Endoscopic-mediated hydrodynamic gene delivery through the biliary system mediates efficient transfection of pig liver

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Supplementary Methods

Gene construction and plasmid preparation

The plasmid, pCMV-HyPBase, was constructed by gene synthesis (BioBasic). The sequence was pig-codon optimized using tools from IDTDNA based on the published hyperactive piggyBac transposase sequence (Yusa K, et al, PNAS 2011).The hFIX expression cassette used in AAV trials (Nathwani AC, et al, Blood 2006) was synthesized into a pUC backbone (BioBasic) with the addition of the human albumin 3' UTR for increased mRNA transcript stability. Pig-codon optimized human Factor IX using tools from IDTDNA and prepared as a gene fragment (Twist Bioscience) and cloned with restriction enzymes to form pT-LP1-hFIX.

Plasmids were prepared for in vitro experiments using maxiprep kits (Qiagen) and for in vivo injection using gigaprep kits (Zymo Research). Ratio of transposon to transposase for injection was 7.5 to 9.2, to try to optimize transposition efficiency (Doherty JE, et al, Human Gene Therapy 2012). Plasmid DNA (pDNA) was diluted in normal saline solution for in vivo experiments in mouse and pigs.

Stool and Tissue analysis

Pigs were euthanized at time points 1- and 3-weeks postinjection, and necropsy was performed within 15 minutes of death. Veterinary and medical pathologists were consulted for proper technique. Biopsies were taken across multiple sites in proximal and distal within each liver lobe. The quadrate lobe was sampled because it connects to the liver hilum with a direct bile duct branch like the 4 other largest lobes, whereas the caudate process was included with right lateral lobe (RLL) for analysis because they share the same biliary duct branch. Preliminary studies found that RLL, right medial lobe (RML), left lateral lobe (LLL), and left medial lobe (LML) all had approximately similar masses within 5%, together accounting for ~95% of the liver mass. Mice were euthanized at 1 week postinjection. For both mice and pigs, tissue was fixed in 10% formaldehyde. The Johns Hopkins Phenotyping Core did tissue embedding and sectioning, along with H&E staining.

The DNeasy Blood & Tissue kit (Qiagen) was used for DNA extraction from tissues, blood, and bile samples. Fecal DNA was purified using a Quick-DNA Fecal/Soil Microbe (Zymo Research). RNA extraction was performed with RNeasy kit (Qiagen), and reverse transcription was performed (SuperScript IV, Thermofisher). PCR (DreamTaq, Thermofisher) was performed using internal primers (Sigma) directed against the synthetic hFIX sequence, Internal FIX For: GATAATAAGGTGGTCTGCTCTTGCACG, Internal FIX Rev: GTCACGTAGGAGTTGAGGACCAG

An antibody against human Factor IX (GAFIX-AP, Affinity Biologicals) was used for western blot detection, as well as staining by immunohistochemistry (IHC) on pig and mouse liver sections. Western blot was performed with 10% SDS-PAGE gel using 2.5 µg/mL antibody dilution, and mouse anti-goat IgG-HRP secondary (sc-2354, Santa Cruz). IHC was performed by VitroVivo Biotech (Rockville, MD) with negative control un-injected pig and positive control, human liver sections. For quantification of transfection efficiency of hydrodynamic injection, whole slide scanning was performed (Olympus) and quantification was performed in ImageJ. Briefly, individual hepatic lobules were identified, and the area of entire lobule and areas of hepatocytes with hFIX staining were outlined in ImageJ, and the percentage area calculated. As the lobule is the functional unit of the liver, the transfection efficiency in lobules would be representative of the entire lobe and liver, respectively.

To calculate the percentage protein of hFIX compared with pig FIX in liver tissue, the beta-actin normalized band intensity was taken for each lobe and the control pig band intensity was subtracted to yield hFIX contribution; this result was divided by the calculated porcine FIX level, determined from the hFIX antibody cross-reactivity (band intensity/6.4%). The average of all 5 liver lobes is presented in the text ($10.11 \pm 4.05\%$).

Hydrodynamic tail vein injection

C57BL/6 mice weighing between 20 and 25 grams were selected for HTVI. Mice were treated with a heat lamp for 5 to 10 minutes until vasodilation was achieved. Eight µg transposon and 1 µg transposase were diluted in 2.2 mL (8-10% body weight) of normal saline solution and injected into mice between 4 and 7 seconds. This dose was selected

to give a matching liver weight-based dosing comparison for pig hydrodynamic studies. Post HTVI, >90% of inject DNA is localized to the liver (Herweijer H, et al, Gene Therapy 2007), such that 8 µg transposon DNA into a 1.5 g mouse liver equates to ~4 to 5.3 mg in a 1 kg pig liver.

Blood collection and analysis

All pigs were anesthetized with ketamine/xylazine before bleeding through the jugular vein by a veterinary technician. Blood was collected into EDTA tubes for plasma proteins, and serum chemistries in SST tubes (Becton Dickinson). Stool was collected while the pigs were anesthetized for phlebotomy by manual rectal examination. Pigs were also weighed during this period. Blood from mice were collected by retroorbital eye bleeding under isoflurane anesthesia and prepared similarly.

ELISA testing for hFIX on cell culture supernatant, human plasma, mouse plasma, and pig plasma was performed according to manufacturer's protocol (AssayMax Human Factor IX ELISA Kit, Assay Pro); the ELISA kit lacks cross-reactivity against mouse hFIX (DeRosa F, et al, Gene Therapy 2016). Discarded, deidentified human plasma was used as a positive control. Serum chemistries and hematology were performed by the Johns Hopkins Phenotyping Core on Diasys Respons®910 chemistry analyzer and Procyte automated analyzer, respectively.

Statistical analysis

GraphPad Prism 7 software (GraphPad Software) was used to perform statistical analysis and generate graphs. Data are presented as mean ± standard error of mean (SEM). Unpaired, parametric, 2-tailed t-tests were used to test mean differences. Significance level used was $P < .05$.

Pig Number	Sex	Weight (kg)	Injection Volume (mL)	Flow Rate (mL/sec)	hFIX transposon plasmid (mg)	hyperPB plasmid (mg)
1	Female	35	30	2	3	0.4
2	Female	35	30	2	3	0.4
3	Female	37	30	2	5.5	0.6
4	Female	36	30	2	5.5	0.6

Supplementary Table 1. Biliary hydrodynamic injection parameters and DNA doses for each pig.

Yorkshire, female pigs weighing between 35.0 – 37.0 kg were used in the study. Each pig was injected at the same flow rate and total volume injected. Two sets of DNA doses were used (3 mg and 5.5 mg) to evaluate transfection response; transposon : transposase ratio was kept similar between doses 7.5:1 versus 9.2:1.

Supplementary Figure 1. Design and validation of the hFIX plasmid vectors in a mouse model. A, The pT-LP1-FIX plasmid encodes terminal repeats (TR) of the piggyBac (pB) transposon to facilitate integration. The LP1 promoter is a composite promoter consisting of human APO-HCR enhancer and hAAT promoter. The SV40 intron located in the 5' UTR enhances expression. The hFIX gene is codon optimized for pig expression. The 3' UTR from the human albumin gene enhances mRNA transcript stability. The SV40 late polyadenylation (poly-A) sequence completes the expression cassette. B, A second plasmid encoding a hyperactive piggyBac transposase (HyPBase) is driven by a cytomegalovirus (CMV) promoter to mediate high level, transient expression in the liver. C, Mouse HTVI of these plasmids yields high level hFIX levels in plasma (n=4) and (D) frequent hFIX-positive hepatocytes by cytoplasmic immunostaining. E, Individual lobules were quantified in three injected mice, and stained area quantified (n=6 lobules per mouse). Mean ± SEM are depicted.

Supplementary Figure 2. Vital signs were monitored, and ultrasound was performed during the hydrodynamic procedure displaying minimal disturbance pre- and postprocedure. A, Ultrasound monitoring during procedure reveals no abnormalities from the hydrodynamic procedure. Preprocedure ultrasound images revealed a normal-sized gallbladder. After the procedure, the gallbladder showed no increase in size suggesting successful balloon placement upstream of the cystic duct. No other liver abnormalities were seen. B, Different vital signs were monitored during the entire endoscopic procedure, from entry of endoscope to withdraw after the biliary

hydrodynamic injection, demonstrating minimal fluctuations. Representative anesthesia record was shown for pig no. 3.

Supplementary Figure 3. hFIX DNA delivered by biliary hydrodynamic injection is detected long-term in pig liver. Genomic DNA was extracted from liver tissue of pig no. 1, no. 2, and no. 4 at 3 weeks after injection. PCR was performed for hFIX DNA and revealed the expected band size (550 bp) for hFIX DNA in all liver lobes tested.

Supplementary Figure 4. Human FIX antibody mediates low-level cross-reactivity to porcine FIX. A, Western blot of human plasma and pig plasma was performed, demonstrating that the hFIX antibody stains human plasma intensely at the correct molecular weight (70 kDa), while demonstrating minimal binding to porcine FIX. B, The band intensity on the western blot was quantified, using normalization of the input protein levels in each well (1:0.66 human plasma to pig plasma protein).

Supplementary Figure 5. Distribution of hFIX immunostaining in pig liver sections after biliary hydrodynamic injection. Immunohistochemistry images for the hFIX-injected pig from the distal biopsy site of the right medial lobe of pig no. 3 are presented. An individual liver lobule demonstrates the most intense expression around the central vein radiating toward the fibrous lobule borders (bar = 100 µm). Positive hFIX-hepatocytes were seen in all functional zones, although most abundant in zone 3 (bar = 50 µm). hFIX-expression was most intense and uniform around the central vein (bar = 20 µm), although numerous positive cells can also be seen near the portal triad (bar = 50 µm).

Supplementary Figure 6. A representative image of mouse liver after hydrodynamic tail vein injection of hFIX plasmid DNA. A representative image of mouse liver after hydrodynamic tail vein injection (HTVI) of hFIX plasmid DNA plus pCMV-HyPBase is presented, demonstrating positive hFIX-hepatocytes in every liver lobule (bar = 200 μm).