i.v. (2×/day [b.i.d.]) was added. The anesthesia setup was as follows: The animal was positioned in a dorsal recumbent position and on a closed-circuit anesthesia system. The heart rate was monitored via electrocardiography (ECG), the respiratory rate via IPPV (intermittent positive pressure ventilation), blood pressure via an arterial catheter (A. femoralis), and body temperature via an esophageal thermometer. The capnography monitor displayed the concentration of carbon dioxide in the respiratory gases. Isoflurane was used as the inhalation gas and for ventilation, a mixture of oxygen and air was used. Infusion pumps were used for controlled analgesia. A contrast injector, the Liebel-Flarsheim Angiomat 6000, was used, which allowed for controlled analgesia. A control panel allowed us to inject naked DNA via pressure. A control panel allowed the adjustment of pressure, volume, and flow rate.

**Surgical procedure for HRII**

Laparotomy was performed from the xyphoid to the umbilicus, followed by isolation/separation of the hepatic arteries of the hepatic ligament. The portal vein was prepared distal from the hepatic ligament and separated from the pancreas over a distance of 1 cm to place a clamp. Then, a 2-cm longitudinal enterotomy was performed. A 2-cm longitudinal enterotomy was performed next to the insertion of the common bile duct into the duodenum. A 7-Fr single-lumen catheter was placed through the major duodenal papilla in the common bile duct. The position of the catheter was initially monitored by injecting contrast medium (pre- and post-injection; Accupaque 300 mg, GE Healthcare AG, Glattbrugg, Switzerland) via real-time X-ray (Figure 1A). Once established, we injected the catheter without contrast medium and ensured manually that the tip of the catheter was placed into the common hepatic duct 1 cm distal to the junction of the left and right hepatic ducts. This allowed us a controlled injection of naked DNA MC vectors (between 2 and 12 mg naked DNA diluted in a volume of 100 mL Ringer’s solution injected within 10 s according to Table 1 into the entire liver by using the Liebel-Flarsheim Angiomat 6000. The catheter was tightly fixed with two clamps (Atraumata Bulldog Clamps FB364R and FB366R), which also temporarily closed the enterotomy. For all of the experiments, clamps were placed at the cystic duct to avoid backflow to the gallbladder, the hepatic arteries, and the portal vein, allowing hepatic inflow obstruction (Figure 1A). Likewise, the caudal cava vein was clamped to prevent venous outflow from the liver during the injections. The total clamping time was kept short—no longer than 1–2 min, given the known vulnerability of the pig intestine to outflow obstruction. After injection, all of the clamps were removed in the following order: caudal cava vein, portal vein, hepatic arteries, and cystic duct. The catheter was removed and the enterotomy was closed with absorbable running sutures. Patency and tightness were tested. Urine was collected for analysis by needle puncture of the bladder, and thereafter, the abdominal cavity was flushed with Ringer’s lactate to reduce contamination. The abdominal cavity was closed stepwise (muscle layer, subcuticular layer) with absorbable running sutures. The wound was covered with a standard dressing. Piglets returned to a loose barn with piglets within 3–4 h after the end of anesthesia.

**Collection of samples**

Blood and urine samples for various biochemical analyses and naked-DNA MC-vector determination were collected on the day of surgery and on the day of sacrifice. Pigs were sacrificed under deep anesthesia with a combination of xylazine and ketamine and released with pentobarbital 6 h, 3 days, or 10 days after injection. The entire liver was resected to collect 75 individual tissue samples that represent all 5 liver lobes (Figures 3 and 4).
Cryo-sections, H&E staining, immunohistochemistry, and cytokine analysis

For histological evaluation, liver tissue from representative lobes was either snap-frozen immediately for cryosectioning (3–5 μm, Presto-CHILL, Kalamazoo, MI, USA) or fixed in formalin (4%) for routine paraffin embedding. Histological sections (3–5 μm) were prepared and routinely stained for H&E and PAS reaction. The latter was used to detect hepatocellular glycogen (polysaccharide) content in liver sections. Immunohistological evaluation was performed on cryosections to detect luciferase-positive cells using an anti-luciferase antibody (ab 181640, Abcam, Cambridge, UK) with Dako autostainer (Dako, Glostrup, Denmark) system. Briefly, antigen retrieval was performed in the antibody using citrate buffer (pH 6) at 98°C for 20 min or EDTA buffer (pH 9) at 98°C for 20 min and CCl1 buffer (pH 8.4). Subsequently, endogenous peroxidase activity was quenched with hydrogen peroxidase for 10 min. Primary antibodies were incubated for 1 h, followed by secondary antibody application. For detection, the Dako EnVision kit was used. Finally, sections were counterstained with H&E for 40 s and mounted. Luciferase-positive pig liver served as a positive control for the luciferase antibody, as negative controls served sections omitting the primary antibody. The serum cytokine analysis for TNF-α, IL-1β, and IL-6 was completed by Cytolab (Regensdorf, Switzerland).

Transmission electron microscopy (TEM)

Samples of liver for TEM evaluation were trimmed immediately after liver exenteration into 1-mm square blocks and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) buffered in 0.1 M Na-phosphate buffer, post-fixed in 1% osmium tetroxide (Sigma-Aldrich, St. Louis, MO, USA), and dehydrated in ascending concentrations of ethanol, followed by propylene oxide and infiltration in 30% and 50% EPON (Sigma-Aldrich). At least three 0.9 mm toluidine blue-stained semi-thin sections per localization were produced. Representative areas were trimmed, and 90 nm lead citrate (Merck, Kenilworth, NJ, USA) and uranyl acetate (Merck)-contrasted ultrathin sections were produced and viewed under Phillips (Eindhoven, the Netherlands) CM10 operating with a Gatan Orius Sc1000 (832) digital camera (Gatan Microscopical Suite, Digital Micrograph, version 230.540; Gatan, Pleasanton, CA, USA).

Vector DNA analysis in pig liver

For liver tissue homogenization, a TissueLyser II (Qiagen, Hombrechtikon, Switzerland) was used. DNA extraction from liver homogenates was performed according to the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). The purity and quality of DNA was determined with the NanoDrop ND1000 (Thermo Fisher Scientific, Waltham, MA, USA). Saturating PCR for the detection of vector DNA was performed using isolated tissue samples. All primer DNA was synthesized by Microsynth AG (Balgach, Switzerland). For the amplification and detection of vector DNA (MC.P3Luc3 and nSMARter.P3Luc1), forward primer f2 luc (5’-CAGTTCGTCACATCTCATCTACC-3’) and reverse primer r3 luc (5’-TGAGCCCCATATCTTGCGTATA-3’) were used to amplify the luciferase transgene. PCR for 42 cycles using HOT FIREPol polymerase (Solis BioDyne, Lucerne, Switzerland) was performed at a denaturation temperature of 95°C, an annealing temperature of 63°C, and an extension temperature of 72°C. The amplified fragment had an expected length of 533 bp. As a positive control, MC.P3Luc3 or nSMARter.P3Luc1 was used, and as the negative control, isolated DNA from untreated/non-infused pig liver was used.

Assay for MC copy number

Genomic DNA (gDNA) from all 75 liver samples was isolated by using a DNeasy blood and tissue kit from Qiagen GmbH. According to the manufacturer’s manual, we used 100 ng gDNA from each sample as a template. Serially diluted DNA vector with various copy numbers (2 × 107–20 copies) for MC.P3Luc3, along with 100 ng non-infused control gDNA were generated to plot standard curves cycle threshold (Ct; y axis) against log vector copy number (x axis) for each vector infused in pig livers (see Table S2 for a list of standard curves for all pigs). To determine the number of vector genomes per cell in liver tissue, we performed absolute quantitative PCR (qPCR) analysis by either using a TaqMan gene expression assay corresponding to luciferase (M03987587_mr, Life Technologies, Carlsbad, CA, USA) or primers and probe bound to BGHPa in all liver tissue samples. The following primers and probes were used: BGHPa forward primer 5’-GCCTTCTAGTTGCCACCCAT-3’, BGHPa reverse primer 5’-G GCACCTTCCAAGGTCAG-3’, and probe 5’-FAM-TGTTGGCC CTCCCGTGC-TAMRA-3’. qPCR analysis for nSMARter.P3Luc1 was performed using PowerUp Sybr Green Master mix (Applied Biosystems, Waltham, MA, USA) and primers that bind to minimal bacterial backbone (i.e., MBB forward primer 5’-AAGTCTGATCG CAGTGGTG-3’ and MBB reverse primer 5’-CCTCTCGAGGA CATGGTGA-3’). For qPCR measurement, we used ABI PRISM 7900 sequence detector (Applied Biosystems), and for analysis Sequence Detection System (Life Technologies). The haploid genome size of the pig is estimated to be 2,800 Mb (according to the NCBI Genome Database), and the mass of a single diploid copy is 6.14 pg (calculated according to the description from Life Technologies). Consequently, 100 ng gDNA contains 16,287 copies of the diploid genome (1 × 105 pg/6.14 pg).

Luciferase activity

Luciferase activity was measured in liver tissue samples as RLU/μg protein lysate. The threshold was defined as ≥ 0.08 RLU/μg protein lysate. For activity determination, the Luciferase Assay System (Promega, Dübendorf, Switzerland) was used on the microplate reader infinite F200 (Tecan Group, Männedorf, Switzerland). For data analysis, i-control 1.10 software (Tecan Group) was used. As positive control, liver tissue lysates from MC.P3Luc3-injected mice with MC-DNA vector expressing luciferase were used, and as the negative control, a liver tissue lysate of an untreated/non-infused pig liver was used.
Statistical methods
All of the values were presented as absolute numbers for all of the individual samples and each animal—in other words, no pooling of data for statistical analysis was done.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.01.006.

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AUTHOR CONTRIBUTIONS
T.C., H.M.G.-C., P.S., U.S., U.H., N.R., T.S., B.S., S.K.R., J.H., and B.T. performed the experiments and analyzed the data. M.B., R.H., J.A.W., and X.S. contributed analyses and/or tools. T.C., H.M.G.-C., and B.T. conceived and managed the project. T.C. and B. T. wrote the manuscript, with the contribution of all of the authors. All of the authors read and approved the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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