



Lentiviral Vector-mediated Gene Therapy of Hepatocytes *Ex Vivo* for Autologous Transplantation in Swine

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Abstract

Gene therapy is an ideal choice to cure many inborn errors of metabolism of the liver. *Ex-vivo*, lentiviral vectors have been used successfully in the treatment of many hematopoietic diseases in humans, as their use offers stable transgene expression due to the vector's ability to integrate into the host genome. This method demonstrates the application of *ex vivo* gene therapy of hepatocytes to a large animal model of hereditary tyrosinemia type I. This process consists of 1) isolation of primary hepatocytes from the autologous donor/recipient animal, 2) *ex vivo* gene delivery via hepatocyte transduction with a lentiviral vector, and 3) autologous transplant of corrected hepatocytes via portal vein injection. Success of the method generally relies upon efficient and sterile removal of the liver resection, careful handling of the excised specimen for isolation of viable hepatocytes sufficient for re-grafting, high-percentage transduction of the isolated cells, and aseptic surgical procedures throughout to prevent infection. Technical failure at any of these steps will result in low yield of viable transduced hepatocytes for autologous transplant or infection of the donor/recipient animal. The pig model of human type 1 hereditary tyrosinemia (HT-1) chosen for this approach is uniquely amenable to such a method, as even a small percentage of engraftment of corrected cells will lead to repopulation of the liver with healthy cells based on a powerful selective advantage over native-diseased hepatocytes. Although this growth selection will not be true for all indications, this approach is a foundation for expansion into other indications and allows for manipulation of this environment to address additional diseases, both within the liver and beyond, while controlling for exposure to viral vector and opportunity for off-target toxicity and tumorigenicity.

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Disclosures

The authors have nothing to disclose.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58399/>

Keywords

Medicine; Issue 141; Gene Therapy; Lentiviral Vector; Hepatocyte Isolation; Pig Surgical model; Autologous Transplantation; Inborn Errors of Metabolism

Introduction

Inborn errors of metabolism of the liver are a family of genetic diseases that collectively affect as many as 1 in 800 live births¹. Many of these diseases are single gene defects² and can be functionally cured by introducing a single corrected copy of the affected gene into a sufficient number of hepatocytes³. The actual percentage of hepatocytes that needs to be corrected varies by the disease⁴ and is largely dependent on the nature of the protein it encodes, for example, excreted proteins versus cytoplasmic. In most cases, efficacy of any treatment for metabolic disease is easily assayed through the presence of biomarkers often available in the circulation.

HT-1 is an inborn error of metabolism of the liver that results from a defect in fumarylacetoacetate hydrolase (FAH)⁵, the last enzymatic step in tyrosine metabolism⁶. FAH deficiency leads to the build up of toxic metabolites in the liver that can cause acute liver failure and death or in the chronic form of the disease can cause cirrhosis and hepatocellular carcinoma. The disease is clinically managed by administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a small molecule inhibitor of an enzyme upstream of FAH in tyrosine metabolism. The disease provides an ideal environment in which to test gene therapy methods, as successful correction of even a small number of hepatocytes will eventually result in the repopulation of the entire liver with corrected cells in both small and large animal models^{7,8}. This occurs because corrected cells have a profound survival advantage over uncorrected cells due to the accumulation of toxic metabolites in the latter. The loss of uncorrected hepatocytes allows for selective expansion of corrected hepatocytes consistent with the regenerative capacity of the liver. Treatment can be easily followed by measuring the decrease in circulating tyrosine and succinylacetone levels following transplantation.

In order to justify the invasive nature of the procedure, which includes a partial hepatectomy, the goal of this approach must be a durable cure. Therefore, replication incompetent lentiviral vectors are used because they will stably integrate into the hepatocyte genome⁹, ensuring delivery of the corrected gene to all daughter cells as the liver grows and expands to replace the rapid loss of uncorrected cells. This is advantageous over adeno associated viral (AAV) vectors, which primarily exist as episomes that can only be passed to a single daughter cell during mitosis¹⁰ thereby losing any effect of the therapy in a matter of weeks.

Although a growing body of literature supports the safety of lentivirus¹¹, concerns over genotoxic events are mitigated by limiting the transduction of host cells to a controlled *in vitro* environment. Free vector is never systemically introduced to the host when this method is performed, limiting exposure to the hepatocytes that will be re-introduced with autologous transplant via the portal vein.

This report describes the method of the surgical and *ex vivo* procedures used to isolate hepatocytes for gene therapy *ex vivo* and subsequent autologous transplantation¹² for the treatment of the HT-1 pig⁸. The full process includes 1) a partial hepatectomy that serves as a source of hepatocytes and a growth stimulus for the host's liver, 2) isolation of hepatocytes from the excised liver followed by *ex vivo* gene correction, and finally 3) reintroduction of the corrected hepatocytes back into the host. The method described is applicable to all large animal models with some modification, but only the FAH-deficient pig¹³ will have the advantage of the selective environment for corrected hepatocytes.

Protocol

All animal procedures were performed in accordance with institutional guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) prior to study conduct. Procedures described here were performed on male and female large white farm pigs (50% Landrace/50% Large White genetic background) up to 3 months of age that are deemed healthy and suitable for surgery. Animals are socially housed unless considered incompatible by institutional veterinary care staff. Animals are fed appropriate levels of chow twice daily and observed for clinical signs by care staff at least once daily.

1. Preparation for Surgery

1. Administer 5 mg/kg telazol and 2 mg/kg xylazine intramuscularly to induce sedation. Administer buprenorphine SR subcutaneously at a dose of 0.18 mg/kg for post-operative analgesia (anticipated analgesia from this dose will be up to 72 h). Manually check the animal for responses to verify sedation.
2. Once sedated, insert an intravenous catheter into a peripheral ear vein for pharmacologic access.
3. Administer intravenous 25 mg/kg cefazolin and intramuscular 5 mg/kg of ceftiofur pre-operatively.
 1. Administer normal saline intravenously to keep blood pressure and heart rate within normal physiologic limits throughout the procedure.
4. Place an orogastric tube to decompress the stomach. Perform endotracheal intubation with an appropriately sized endotracheal tube, verify proper placement by compressing the chest manually to detect exhalation, and then mechanically ventilate the animal with 1–3% isoflurane.
5. Place electrocardiogram (ECG) leads, blood pressure cuff, temperature probe, and pulse-oximeter to monitor and record vital signs. Place the animal in a supine position, scrub the abdomen from rib cage to pelvis with betadine, and drape sterilely.
6. Ventilate subject with 1–3% isoflurane during the procedure to maintain a surgical plane of anesthesia. Continue to monitor vital signs for changes and adjust isoflurane accordingly to maintain heart rate at pre-incision levels. If blood pressure drops dramatically, reduce isoflurane and initiate institutionally-approved agents to restore vitality, such as intravenous epinephrine.

1. Record heart rate, respiration rate, blood pressure, and body temperature on a surgical procedure record to track and maintain animal welfare as per institution-specific policy to ensure compliance with large animal welfare standards.

2. Laparoscopic Partial Hepatectomy

1. Perform initial port site entry using an open Hassen technique cephalad to the umbilicus. When peritoneum is visualized, safely introduce a 12 mm trocar into the abdominal cavity. Pass a 5 mm, 30°, scope through this port.
2. Insufflate the abdomen with CO₂ to 15 mmHg. Under direct visualization with the laparoscopic camera, place two additional 5 mm ports triangulating on the left lateral lobe of the liver. Exact placement of the ports will depend on size and anatomy of the animal.
 1. At this point, place the laparoscope in one of the 5 mm ports.
3. Identify the left lateral segment of the liver at the point of the major fissure of left lobe. This fissure separates the medial and left lateral segments. Secure the portal structures along with the hepatic vein along this fissure using a surgical stapler (see Table of Materials). Transect the parenchyma through this fissure using sequential firings from the stapler using 60, 45, or 30 mm long vascular loads.
 1. When parenchymal resection is complete, assess the remnant liver to ensure adequate hemostasis. Control any bleeding from the parenchyma with cautery or suture.
4. Retrieve the liver section using endoscopic graspers and an endoscopic tissue retrieval bag.
5. Remove the ports and close the 12 mm port incision in three layers with interrupted size 0 suture for the midline fascia, a running 2–0 suture for the deep dermal layer and a running 4–0 suture for the subcuticular layer. The 5 mm ports may be closed in one layer with 2–0.
6. Place a sterile dressing on the incisions (see Table of Materials).
7. When cells will be ready in less than 4 hours, maintain the animals under general anesthesia post-operatively until the time of autotransplantation.
8. Alternatively, if cell manipulations require longer periods (such as to allow formation of hepatocyte spheroids or longer transduction/selection periods based on individual applications of this method) allow the animal to recover from anesthesia and repeat the anesthetic induction steps prior to autotransplantation when the cells are ready.

3. Hepatocyte Isolation

1. Connect a peristaltic pump with perfusion set to deliver warm dispersion solutions (maintained in 43 °C water bath) to catheters to be placed in the large

exposed vessels of the liver section, such that solution temperature is 38 °C upon introduction to the tissue. All tubing, equipment, and solutions must be maintained sterile to ensure the cells are suitable for transplantation.

1. Prime the pump and tubing with Per II up to a stopcock positioned to switch between Per I and Per II delivery to the tissue. Switch the stopcock to the Per I position and prime the rest of the tubing set, filling an in-line bubble trap completely to prevent introducing air bubbles into the tissue.
2. Prepare a clean transverse cut perpendicular to the hepatic circulation to reveal cross sections of portal vein branches and hepatic veins for catheterization.
3. Catheterize the available exposed veins with a snug-fitting catheter to avoid leakage of the perfusate. Cannulate at least 1 portal and 1 hepatic vein to ensure adequate perfusion of the tissue.
 1. Ensure that the catheter(s) are primed/free of air before placing the catheter into a vein.
4. Perfuse with warm Per I at 100 mL/min, moving the outlet tube from vein to vein every 30 seconds to 1 min. Cycle through all available veins for 15–20 min. During Per I infusion, set an evacuation tube to empty the procedure tray into a waste container under vacuum.
5. Switch to Per II at 100 mL/min, cycling through the veins as with Per I, for a total of 30 min. During Per II, use a return pump to recycle Per II back to the reservoir for re-administration to conserve preparation of the active enzyme. Set the return pump to less than the outflow pump (*i.e.*, 94 mL/min), being careful not to return excessive air to the source bottle.
 1. If the liver capsule breaks, this time can be reduced.
 2. If the liver is not fully digested (does not remain dimpled with gentle focal pressure) an additional 5 min of perfusion can be performed.
6. Occasionally (approximately every 5 minutes) document surface temperature of the bubble trap and/or liver to ensure the hepatocytes are not getting cold. If the liver is getting cold (<35 °C) increase the heat of the water bath to restore the liver temperature closer to 38 °C. The liver should blanch as the perfusion proceeds.
7. Remove liver to sterile plate or Petri dish for transport to cell culture hood. Cover sterile field with a sterile drape to maintain integrity during the hepatocyte processing.
8. Expose the liver section from sterile drape and submerge the liver with cold hepatocyte wash media (HWM). Disrupt the capsule by dragging scissors across the top. Wearing sterile gloves, gently massage the liver to release the hepatocytes into the medium.

9. Filter the HWM containing hepatocytes through sterile gauze into large (~200 mL) centrifuge bottles. Wash the hepatocytes through the following procedure in triplicate, combining the cell pellet from the multiple bottles after the first resuspension (as applicable):
 1. Centrifuge at $50 \times g$, 5 min, 4 °C.
 2. Aspirate and resuspend in 150 mL of HWM.
 3. Leave the pellet in HWM after the 3rd wash.
10. Count cell concentration using a hemocytometer or other device, as available. These cells are now ready for *ex vivo* manipulation of interest, including gene therapy, cell sorting, or other specialization prior to autotransplantation. Final volume of HWM can be adjusted to target a specific concentration (cells/mL).

4. Hepatocyte Transduction

1. Resuspend hepatocytes in media (Dulbecco's modified eagle medium [DMEM], 10% fetal bovine serum [FBS], Penicillin-Streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], dexamethasone, epidermal growth factor [EGF], and NTBC) at 1–2 million cells per mL in a conical tube on ice.
2. Thaw lentiviral vector at room temperature and add to conical tube on ice at target 20 multiplicity of infection (MOI) to bind the cells. Rotate cells at 4 °C at 10 rpm for 90 min to bind the viral vector to the cell surface.
3. Transfer tubes to 37 °C for 30–40 min. Invert to mix every 5 minutes to facilitate the vector transducing the bound cells.
4. Centrifuge cells at $50 \times g$ at 4 °C for 4 min. Resuspend cell pellet in saline at desired concentration on ice. Repeat this wash to ensure removal of any unbound vector from the preparation.
5. If possible, plate sufficient aliquots of cells (*i.e.*, 500,000 cells per well in a 6 well cell culture plate) to check for viability, adhesion, transduction efficiency, *etc.*

NOTE: It is often not possible to assess these parameters prior to autotransplantation, especially for same-day procedures. For example, the procedure described herein employed an untagged *Fah* cDNA under the control of the alpha-1 antitrypsin liver-specific promoter, which was not readily assayable in the hepatocytes prior to transplantation. However, these plated cells can be assayed subsequent to the autotransplantation as an indicator of the success of transduction (*i.e.*, via Western blotting) or a predictor of the general success of the procedure.

5. Hepatocyte Transplantation

1. Identify the portal vein via ultrasound using a 2 to 5 MHz transducer. Direct an 18 G, 5 inch needle towards the main portal vein proximal to its bifurcation.

2. Begin slow manual infusion of up 1×10^9 hepatocytes (approximately 10 g) using a syringe. Monitor portal pressures during transplantation using an infusion catheter.
 1. If portal pressures increase more than 8 mmHg above baseline, pause infusion and allow the pressure to return to baseline levels.
 2. If portal pressures do not return to baseline after pausing infusion for five minutes, discontinue the infusion.
3. After transplantation and catheter removal, use the ultrasound to evaluate for the presence of thrombotic events and forward flow in the portal vein.

6. Postoperative Recovery and Maintenance

1. Move subject to a proper recovery area and observe until the animal is fully ambulatory, monitoring heart rate, oxygen saturation and body temperature every 15–30 min. Maintain body temperature with warm blankets or an air-heated wrap, as needed.
2. Administer 1 mg/kg omeprazole by mouth daily (through the end of the study) to reduce the chances of gastric ulceration that can occur with bouts of inappetence.
3. Postoperatively, the animal is monitored closely by lab and veterinary staff and typically does not require additional pain medication separate from the pre-operative buprenorphine dose. If signs of distress/pain are observed by qualified veterinary care staff (incision guarding, inappetence, lethargy), anti-inflammatory agents (i.e., Carprofen) may be administered.

7. Recipes

1. See Table 1 for recipes used in this protocol.

Representative Results

The liver resection and autologous transplantation are represented schematically in Figure 1. In a representative cohort of 5 pigs that underwent hepatic resection, most had yields of $>1 \times 10^9$ hepatocytes with approximately 80% viability (Table 2), providing plenty of cells for any type of desired manipulations, including gene therapy. Subsequent culture of the non-transplanted portion of prepared hepatocytes from each of those 5 pigs showed good viability and adhesion, with typical hepatocyte morphology 46 hours after transduction and initial plating (Figure 2). These results are representative of a successful preparation, while poor results would be indicated with low numbers of cells, viability, or minimal adherence of cells in a monolayer.

A liver biopsy prior to treatment of the *Fah*^{-/-} pig shows no expression of FAH via immunohistochemistry (Figure 3). Initial engraftment will vary by the amount of cells reintroduced during transplantation. Transduction frequencies of porcine hepatocytes *in vitro* using lentivirus at an MOI of 10 TU/hepatocyte is typically 70–100%. The engrafted cells will clonally expand in the *Fah*^{-/-} liver until the entire liver is repopulated by the corrected

cells. Representative biopsies at 2, 6, and 12 months demonstrate a timeline of FAH-positive cell expansion, which is typically complete at 12 months after transplantation (Figure 3). Even a low percentage of engraftment would be expected to eventually repopulate the liver, although actual initial engraftment rates were not evaluated in this experiment.

Pigs are carefully monitored post-transplantation for weight gain, as failure to thrive is a sign that not enough corrected hepatocytes are present. In this case animals are cycled back on NTBC as needed until the corrected hepatocyte population is sufficient to allow complete weaning from the drug. Evaluation of circulating biomarkers provides easy access to follow the therapy. Once an animal has achieved about 20% repopulation of corrected hepatocytes in the liver, tyrosine and succinylacetone levels will normalize when compared to wild-type animals (Figure 4A). Furthermore, the under-treated or untreated *Fah*^{-/-} pig demonstrates similar fibrotic liver changes seen in affected humans, which can be followed by Masson's trichrome staining of serial biopsies⁸. Surrogate markers of ongoing liver injury can be evaluated by assaying circulating liver enzymes, such as aspartate aminotransferase and alkaline phosphatase. While uncorrected animals show significant elevation in both parameters compared to wild type animals, *ex vivo* gene therapy returns these serum values to normal (Figure 4B and 4C). Lastly, general liver metabolic health is disrupted in untreated *Fah*^{-/-} pigs, as indicated by elevations in circulating ammonia. Repopulation of the liver with corrected cells restores wild-type levels of ammonia (Figure 4D).

Discussion

This report describes an *ex vivo* autologous gene therapy approach to cure a porcine model of HT-1. It involves a partial hepatectomy, followed by *ex vivo* hepatocyte isolation and transduction of isolated hepatocytes with lenti virus carrying the corrective transgene. Corrected autologous hepatocytes are then transplanted back to the FAH deficient animal through the portal vein⁸. Although the method described is applicable to all large animal models with some modification, the FAH-deficient pig has the unique advantage of a highly selective environment for corrected cells^{13,15}. This method is an effective cure for HT-1, as dosed animals show NTBC independent growth with normalization of biochemically measured metabolites and inflammatory liver biomarkers, prevention of cirrhosis and HCC and the complete reversal of early fibrosis seen with NTBC cycling. Furthermore, a more recent study involving extensive histological analysis has demonstrated no detectable uncorrected cells, fibrosis, cirrhosis or tumorigenicity 3 years post therapy (manuscript in review). However, the utility of the FAH-null background can serve as a tool to allow evaluation of a wide array of interrogations in hepatocyte physiology and disease indications beyond HT-1.

Relative success of the procedure can be evaluated by the number of cycles of NTBC required before the subject can be completely weaned from this protective drug. In numerous iterations with this model and in other small animal models of HT-1, approximately 20% correction is required before NTBC independent growth is achieved. More cycling with NTBC is indicative of poor initial engraftment/viability, advanced liver disease at the time of initial engraftment, or poor transgene expression, although other factors may also play a role. Biochemical markers of liver health (AST, ALT, and ammonia)

are readily available and offer insight into the extent of corrected hepatocyte expansion, but ultimate verification of phenotypic cure is best proven by the normalization of serum tyrosine and succinylacetone and histologic confirmation of FAH-expressing hepatocytes in the absence of fibrosis. This is achieved when the liver has been repopulated with corrected hepatocytes.

The efficacy of the procedure relies most heavily upon the integrity of the hepatocytes, which must be maintained sterile and viable throughout the entire isolation, transduction and transplantation process. Reintroduction of non-viable or uncorrected hepatocytes will not rescue the phenotype, and an unsuccessful procedure could take months of subject observation to verify.

In this model, good transgene expression of the functional FAH enzyme is a minimum requirement for liver repopulation. Lentivirus is only one way to deliver an effective copy of the transgene. This method will allow the possibility for the use of other delivery systems including nonviral systems and those aimed at editing the specific genomic defect. Ultimately, this model will allow for a vast array of possibilities, including the correction of other defects in the FAH deficient bioreactor. So long as the transplanted cell expresses a functional FAH enzyme, any other modification of the cells *ex vivo* would also be propagated. This would allow any of the inborn errors of metabolism of the liver to be corrected in the *FAH*^{-/-} background. As the expansion of corrected hepatocytes ultimately repopulates the liver, relevant numbers of hepatocytes for any disease indication can be achieved, which underscores the value of this model and procedure as a basic science and preclinical therapy model.

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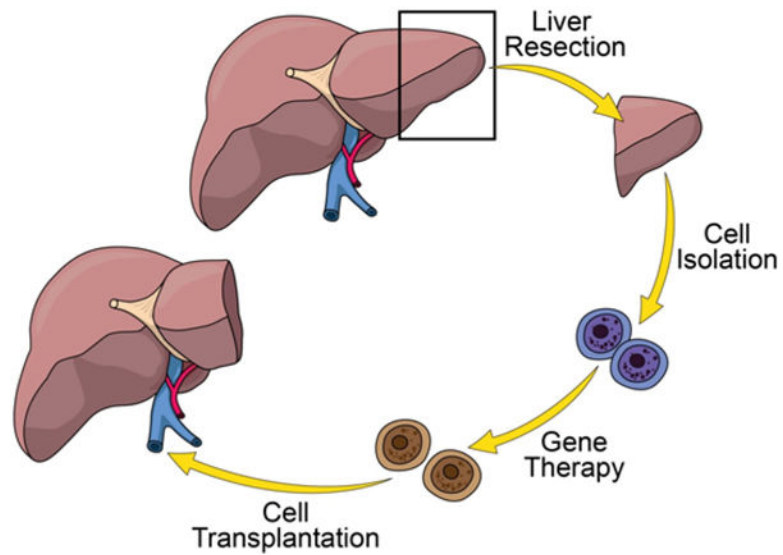


Figure 1: Hepatectomy and Autologous Transplantation.

(Clockwise from top) A partial liver resection is performed on the subject to provide a source of hepatocytes and stimulate liver regeneration. Hepatocytes are isolated from the resected liver (blue cells), transduced *ex vivo* with the lentiviral vector containing the transgene of interest (brown cells), and then the transduced cells are autologously transplanted back to the source animal via portal vein injection. Please click here to view a larger version of this figure.

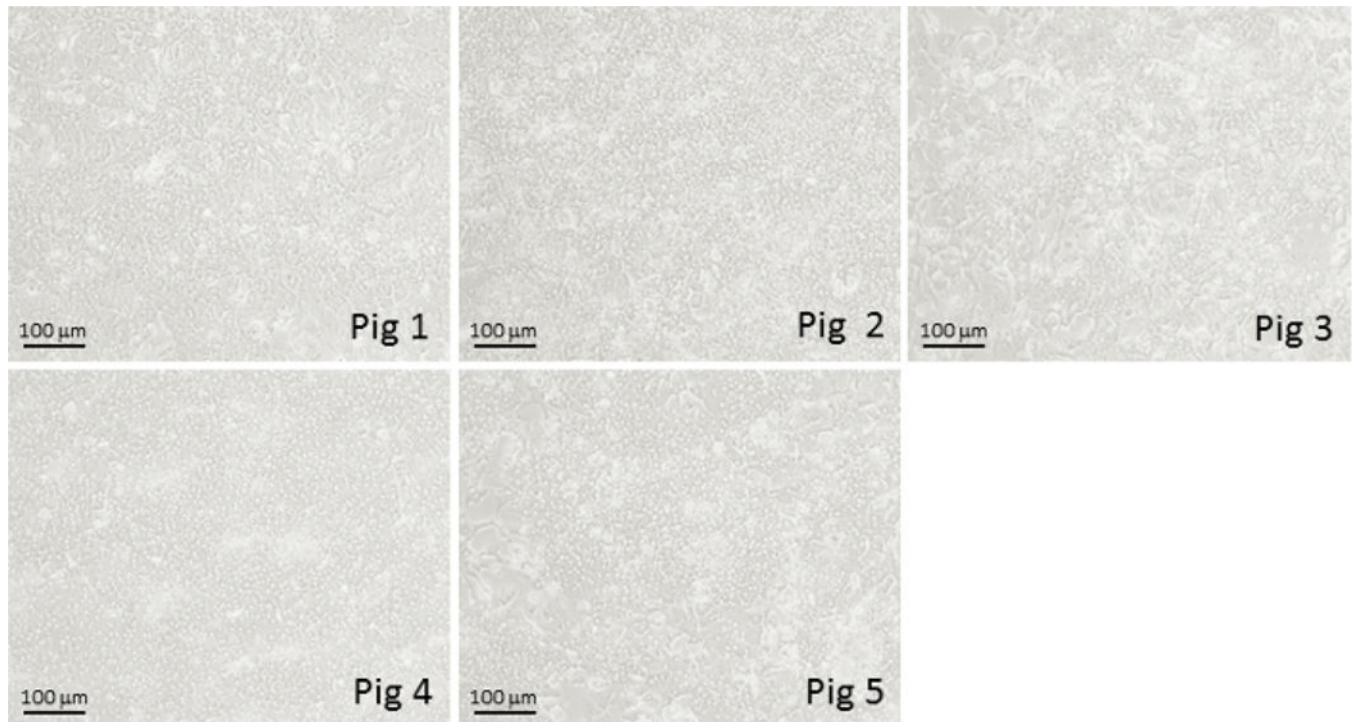


Figure 2: Primary Pig Hepatocytes from Liver Sections.

Hepatocytes were cultured from liver resections from 5 different pigs, transduced with lentiviral vector, and allowed to grow for 48 h to demonstrate morphology, viability, purity, and adhesion to culture dishes. These *in vitro* qualitative assessments serve as surrogate indicators for the likelihood of successful engraftment *in vivo* for each preparation. Please [click here to view a larger version of this figure](#).

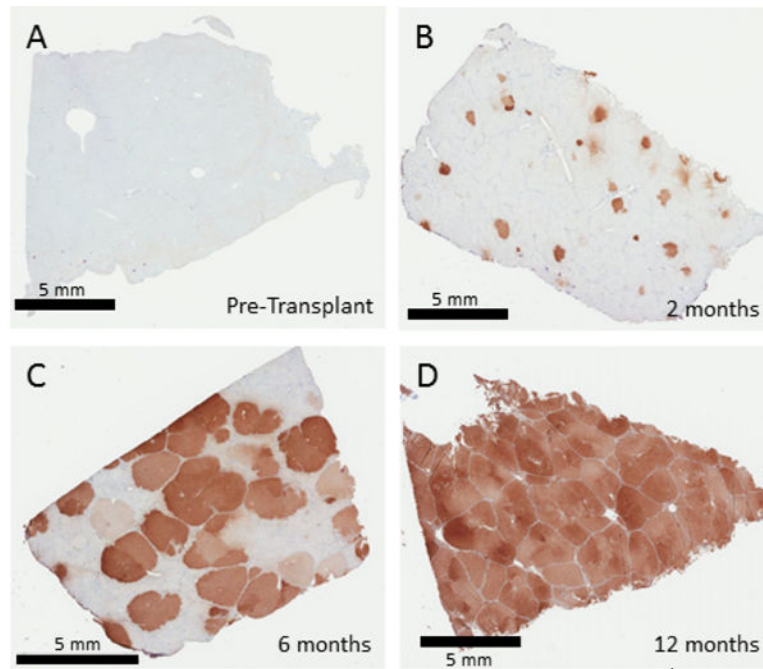


Figure 3: Corrected Hepatocytes Repopulate the *Fah*^{-/-} Pig Liver.

FAH immunohistochemistry from liver biopsies 0, 2, 6 and 12 months after *ex vivo* gene therapy of autologous hepatocytes. Untreated *Fah*^{-/-} pigs show no FAH-positive cells in the liver (A). Two months after transplantation (B), individual foci of FAH positive hepatocytes are seen, which then undergo expansion to replace 50–60% of the liver at 6 months (C) followed by complete replacement, seen at 12 months (D). Please click here to view a larger version of this figure.

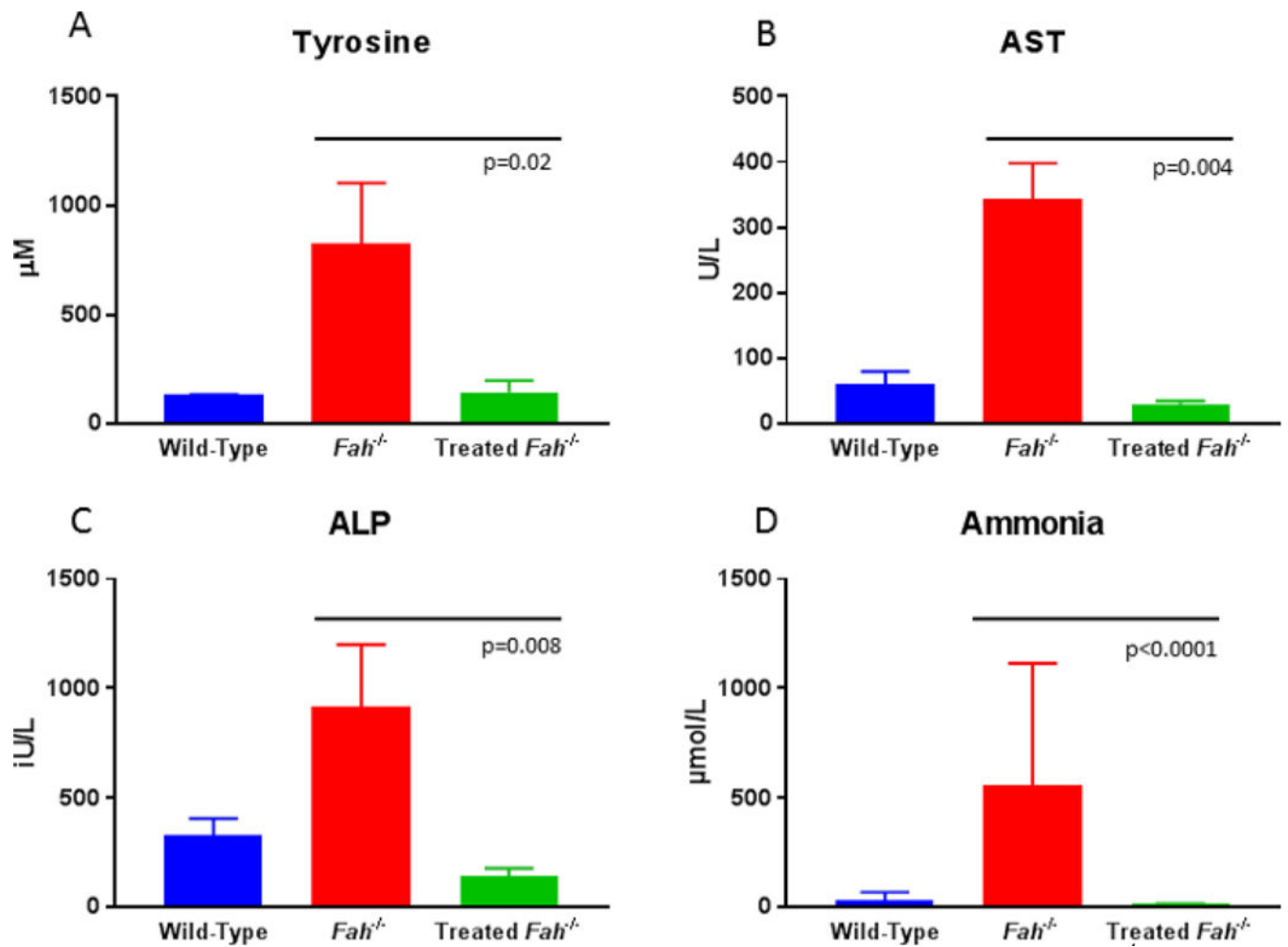


Figure 4: Serum Biochemistry at 10 months post ex vivo gene therapy in *Fah*^{-/-} Pigs.

10 months after therapy, tyrosine, aspartate aminotransferase¹⁴, alkaline phosphatase (ALP) and ammonia levels are significantly lower than untreated FAH deficient animals and are indistinguishable from wild-type levels. Data were analyzed for significance using the Mann-Whitney *U* test (*p* values as presented). Please click here to view a larger version of this figure.

Table 1:

Recipes for Solutions Used in Hepatocyte Isolation from Liver Sections.

Reagent	HWM	Reagent	Per I (10x)	Per II (10x)
Williams'-E Powder (g/L)	10.8	NaCl (g/L)	83	39
NaHCO ₃ (g/L)	2.2	KCl (g/L)	5	5
HEPES (g/L)	2.6	HEPES (g/L)	24	240
Pen/Strep (100x, mL/L)	10	EGTA (g/L)	9.5	-
Fetal Bovine Serum (mL/L)	100	N-acetyl-L-cysteine	8	8
pH	7.3	(N-A-C, g/L)		
		Nitroglycerin (mL/L)	5	5
		CaCl ₂ 2H ₂ O (g/L)	-	7
		Collagenase D (mg/mL)	-	0.2
		PH	7.4	7.6

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Table 2:

Representative hepatocyte results from primary isolations from 5 pigs.

Pig	Total Cells (x 10 ⁶)	Live Cells (x 10 ⁶)	Viability (%)
1	1,381	1,160	84
2	1,000	770	77
3	1,213	995	82
4	789	671	85
5	1318	975	74
Average	1,140	914	80
St Dev	244	194	5

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Materials

Name	Company	Catalog Number	Comments
2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC)	Yecuris	20-0027	
12 mm Trocar	Covidien	B12STS	
5 mm Trocar	Covidien	B5SHF	
Endo Surgical Stapler 60	Covidien	EGIA60AMT	
Endo Surgical Stapler 45	Covidien	EGIA45AVM	
Endo Surgical Stapler 30	Covidien	SIG30AVM	
Endo catch bag	Covidien	173050G	
0 PDS	Ethicon	Z340H	
2-0 Vicryl	Ethicon	J459H	
4-0 Vicryl	Ethicon	J426H	
Dermabond	Ethicon	DNX12	Sterile Dressing
Williams'-E Powder	Gibco	ME16060P1	
NaHCO ₃	Sigma Aldrich	S8875-1KG	
HEPES	Fisher	BP310-1	
Pen/Strep	Gibco	15140-122	
Fetal Bovine Serum	Corning	35-011-CV	
NaCl (g/L)	Sigma Aldrich	S1679-1KG	
KCl (g/L)	Sigma Aldrich	P3911-500G	
EGTA (g/L)	Oakwood Chemical	45172	
N-acetyl-L-cysteine	Oakwood Chemical	3631	
(N-A-C, g/L)	Sigma Aldrich	A9165-100G	
CaCl ₂ 2H ₂ O (g/L)	Sigma Aldrich	223506-500G	
Collagenase D (mg/mL)	Crescent Chemical	17456.2	
Dulbecco's modified eagle medium (DMEM)	Corning	15-013-CV	
Dexamethasone	Fresenius Kabi	NDC6337	
Epidermal Growth Factor	Gibco	PHG0314	