A novel method of drug-mediated in vivo selective hepatocyte expansion for liver-directed gene and cell therapy

By

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A Dissertation

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LIST OF ABBREVIATIONS

AAT	α-1 antitrypsin
AAT-Z	Z-allele AAT
ABS	Antley-Bixler syndrome
Alb	Murine albumin gene
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APAP	Acetaminophen; N-acetyl-p-aminophenol
ApoE	Apolipoprotein-E
AST	Aspartate aminotransferase
ATP7B	Copper-transporting ATPase 2
AUC	Area under the curve
BCL-2	B-cell lymphoma 2
BH4	Tetrahydrobiopterin
BSEP	Bile Salt Export Pump
BUN	Blood urea nitrogen
C	Celsius
CAG	Cytomegalovirus enhancer/chicken beta-actin promoter
CAR-T	Chimeric antigen receptor **T**-cell
Cas9	CRISPR associated protein 9
Casp8	Caspase 8
cDNA	Complementary DNA
CEHPOBA	4-[(2-carboxyethyl)-hydroxyphosphinyl]-3-oxobutyrate
CO_2	Carbon dioxide
CPR	Cytochrome P450 reductase
CRISPR	Clustered regularly interspaced short palindromic repeats
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2
CYP2C9	Cytochrome P450 Family 2 Subfamily C Member 9
CYP2C19	Cytochrome P450 Family 2 Subfamily C Member 19
CYP2D6	Cytochrome P450 Family 2 Subfamily D Member 6
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
CYP21A2	Cytochrome P450 Family 21 Subfamily A Member 2
Cypor	Cytochrome P450 reductase
Cyps	Cytochrome p450 enzymes
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid

DNAJC12	DNA J-domain containing protein member C12
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ENU	*N*-ethyl-*N*-nitrosourea
ER	Endoplasmic reticulum
ERT2	Tamoxifen-responsive mutated estrogen receptor
F9	Factor 9
FAA	Fumarylacetoacetate
FAD	Flavin adenine dinucleotide
FAH	Fumarylacetoacetate hydrolase
Fah-/-	Homozygous Fah deficient mice
FDA	Food and Drug Administration
FIX	Factor 9
FKBP	FK506 (tacrolimus) binding protein
FMN	Flavin mononucleotide
GC-1	Sobetirome, a thyroid hormone receptor agonist
gDNA	Genomic DNA
GFP	Green fluorescent protein
GGT	Gamma-glutamyl transferase
gRNA	Guide RNA
GS	Glutamine synthetase
GSD	Glycogen storage disease
H&E	Hematoxylin and eosin
HBEGF	Heparin-binding EGF-like growth factor (HB-EGF)
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HDV	Hepatitis delta virus ribozyme
hF9	Human factor 9
HGD	Homogentisate 1,2-dioxygenase
HGF	Hepatocyte growth factor
HH	Hammerhead ribozyme
HPD	4-hydroxyphenylpyruvate dioxygenase
HSV	Herpes simplex virus
HSVtk	Herpes simplex virus thymidine kinase
HT1	Hereditary tyrosinemia type I
IF	Immunofluorescence
indels	Insertion or deletion mutations
IP	Intraperitoneal
iPSC	Induced pluripotent stem cell
ITR	Inverted terminal repeat

IU/L	International units per liter
ko	Knockout
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein receptor
LTR	Long terminal repeat
MAI	Maleylacetoacetate isomerase
MDR3	Multidrug resistance protein 3
MHC	Major histocompatibility complex
MSUD	Maple syrup urine disease
mTmG	Membranous Tomato/membranous GFP fluorescent reporter
mTTR	Mouse transthyretin promoter
MUT	Methylmalonyl-CoA mutase
n	Number of animals
NAC	N-acetyl cysteine
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-p-benzoquinone imine
ns	Not significant
O_2	Molecular oxygen
OHSU	Oregon Health & Science University
OTC	Ornithine transcarbamylase
P1	Postnatal day 1
P2A	Porcine teschovirus 2A self-cleaving peptide sequence
PAH	Phenylalanine hydroxylase
Pah$^{enu2/enu2}$	Mouse model containing homozygous *Pah* ^{enu2} alleles
Pah$^{\Delta exon1/\Delta exon1}$	Mouse model containing homozygous *Pah* $^{\Delta exon1}$ alleles
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Phe	Phenylalanine
PKU	Phenylketonuria
polyA	Bovine growth hormone polyadenylation signal
POR	Cytochrome P450 reductase
rAAV	Recombinant adeno-associated virus
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleic acid-protein complex
RT-PCR	Real time PCR
SB100X	Hyperactive Sleeping Beauty transposase
SD	Standard deviation
sgRNA	Single guide RNA

shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SpCas9	*Streptococcus pyogenes* Cas9 enzyme
T3	Triiodothyronine; thyroid hormone
TAT	Tyrosine aminotransferase
TCO_2	Total carbon dioxide
tdTomato	Tandem dimer Tomato
TIDE	Tracking of Indels by Decomposition
TU	Transduction units
Tyr	Tyrosine
U/L	Units per liter
uPA	Urokinase-type plasminogen activator
US	United States
vg/kg	Vector genomes per kilogram body weight
VSV-G	Vesicular stomatitis virus G protein
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
WT	Wild-type
YAP	Yes-associated protein 1

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DISSERTATION ABSTRACT

Liver-directed gene and cell therapies represent a promising therapeutic strategy for many genetic disorders. However, they are hampered by inefficiency. The problem of inefficiency could be overcome by providing a selective growth advantage to corrected cells to allow them to repopulate the liver. This is theoretically possible in the liver due to its regenerative nature: mature hepatocytes can divide many times to replace cells lost to injury. In fact, this effect is seen in several genetic liver disorders where the mutant state confers cellular injury and restoration of wild-type gene expression is protective. However, most genetic liver disorders do not exhibit a selective effect and could benefit from an approach that allows expansion of therapeutic cells regardless of genetic background.

My dissertation aimed to characterize a novel method for in vivo selective expansion of hepatocytes using the common medication acetaminophen (APAP). In the liver, APAP is metabolized by cytochrome P450 enzymes to form a hepatotoxic intermediate. Cytochrome P450 enzymes require a cofactor, cytochrome P450 reductase (Cypor), for activity. Therefore, knockout of Cypor prevents APAP toxicity. We hypothesized that this would allow a rare population of Cypor-deficient cells to be selectively expanded in vivo by APAP treatment. If a therapeutic gene is linked to Cypor deficiency in the same gene therapy vector

or therapeutic cell, we hypothesized that APAP treatment would allow corrected cells to be expanded until they reach a corrective threshold.

Chapter 2 of this dissertation provides proof-of-concept for this selection system and its applications to gene therapy with integrating lentiviral vectors. I first demonstrated that knockout or knockdown of Cypor is sufficient to allow a rare population of Cypor-deficient cells equivalent to <1% of the murine liver to expand to as much as 40-50% of the liver with repeated APAP treatment. Selectable lentiviral vectors, chosen due to their integrating nature and corresponding ability to persist in dividing cells, were designed for several disease indications. Vectors including a selectable Cypor-targeting guide RNA or short hairpin RNA and a human factor 9 transgene in cis were administered to mice. APAP administration allowed up to a 90-fold increase in blood human factor 9 levels. Additionally, a selectable lentiviral vector was created containing a phenylalanine hydroxylase transgene, therapeutic for the inborn error of metabolism phenylketonuria (PKU). This vector was administered to a mouse model of PKU, which exhibits very high blood phenylalanine. Following repeated APAP administration, partial to full correction of blood phenylalanine was achieved.

Chapter 3 expands the applications of the APAP selection system to selective expansion of transplanted hepatocytes. PKU mice were transplanted with Cypor-knockout hepatocytes. Following APAP selection, blood phenylalanine levels fell to within the fully corrected range and remained stable for >250 days. Blood

chemistry, liver histology, and assessment of drug metabolism in the corrected mice showed no significant side effects of partial Cypor deficiency.

Taken together, the data described in this dissertation show compelling efficacy and safety data for acetaminophen-selection of gene edited cells as a therapeutic. This system has the potential to allow the application of previously sub-therapeutic gene and cell therapy approaches to a wide variety of genetic liver disorders.

CHAPTER 1

Introduction

Liver Physiology

Liver structure and function

The liver is a key metabolic organ that performs a wide variety of essential functions. Situated at the top of the abdominal cavity, the liver receives blood from two different sources. Roughly 25% of the liver's blood is highly oxygenated and arrives directly from the aorta via the hepatic artery. The other 75% is nutrient-rich, oxygen-poor blood that has already passed through organs of the digestive system and is carried to the liver through the portal vein. The liver serves to metabolize nutrients and toxins in the blood before it returns to circulation. After entering the liver, the blood flows through the highly fenestrated liver vasculature known as sinusoids, where it is directly exposed to hepatocytes. Blood then drains from the liver through the central vein into the inferior vena cava [1].

A majority of the functions attributed to the liver are carried out by hepatocytes. Hepatocytes are cuboidal epithelial cells that make up most of the liver parenchyma (an estimated 60% by cell number and 80% by mass) [2] and conduct many different metabolic functions. These include amino acid metabolism, cholesterol metabolism, bile acid synthesis, secretion of serum proteins, drug metabolism, fatty acid metabolism, and glucose homeostasis, among many others. Hepatocytes are frequently polyploid and may be binucleated. Up to 40% of human hepatocytes and 90% of rodent hepatocytes are polyploid [3].

Hepatocytes are responsible for synthesizing bile acids and for reuptake of bile acids from the portal blood. Cellular communication between the intestine and the liver ensures that bile is produced at a rate such as to maintain homeostasis in the total bile acid pool [4]. From the hepatocyte, bile is secreted into bile canaliculi and from there into the common bile duct, where it exits the liver for storage in the gall bladder and use in fat digestion in the intestine.

The remainder of the liver that does not consist of hepatocytes is made up by a variety of cell types, including liver-resident macrophages known as Kupffer cells, epithelial cell types lining the ducts, sinusoidal endothelial cells, hematopoietic cells, fibroblasts, and vitamin A-metabolizing cells called hepatic stellate cells [5].

The liver is unique in its ability to regenerate. Under normal conditions in the adult liver, hepatocytes are largely quiescent with a lifespan of approximately 400 days. In the adult rat liver, less than 1% of hepatocytes are actively replicating at any given time [6]. However, following liver injury or resection, a massive wave of proliferation allows replacement of liver mass. Following resection of up to 2/3rds of the liver (partial hepatectomy), the liver returns to its original size within 7 days in mice and 3 months in humans [7]. Mature hepatocytes serve as the source of new hepatocytes, and no dedicated stem cells are required for this regeneration [8, 9].

Liver Zonation

The liver is organized in hexagonal repeating units known as hepatic lobules [10] (Figure 1-1). The edges of the lobules are defined by the portal triad, which is composed of a portal vein and a hepatic artery, through which blood enters the liver, as well as a common bile duct through which bile exits the liver. The center of each lobule is defined by the central vein, through which blood exits the liver. The direction of bile drainage across the hepatic lobule is retrograde relative to the direction of blood drainage.

Within the lobule, various signaling gradients initiate spatial gene expression patterns that define subsets of hepatocytes. This includes a Wnt signaling gradient originating from the central vein [11] and a glucagon gradient extending from the portal vein [12]. These hepatocyte subsets are broadly defined as belonging to one of three "zones". Zone 1, or periportal, hepatocytes are adjacent to the portal triad and experience the highest oxygen concentration. Zone 3, or pericentral or centrilobular, hepatocytes are adjacent to the central vein and are oxygen-poor. Zone 2 mid-lobular hepatocytes separate zones 1 and 3. The three zones are broadly defined, and additional gradation exists within zones. For example, the pericentral gene *glutamine synthetase* (*GS*) is expressed only in 2-3 layers of hepatocytes immediately adjacent to the central vein [13], whereas the pericentral gene CYP2E1 shows expression extending outward to mid-lobular hepatocytes [14]. The variations in gene expression along this spatial gradient

lead to specializations in function. Functions enriched in zone 1 hepatocytes include ammonia metabolism, fatty acid oxidation, cholesterol synthesis, and gluconeogenesis. Zone 3 performs the majority of drug metabolism, bile acid synthesis, lipogenesis, and glycolysis [15].

Because of their differences in function, hepatocytes display differences in susceptibility to toxins based on zonation. As the location of the majority of xenobiotic metabolism, zone 3 has an increased susceptibility to drug induced-liver injury. In contrast, zone 1 shows increased susceptibility to certain immunologic injuries and toxins such as allyl alcohol [16, 17] (Figure 1-1).

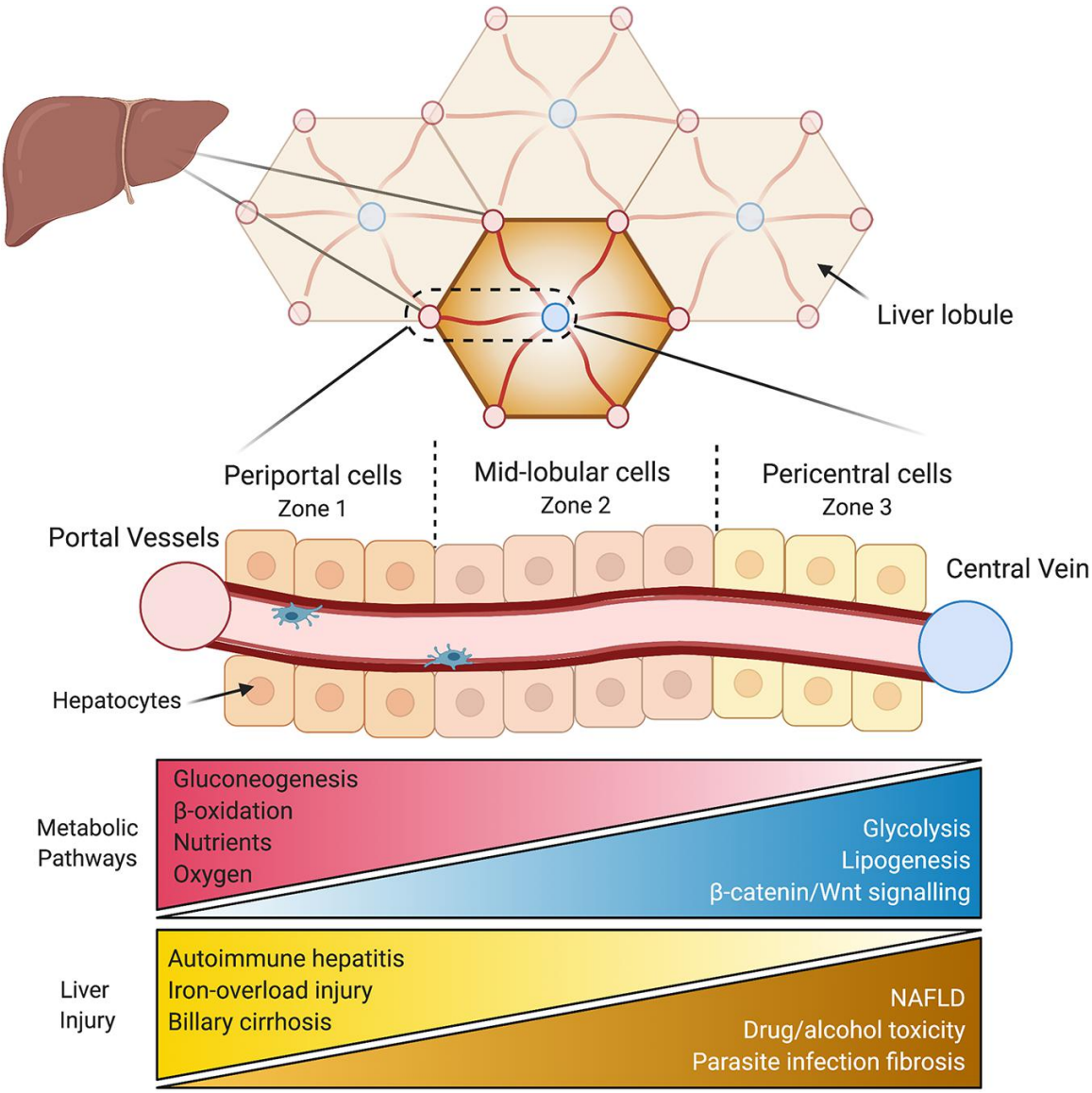


Figure 1-1. Structure and function of the hepatic lobule. The liver is composed of repeating hexagonal units known as hepatic lobules. The portal triad (portal vein, hepatic artery, and common bile duct) makes up the corners of the hepatic lobules, and the central vein marks the center. Hepatocytes are functionally zonated based on their location within the lobule. Certain cellular toxicities are also zonated as a result. Reproduced with permission from [16].

Gene and Cell Therapies for Liver Disorders

Liver-directed gene therapy

Gene therapy refers to the introduction or modification of nucleic acids as a therapeutic strategy. Typically, this involves the use of a viral vector or lipid nanoparticle to carry therapeutic cDNA expression cassettes, gene editing machinery, or therapeutic RNAs to target cells. Many different disorders are amenable to correction by targeting gene delivery to hepatocytes. This includes disorders that present with a liver phenotype (examples include hereditary tyrosinemia type 1 and Wilson's disease) and disorders wherein loss of physiologic hepatic gene expression results in an extrahepatic phenotype (examples include phenylketonuria, Alkaptonuria, and hemophilia B) [18].

The liver has a storied history within the field of gene therapy. One of the early attempts at gene therapy by in vivo delivery of viral vectors in humans aimed to correct ornithine transcarbamylase (OTC) deficiency, a urea cycle disorder that results from loss of OTC expression in hepatocytes. This phase I clinical trial utilized an adenoviral vector encoding an OTC gene delivered by infusion of the hepatic artery [19]. Sadly, this attempt ended in tragedy with the death of a patient in 1999 from a systemic immune reaction to the adenoviral vector. This outcome initiated a hiatus in attempts at in vivo gene therapy. Development of a novel type of viral vector with much reduced immunogenicity allowed in vivo gene therapy attempts to resume [20]. This vector, recombinant adeno-associated virus or rAAV (not a close relative of adenovirus despite its name), has been the

subject of a myriad of clinical trials over the past two decades. The first FDA approval for an rAAV gene therapy came in 2017 for treatment of congenital retinal degeneration. The first FDA approval for an rAAV therapy targeting hepatic expression occurred in 2022 with the approval of Hemgenix, a gene therapy product for hemophilia B [21]. With a price tag of $3.5 million for a one-time infusion of $2x10^{13}$ vg/kg, equivalent to approximately 10^{15} total viral particles, this drug is currently the most expensive on the market [22].

As a target for in vivo gene therapy approaches, the liver presents both advantages as well as unique challenges. Compared to many other tissue types, delivery of viral vectors to the liver is relatively straightforward. Due to the fenestrated vasculature of the liver, a large proportion of any vector delivered systemically tends to transduce the liver [23]. Another advantage is that adult hepatocytes are fairly long-lived cells and therefore show relatively long-term expression of transgenes expressed by episomal (i.e., non-integrated) vectors. Liver-targeted rAAV gene therapy for hemophilia B has been shown to be efficacious for >8 years in adult patients dosed in clinical trials [24].

Despite its successes, continued challenges limit the efficacy of rAAV gene therapies. Although immune responses to rAAV are low relative to other virus types, the extremely high doses required for therapeutic efficacy have elicited immune responses in patients. rAAV doses ≥ $5x10^{13}$ vg/kg have been implicated in causing potentially dangerous immune responses, and doses ≥ $1x10^{14}$ vg/kg

have been associated with multiple deaths in recent years [25]. Dividing cell types present another challenge for rAAV therapies. Hepatocytes in young children are rapidly dividing, meaning that the genomes of episomal rAAV vectors are lost quickly in pediatric patients and may be gradually lost in adult patients [26]. Additionally, development of hepatocellular carcinoma (HCC) due to low-frequency semi-random integration of rAAV vectors into the host genome is well characterized in mouse models [27]. Whether a similar oncogenic effect will be seen in large animals [28] and humans [29, 30] is controversial.

To allow liver-directed viral gene therapy to be applied to the rapidly dividing cells of the pediatric liver, integrating viral vectors are required. Integrating viral vectors were first developed from a mouse retrovirus in the early 1980s [31] and by the end of the decade were being successfully applied in the clinic for ex vivo delivery to proliferative hematopoietic cells [32]. Similar to in vivo episomal gene therapies, ex vivo integrating gene therapies also faced a historic reckoning that required new generations of safer vectors to be developed. The initial generation of integrating viral vectors, called gamma-retroviral vectors, were based upon the genome of the Moloney Murine Leukemia Virus. This virus contained a strong promoter element that led to the activation of oncogenes and subsequent leukemia development in several patients treated for X-linked severe compromised immunodeficiency [33]. The subsequent generation of integrating vectors, known as lentiviral vectors, are based on the genome of Human Immunodeficiency Virus [34]. Current “third generation” lentiviral vectors include

several modifications to improve their safety profile, including separation of the integrating cargo and essential viral packaging elements onto different plasmids [35] and deletion of the virus' native promoter element in the 5' long terminal repeat (LTR) [36]. They also have a safer integration profile relative to gamma-retroviruses, preferring gene bodies to promoter regions [37].

Unlike gamma-retroviruses, lentiviruses are able to transduce non-dividing cells [34]. Pseudotyping with vesicular stomatitis virus G protein (VSV-G), which allows endocytosis by the low density lipoprotein receptor (LDL-R) [38], gives lentiviruses broad tropism for a variety of cell types including hepatocytes. These features broaden their potential applications to include in vivo use for liver-directed gene delivery. Studies in animal models have demonstrated the potential of in vivo liver-directed lentiviral gene therapy for treating disorders such as hemophilia A [39]. However, clinical applications of lentiviral vectors have largely been limited to ex vivo hematopoietic gene therapy applications such as creation of CAR-T cells [40] and correction of sickle cell anemia [41]. Factors that have constrained the clinical use of lentiviral vectors in vivo include the low efficiency of transduction and extremely high expense of viral production.

As an alternate option for integrating in vivo gene therapy, rAAV vectors with arms of homology targeting viral integration to a specific locus in the host genome have been developed [42]. This has the potential to avoid concerns of insertional mutagenesis, but the targeted integration is a highly inefficient

process and thus reaching therapeutic cell thresholds with this strategy is extremely challenging.

Hepatocyte transplantation

A potential alternative to viral gene therapy approaches targeting the liver is hepatocyte transplantation. Hepatocyte transplantation is a cell therapy approach that involves the delivery of hepatocytes into portal circulation, where they engraft in the liver and support liver function.

The first step of hepatocyte transplantation is to obtain hepatocytes as a single-cell suspension by collagenase perfusion of the donor liver. This protocol was developed in 1976 [43] and has been adopted as standard practice. The typical source of hepatocytes is a liver of an allogenic donor, either cadaveric or explanted. Following perfusion, hepatocytes are delivered into circulation where they enter the liver through the portal vein. In rodent models, this is typically done by a minimally invasive injection of the spleen and can also be done by cannulation of the portal vein. In humans, infusion of the portal vein, splenic artery or, in children, the umbilical vein can deliver hepatocytes into portal circulation. After entering the liver, the hepatocytes travel through the branching portal vasculature before becoming “stuck” in the sinusoids which are narrower than the hepatocytes. This causes portal hypertension, and clinical protocols involve the monitoring of portal pressure [44]. Although up to 70% of transplanted cells remain trapped in the vasculature before eventually being cleared by

phagocytic cells [45], a minority is able to migrate into the liver parenchyma and form connections with host hepatocytes [46].

Hepatocyte transplantation was first performed in an animal model in 1976 in a rat model of Crigler-Najjar syndrome [47]. Correction of the Crigler-Najjar phenotype of hyperbilirubinemia was seen, implying successful cell engraftment. Due to difficulty in distinguishing host hepatocytes from donor hepatocytes, it was not definitively known that transplanted hepatocytes engraft in the liver parenchyma until this was demonstrated in a mouse model in 1991 [48]. The first attempt at autologous hepatocyte transplantation in humans was conducted in 1992 [49], followed by the first allogenic transplant in 1994 [50]. Over 150 human hepatocyte transplantation attempts have been reported to date [51]. Disease indications includes genetic diseases such as inborn errors of metabolism and excreted protein disorders as well as acquired conditions such as alcohol-induced liver failure and mushroom poisoning. Although results have been mixed, definitive phenotypic improvement has been shown for multiple conditions, including Crigler-Najjar syndrome, phenylketonuria, glycogen storage diseases, and urea cycle disorders [52]. Thus far, all benefits from clinical hepatocyte transplantation applications have been anecdotal, and no double-blind placebo controlled clinical trials have been conducted. No serious adverse outcomes have been reported in any patients treated with hepatocyte transplantation, including both adults and children as young as 1 day old [53].

Despite its promises, several factors have limited the clinical implementation of hepatocyte transplantation. One major problem is the low initial engraftment frequency of transplanted cells. In rodent models, one infusion of hepatocytes typically achieves ≤1% replacement of the recipient liver [48, 54]. Repeat infusions may be done, but cell sourcing is limiting. This problem could be overcome by providing a selective advantage to the transplanted cells to induce cell division until a therapeutic threshold is reached. This phenomenon is known to occur naturally in certain liver disorders, but improved approaches are needed to allow this to be broadly applied as a therapeutic approach.

Selective hepatocyte expansion

It is well known that mature hepatocytes are proliferative and are able to divide to replace lost cells. Although the precise mechanisms underlying this process remain unclear, it has been robustly shown that following hepatocyte loss, remaining hepatocytes are stimulated to divide and then stop proliferation once the liver reaches its original mass [55]. As previously discussed, hepatocytes are rapidly replaced following surgical resection by partial hepatectomy. Similarly, when a subset of hepatocytes succumbs to a cellular injury, the remaining hepatocytes divide to replace the damaged cells. Therefore, when a particular genetic state confers protection from a certain cellular injury, it also confers a selective growth advantage during the injury response. In this way, the liver can be selectively repopulated by injury resistant cells. This expansion effect can

allow a therapeutic cell replacement threshold to be reached even by gene and cell therapy approaches with very low starting efficiencies.

Several conditions must be met in order for a selective advantage to be conferred by a genetic correction. Firstly, the genetic deficiency in hepatocytes must cause a cellular injury to the hepatocytes themselves, rather than conferring injury to a secondary tissue type due to high circulating levels of a metabolite. Secondly, the injury must result in liver regeneration. Lasty, the injury must be cell autonomous. In other words, the genetic deficiency must confer a cellular injury only to the affected cells without affecting surrounding cells. In conditions where these criteria are met, selective expansion of non-mutant hepatocytes is seen. However, most disorders do not meet these criteria and therefore do not give mutation-corrected cells a survival advantage. Selective liver repopulation as is seen in disorders with genetic selection has the potential to be a powerful tool for enabling a therapeutic cell threshold to be reached with gene and cell therapy approaches.

Because proliferation is a requirement for genetic selection, the liver is unique among the solid organs in experiencing selective cell expansion. However, a selective advantage for corrected cells has been observed in several genetic disorders affecting other proliferative cell types, including hematopoietic cells and skin [56].

Liver disorders with genetic selection

Multiple genetic disorders affecting the liver that confer a selective advantage to corrected hepatocytes have been described in animal models. This includes including hereditary tyrosinemia type I (HT1) [57], methylmalonic acidemia [58], Z-allele alpha-1 antitrypsin (AAT) deficiency [59], Wilson's disease [60], and progressive familial intrahepatic cholestasis [61, 62]. The mechanisms driving selection in these disorders are summarized in Table 1-1. Broadly speaking, all involve a buildup of a toxic compound or metabolite in mutant cells that can be reversed by expression of the wild-type gene. Most are due to loss of function mutations, with the exception of Z-allele AAT deficiency which results from a toxic gain-of-function protein product. Liver repopulation by revertant hepatocytes in HT1 patients is well documented [63-65]. Whether other disorders that show a selective effect in animal models will translate to humans remains to be seen.

Table 1-1. Liver disorders showing genetic selection for corrected hepatocytes:

Disorder	**Selective mechanism**
Hereditary tyrosinemia type 1	Accumulation of toxic metabolite FAA due to loss of metabolizing enzyme FAH [57]
Methylmalonic acidemia	Accumulation of toxic metabolites due to loss of metabolizing enzyme MUT [58]
Z-allele Alpha-1 antitrypsin deficiency	Endoplasmic reticulum (ER) stress due to accumulation of misfolded AAT protein [59]

Wilson's disease	Accumulation of copper due to loss of transporter ATP7B [60]
Progressive familial intrahepatic cholestasis type 3	Bile salt induced damage from lack of phospholipids in bile due to loss of phospholipid translocator MDR3 [61]
Progressive familial intrahepatic cholestasis type 2	Bile acid accumulation from loss of bile salt export transporter BSEP [62]

Of these disorders, HT1 has the best described and has the most robust selective affect. HT1 is caused by a genetic deficiency in the gene fumarylacetoacetate hydrolase (FAH). FAH is responsible for the breakdown of the metabolite fumarylacetoacetate (FAA) as part of the tyrosine and phenylalanine metabolic pathway (Figure 1-2). Accumulation of the highly electrophilic FAA leads to cellular toxicity. Hepatocytes in an FAH-deficient liver can be protected from FAA toxicity through restoration of FAH expression by gene addition or gene repair therapies. Hepatocytes repaired in vivo or wild type hepatocytes delivered by transplantation thus have a selective advantage in the FAH-deficient liver. Just 1000 wild-type hepatocytes are sufficient to repopulate the $Fah^{-/-}$ mouse liver, and repopulation of >90% of the liver can be achieved in this model [57]. In livers of HT1 patients, revertant hepatocytes that have regained FAH activity through random mutation frequently repopulate the liver, leading to somatic mosaicism [63-65].

Transgenic models of hepatocyte selection

In addition to disorders with a naturally occurring selective effect, multiple transgenic animal models for selective liver repopulation have been created as experimental tools. These models are summarized in table 1-2.

The Albumin-Urokinase mouse provided the first evidence for the feasibility of selective liver repopulation [66]. This mouse model expresses the urokinase plasminogen activator (uPA) protein driven by the endogenous promoter of albumin (*Alb*), a highly-expressed liver-specific gene. This mouse was created in 1991 with the intention of investigating the effect of uPA on hemostasis. However, the investigators instead observed a revertant phenotype where mice lost the effect of the uPA transgene with age. Liver histology revealed wide-spread cellular injury dotted with nodules of healthy hepatocytes. These nodules were found to be revertant hepatocytes that had silenced expression of the toxic transgene, and these cells expanded to repopulate over 90% the liver as the animals aged. Along with the Fah-deficient mouse, the Alb-uPA mouse is one of the most widely used animal models of hepatocyte expansion due to the robust selective effect.

A variety of other transgenic models of hepatocyte selection have been described (Table 1-2). All involve liver-specific expression of cytotoxic or pro-apoptotic transgenes, often driven by the murine *Alb* promoter. Several of these

toxicities are inducible upon administration of a substrate or ligand while others are constitutive.

Table 1-2. Transgenic mouse models showing genetic selection for corrected hepatocytes:

Transgene	Selective mechanism
Alb-uPA	Liver-specific uPA expression causes proteolytic damage through activation of plasmin. [66]
Alb-HSVtk	Liver-specific HSV thymidine kinase expression induces toxicity upon administration of the substrate ganciclovir. [67]
Alb-FKBP-Casp8	Liver-specific inducible caspase-8 fusion protein expression causes apoptosis upon induction by AP20187. [68]
AAT-Z	Multiple copies of the human Z-allele AAT protein driven by its native liver-specific promoter cause ER stress. [69]
Alb-hHBEGF	Liver-specific expression of human HBEGF gene confers susceptibility to diphtheria toxin. [70]

Non-genetic selection

All of the above selective mechanisms rely on a certain genetic background, whether naturally occurring or transgenic, to create a cell autonomous genetic liver injury that confers a selective advantage to wild-type cells. However, this effect is not seen in most genetic liver disorders. Therefore, a selective strategy that is applicable in any genetic state is needed.

In hepatocyte transplantation, non-genetic selection methods have also been applied to induce cell division by providing a transient proliferative advantage for transplanted cells. This has generally involved a combination of preconditioning of the host liver, usually with the use of a DNA damaging treatment such as irradiation or alkylating agents, and a general proliferative stimulus such as partial hepatectomy, hepatocyte growth factor, or a thyroid hormone receptor agonist. A general proliferative stimulus alone is insufficient to expand only transplanted cells, as the native cells respond equally and thus the ratio of transplanted to host cells remains unchanged. Preconditioning of the host liver prevents division of native cells and thus gives the transplanted cells a selective advantage in responding to the proliferative stimulus. Approaches to preconditioning and regenerative stimuli are summarized in table 1-3. Although a variety of techniques for both preconditioning and stimulating proliferation have been described in animal models (table 1-3), hepatic irradiation [71] and partial hepatectomy [72] are currently the only interventions considered safe enough for human application [51].

Despite the inherently transient nature of the selective effect with preconditioning approaches, they have been successful in achieving robust levels of liver repopulation in animal models. For example, in a mouse model treated with irradiation preconditioning and the thyroid hormone receptor agonist GC-1, up to 40% liver repopulation by transplanted hepatocytes was seen [73]. This was successful in reversing hypercholesterolemia in a mouse model of ApoE

deficiency, a genetic background that does not confer any inherent selective advantage to corrected cells.

Table 1-3. Approaches to transient non-genetic selection of transplanted hepatocytes:

Preconditioning approaches	**Mechanism**
X-ray Irradiation	DNA Damage [74]
Retrorsine	DNA Damage [75]
Monocrotaline	DNA Damage [76]

Proliferative Stimuli	**Mechanism**
Partial hepatectomy	Growth stimulus to replace resected liver [77]
Hepatocyte growth factor (HGF)	Mitogen [78]
Thyroid hormone (T3)	Agonist of thyroid hormone receptor [79]
GC-1 (sobetirome)	Agonist of thyroid hormone receptor- less toxicity than T3 [73]

Towards a universal selection system

Non-genetic conditioning approaches allow hepatocyte selective expansion to be accomplished on any genetic background but are limited by being transient. After the injury to the native liver "wears off", repeat administration of the proliferative stimulus will not selectively favor the transplanted cells. It is also not applicable to gene therapy approaches. An ideal universal selection strategy would: a) not rely

on a specific genetic background or disease, and b) allow a selective agent to be applied repeatedly until a therapeutic threshold is reached. A universal genetic selection system that relies on a specific genetic modification in the targeted cells to confer resistance to a hepatotoxin rather than a certain genetic background in the recipient would meet both conditions.

The first such system was described in 1998 and involved repopulation of the wild-type mouse liver by hepatocytes transgenically expressing the human oncogenic protein BCL-2 [80]. This modification caused resistance to apoptosis as induced by the administration of a pro-apoptotic Fas receptor agonist. With repeated administration of the agonist, this allowed up to 16% repopulation of the liver with BCL-2 transgenic transplanted hepatocytes using a cell transplantation strategy. With a gene therapy approach delivering BCL-2 in vivo with an integrating viral vector, up to 85% liver repopulation was accomplished [81], thus demonstrating proof-of-principle for the overall approach. However, the authors acknowledged that toxicities associated with BCL-2 and the Fas agonist made this strategy not ideal for therapeutic use in most cases [80].

A potential alternative approach for hepatocyte expansion is to create a proliferative advantage during normal liver cell turnover rather than a survival advantage in response to a hepatotoxin. One such system was described in 2018 [82]. Rat hepatocytes were transduced ex vivo with a lentivirus expressing a YAP (Yes-associated protein)-ERT2 (tamoxifen-responsive mutated estrogen

receptor) fusion protein. YAP is a pro-proliferative, anti-apoptotic transcription factor. ERT2 fusion allowed YAP to localize to the nucleus only upon tamoxifen administration. These hepatocytes were transplanted into a rat model of Crigler-Najjar syndrome, which shows elevated bilirubin. This approach allowed a tamoxifen-dependent decrease in bilirubin to within a therapeutic range. Liver repopulation up to 14% was seen at 12 months after transplant. This approach is of interest because it provides proof-of-concept for liver repopulation without underlying injury. However, constitutive expression of the oncogene YAP, even in an inducible form, creates concerns of oncogenesis that limit clinical translation.

Because of the powerful selective effect seen in the *Fah*$^{-/-}$ liver, initial attempts to create a universal selection system in the Grompe Laboratory sought to replicate the effect seen in HT1 [83]. In lieu of restoration of Fah expression, genetic inhibition of enzymes upstream of FAA creation in the tyrosine metabolic pathway also prevents FAA accumulation and gives targeted cells a selective growth advantage in Fah-deficient livers (Figure 1-2). A small molecule inhibitor of Fah, CEHPOBA, was administered to wild-type mice in order to phenocopy the pathology seen in HT1. Prior to CEHPOBA administration, mice were dosed with an integrating rAAV vector expressing the therapeutic transgene human Factor 9 as well as a selectable shRNA targeting *Hpd*, an enzyme upstream of FAA creation in the tyrosine metabolic pathway. Thus, upon CEHPOBA administration, *Hpd*-deficient hepatocytes were positively selected (Figure 1-3). This strategy allowed blood levels of human Factor 9 known to be therapeutic for

hemophilia B to be achieved. Additionally, transplanted hepatocytes from a knockout mouse lacking *Hgd*, another enzyme upstream of FAA, were also selectable in the wild-type liver with CEHPOBA administration [84]. These data served as promising proof-of-concept for the creation of a universal system for selective hepatocyte expansion. However, CEHPOBA is not FDA approved and is exceedingly difficult to manufacture, preventing the clinical applicability of this selection system.

Table 1-4. Universal hepatocyte selection systems:

Selectable genetic modification	**Selective agent**
BCL-2 (Transgenic donor)	Anti-Fas antibody (agonist) [80, 81]
YAP-ERT2 (lentiviral delivery)	None (normal cell turnover) [82]
Hpd knockdown (integrating rAAV delivery) or *Hgd* knockout (ko donor)	Fah inhibition by small molecule CEHBOPA [83, 84]

A selection system relying upon a clinically applicable and readily available hepatotoxin is needed to allow clinical translation of a universal selective strategy. We theorized that a well-known common hepatotoxin, acetaminophen, could represent a powerful selective agent to allow in vivo selective expansion of gene-targeted hepatocytes. The next sections of this introduction will describe the principles of acetaminophen metabolism and its applicability to hepatocyte selection.

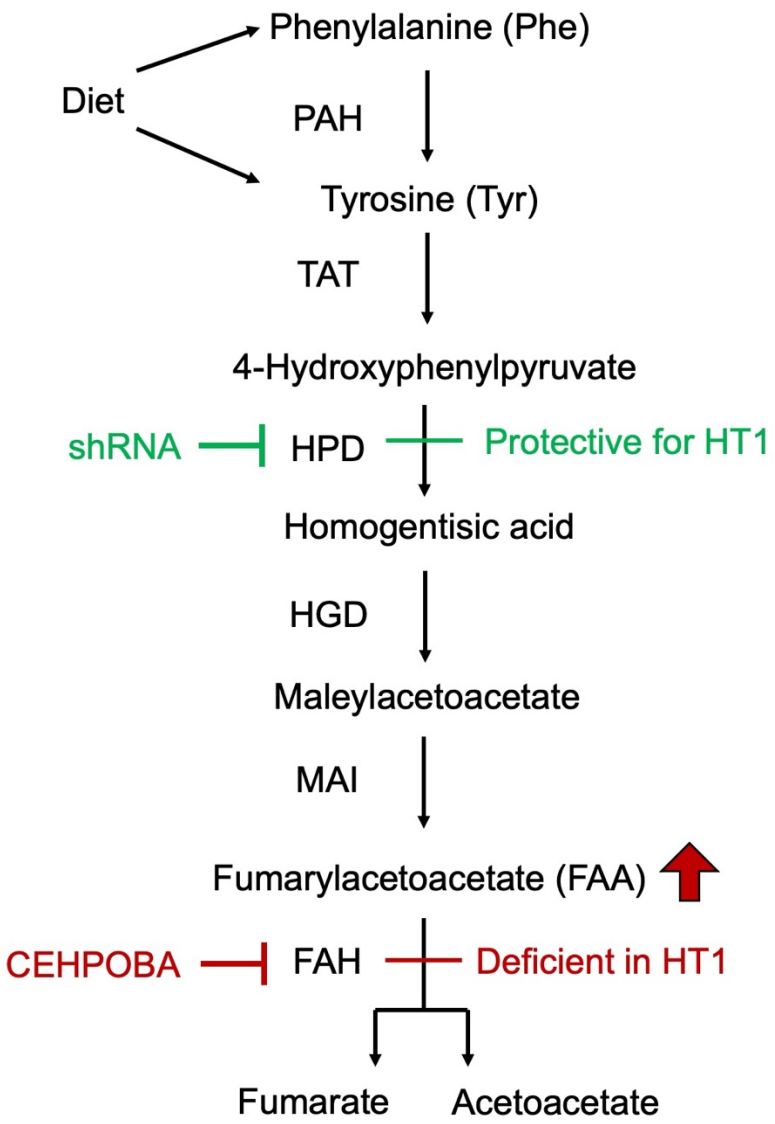


Figure 1-2. The tyrosine and phenylalanine metabolic pathway. FAA is produced as a byproduct of metabolism of dietary Phe and Tyr. FAH metabolizes FAA. In the FAH-deficient liver, FAA accumulates and causes cellular toxicity. Preventing FAA accumulation by either restoring FAH activity or inhibiting the upstream enzyme HPD results in a selective advantage.

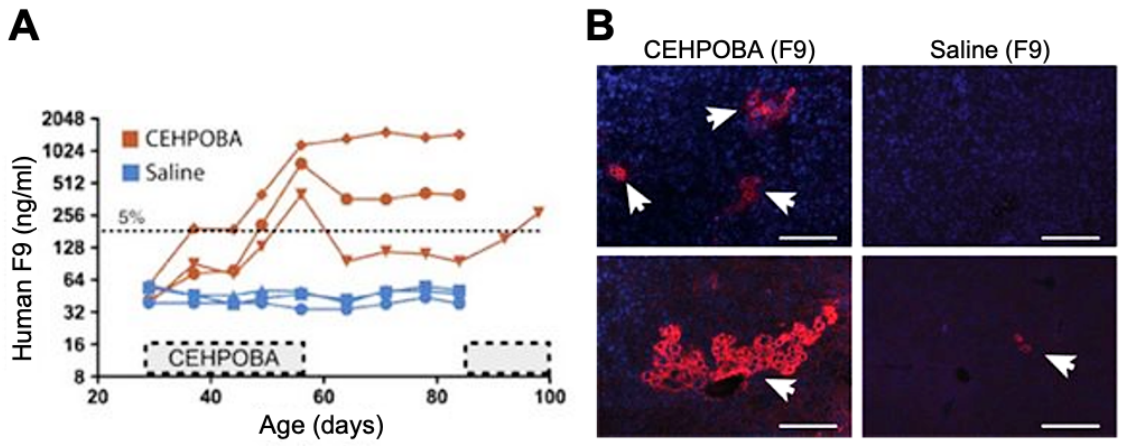


Figure 1-3. **Drug selection of integrating rAAV-hF9-*Hpd* shRNA in wild type mice.** (**A**) Plasma hF9 concentration in mice treated with CEHPOBA or saline control. All mice received rAAV as neonates. Dashed line indicates therapeutic hF9 (5% of normal). The gray rectangle indicates CEHPOBA treatment periods. (**B**) hF9 staining (Red) and nuclear DAPI (blue) showing representative liver nodules from two CEHPOBA-treated mice (left) and two saline-treated controls (right). Arrows indicate hF9-positive hepatocytes. Scale bars, 100 μm. Adapted from [83].

Hepatic Drug Metabolism

The cytochrome p450 enzyme system

The cytochrome p450 enzyme superfamily is a large class of enzymes that carry out a variety of functions, both in hepatic and extrahepatic tissues. In extrahepatic tissues, functions of CYP enzymes include steroid hormone synthesis in the adrenal gland and promoting proper skeletal development. In the liver, CYP enzymes conduct processes such as cholesterol metabolism, bile acid synthesis, and phase I drug metabolism [85]. Cyp enzymes function primarily as monooxygenases to catalyze the transfer of a single oxygen atom from O_2 to their substrates.

Based on amino acid sequence similarity, Cyp enzymes are divided into one of 18 families, denoted by numerals, and further divided into subfamilies denoted by letters [86]. Individual enzymes within the subfamily are identified by an additional numeral resulting in names such as CYP1A2, CYP3A4, etc. CYP families 1-3 are involved in drug metabolism through phase 1 oxidation reactions, primarily in the liver and additionally in the intestine. It is estimated that Cyps in families 1-3 are responsible for 80-90% of oxidative drug metabolism and 50% of all drug eliminations processes [86]. Six CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) are responsible for the majority of this metabolic activity [87]. Of these, CYPs 1A2, 2C19, 2E1, and 3A4 show zone 3-specific expression in the hepatic lobule, and 2C9 and 2D6 are not zonal [88].

CYPs 1A2 and 2E1 are liver-specific, while 2C9, 2C19, 2D6, and 3A4 also show intestinal expression [89].

Although upwards of 50 CYP enzymes exist in the human genome, only a single gene, cytochrome P450 reductase (CYPOR, POR, or CPR), encodes the electron donor required by all CYPs for activity [90]. The CYPOR protein consists of a flavin adenine dinucleotide (FAD) domain and a flavin mononucleotide (FMN) domain connected by a flexible hinge domain [91]. FAD accepts a pair of electrons from the donor nicotinamide adenine dinucleotide phosphate (NADPH) and transfers them to FMN. The FMN domain then transfers the electrons to the Heme catalytic center of a cytochrome P450 enzyme (CYP). In turn, the CYP then catalyzes the monooxygenation of its substrate. CYPOR and CYPs are membrane-bound proteins localized the cytoplasmic surface of the endoplasmic reticulum, which is thought to promote hydrophobic interactions necessary for their function [92].

Acetaminophen metabolism

To interrogate the functions of hepatic Cyp metabolism, mice that lack hepatic expression of Cypor were created [93, 94]. These mice display changes in the metabolism of many drugs [95]. Among these changes is resistance to the common cytochrome p450-metabolized hepatotoxin acetaminophen.

Acetaminophen, colloquially referred to by the commercial name Tylenol and known in some parts of the world as paracetamol, is among the most frequently taken over-the-counter drugs [96] and is used to treat pain and fever. Acetaminophen (abbreviated APAP for the chemical name N-acetyl-p-aminophenol) is metabolized by multiple pathways (Figure 1-4). The primary pathway is through phase II esterification to either glucuronides or sulfates (glucuronidation and sulfation, respectively). This directly results in non-toxic byproducts that are readily excretable in the urine [97]. It is estimated that approximately 90% of APAP is metabolized through these pathways. However, when esterification pathways are saturated, remaining APAP is acted upon by cytochrome p450s. CYP2E1 has the largest contribution [97], and CYP1A2 and CYP3A4 also participate [98, 99]. This phase I reaction leads to the formation of a highly reactive intermediate, *N*-acetyl-p-benzoquinone imine (NAPQI). NAPQI is a potent electrophile that readily binds intracellular proteins. NAPQI is detoxified by conjugation to cellular glutathione by glutathione-S-transferase enzymes. This phase II reaction forms cysteine conjugates and mercapturic acid, which are non-toxic and readily excreted in the urine [97].

When an excess of NAPQI is present following a large dose of APAP, cellular glutathione is depleted. The remaining NAPQI causes cellular injury and eventual necrosis. The binding of NAPQI to mitochondrial proteins has been specifically implicated in the progression of cell death [100]. The histological hallmark of APAP hepatotoxicity is centrilobular (i.e., zone 3) necrosis. This zonal pattern

occurs because CYP2E1, CYP1A2, and CYP3A4, which are responsible for the formation of NAPQI, all have zone 3 specific expression, restricting liver injury to this zone [101]. An antidote to APAP toxicity, N-acetyl cysteine (NAC), is available. NAC treatment restores cellular glutathione and can prevent NAPQI toxicity if administered shortly after APAP ingestion [102].

Due to the hepatotoxic nature of APAP, acute liver failure can occur following high doses. Over 2000 people in the US are hospitalized for APAP-induced liver toxicity each year, split approximately evenly between intentional and accidental overdoses [103]. The injury is acute, either resolving or requiring a liver transplant within 4 to 5 days [104, 105]. Intervention with NAC promotes transplant-free survival. If acute liver failure is avoided, the liver recovers and no permanent injury is seen [104].

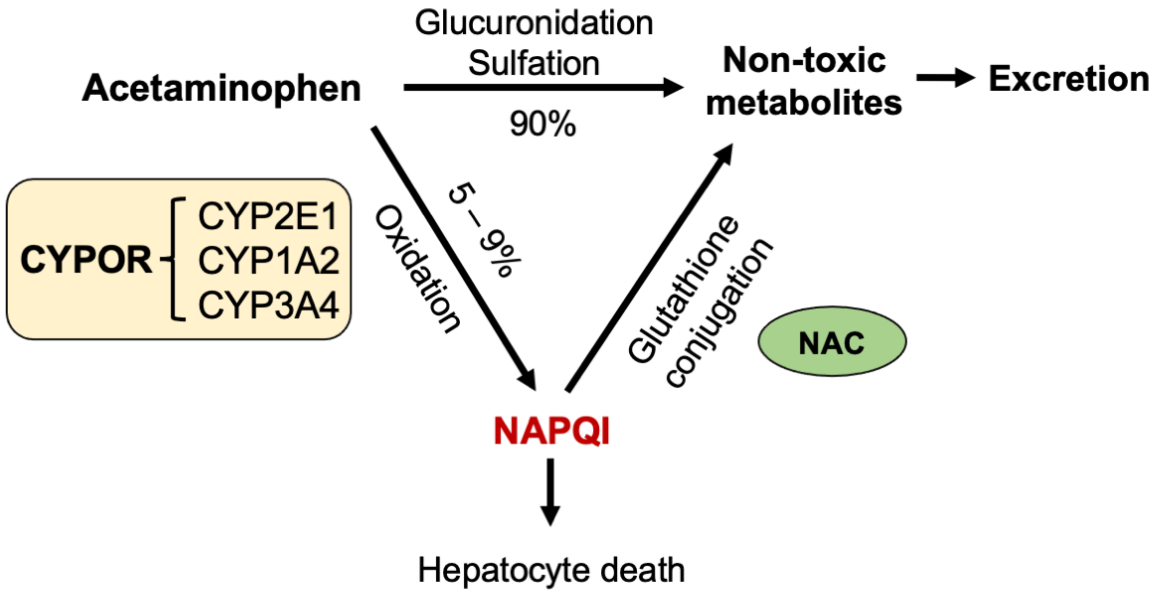


Figure 1-4. Principles of acetaminophen metabolism. (**A**) Pathways of acetaminophen metabolism. Cypor is required for the metabolism of acetaminophen to the hepatotoxic metabolite NAPQI. NAPQI is detoxified by conjugation to glutathione. N-acetylcysteine (NAC) replenishes glutathione.

Acetaminophen as a hepato-selective agent

Based on the mechanisms described above, we theorized that APAP has the potential to be a powerful selective agent for in vivo expansion of gene-modified hepatocytes. Specifically, we hypothesized that knockout or knockdown of Cypor would prevent Cyp activity and thereby prevent APAP-mediated hepatotoxicity. If a liver with rare population of Cypor-deficient hepatocytes is exposed to APAP, the Cypor-deficient cells are expected to expand to replace the wild-type hepatocytes that are susceptible to APAP-induced toxicity (Figure 1-5). If the Cypor deficiency is linked to a therapeutic payload, therapeutic cells could be selectively expanded until they reach the threshold required for phenotypic correction. All hepatocytes in zone 3 were expected to be amenable to this selection system. We anticipated that this system would have broad applicability to allow liver-directed gene and cell therapy to reach a therapeutic threshold for a wide variety of genetic disorders. In the final sections of this introduction, I will describe disease applications for the acetaminophen selection system.

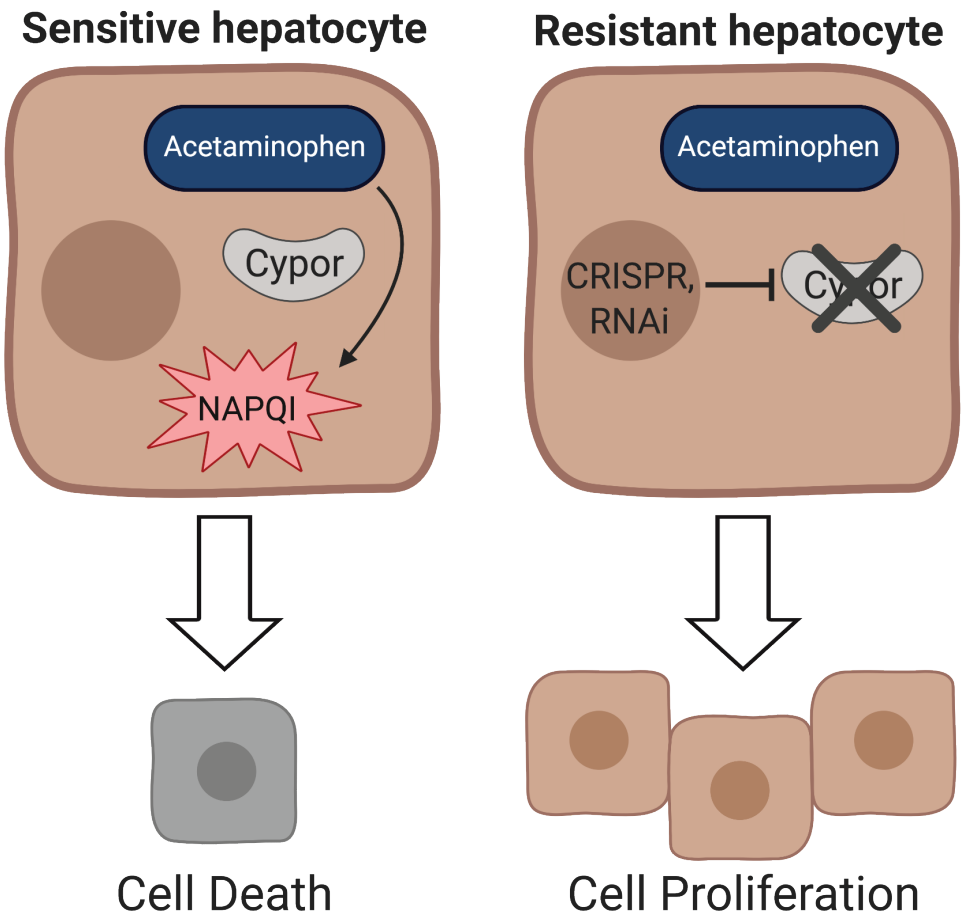


Figure 1-5. Acetaminophen-mediated hepatocyte selection. Mechanism of APAP selection. Disruption of *Cypor* renders hepatocytes resistant to the hepatotoxic effects of APAP, resulting in a selective advantage with APAP administration. Created with www.biorender.com.

Phenylketonuria

Natural history and biochemistry of PKU

Phenylketonuria (PKU) is an inborn error of metabolism resulting from deficiency in breaking down the amino acid phenylalanine into tyrosine. PKU is caused by mutations in the *phenylalanine hydroxylase* (*PAH*) gene and is inherited in an autosomal recessive manner. Deficiency of PAH causes high circulating levels of Phe in the blood, which results in neurologic symptoms including severe mental retardation. In the brain, elevated Phe is known to inhibit production of the neurotransmitters serotonin and dopamine [106], and cause hypomyelination and altered protein synthesis [107]. Hypopigmentation of the hair and skin is also seen due to a deficiency of melanin synthesis [108]. PKU disease presentation is divided into categories of severity based on the degree of hyperphenyalanemia: mild hyperphenylananemia (blood Phe: 120-600 μM), mild PKU (blood Phe: 600-1200 μM) and classical PKU (blood Phe: >1200 μM) [109]. Differing severity of mutant alleles is primarily responsible for these gradations [110].

The liver is the primary site of *PAH* expression and Phe metabolism. *PAH* is expressed throughout the hepatic lobule, with the highest expression seen in zone 1 hepatocytes [111]. Low levels of *PAH* expression are also seen in the kidney and gallbladder. PAH functions as a homotetramer, which is assembled as a “dimer of dimers” [112]. It requires a molecular chaperone known as DNAJC12 for proper protein folding, and its enzymatic activity requires the small-

molecule cofactor tetrahydrobiopterin (BH_4). A recently-described long non-coding RNA is also thought to be required for the enzymatic activity of PAH [113].

The population frequency of phenylketonuria is approximately 1 in 25,000 births worldwide. This rate varies by country, with most of the highest rates seen in Europe and the Middle East [109]. In most developed countries, PKU is detected during newborn screening based on high levels of Phe in the blood [107]. Following diagnosis, infants are placed on a diet with highly restricted levels of Phe. Good compliance with this diet throughout the lifetime of the patient is essential to avoid the bulk of neurologic symptoms associated with PKU. Maintaining blood Phe levels ≤360 μM is the target of dietary therapy. However, many patients struggle to maintain compliance with the diet. Even with good dietary compliance, higher rates of mood and attention disorders are seen in PKU patients relative to the general population [107]. An enzyme substitution therapy has recently become available but must be administered by daily injection [114]. Up to half of patients are responsive to supplementation of the co-factor BH_4, but the most severe cases are not amenable to this [109]. Thus, there is a significant unmet therapeutic need for PKU.

Mouse models of PKU

The first murine models of PKU were generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis in the early 1990s [115]. One of the strains generated, known as $Pah^{enu2/enu2}$, has become widely used as the primary animal model of classical

PKU. The *Pah*$^{enu2/enu2}$ mouse has a single point mutation in exon 7, which leads to a Phe → Serine point mutation in the catalytic domain of the protein. This mouse shows no Pah enzyme activity and has blood phenylalanine levels >1200 μM without treatment.

In gene therapy applications, it is known that the Pah^{enu2} mutant protein has a dominant negative effect by oligomerizing with the wild-type protein and reducing its activity. Additionally, the presence of the mutant protein prevents immunohistochemical methods for detection of corrected Pah as no antibodies are available that are able to distinguish the single amino acid change. To overcome the limitations of the *Pah*$^{enu2/enu2}$ mouse model, the Harding laboratory at OHSU created a novel Pah-null mouse [116]. This model, the *Pah*$^{\Delta exon1/\Delta exon1}$ mouse, has a complete deletion of exon 1 of *Pah* and no Pah protein product. This mouse shows similar blood Phe levels and neurologic pathology as compared to the *Pah*$^{enu2/enu2}$ model. However, due to the absence of the dominant negative protein product, it shows an increased responsiveness to gene therapy [116].

Gene and cell therapy for PKU

Many gene therapy approaches for PKU have been reported in mouse models, with mixed successes [117]. Three human clinical trials of rAAV gene therapies for PKU are currently ongoing in the US. One has been placed on hold following development of HCC in preclinical mouse trials. In the other two trials, correction

of blood Phe to ≤360 μM has been reported in at least one patient (dosed at $6x10^{13}$ vg/kg), although others did not achieve complete correction [118]. All of these trials require high vector doses (> 10^{13} vg/kg).

Two instances of human hepatocyte transplantation for PKU have been published. In the first [119], a 6-year-old patient with severe classical PKU was dosed repeatedly with hepatocytes explanted from a glycogen storage disease (GSD) patient. These cells were available due to the GSD patient having undergone liver transplantation for treatment of their disease. Because the transplanted cells represented only a small percentage of the liver, they were not expected to be sufficient to cause a GSD phenotype. The phenylketonuric child received four cell infusions over a two-day period totaling over 1.5×10^9 hepatocytes. Seven months later, the patient received a final infusion of 0.85×10^9 hepatocytes from a cadaveric donor with no genetic deficiencies. Decreases in blood Phe levels to within the range considered mild hyperphenylalanemia were seen as late as three months following the second infusion. However, this effect was not seen in follow-up beyond this point and blood Phe returned to pre-treatment levels. In another report [71], an adult female patient with classical PKU was treated with irradiation preconditioning to the largest lobe of the liver and infused with 5.0×10^9 hepatocytes from a maple syrup urine disuse (MSUD) patient over a course of three infusions. This resulted in a decrease in blood Phe levels from 1350 μM to 850 μM. This effect was also lost in long-term follow-up. These attempts serve as proof-of-concept for the use of hepatocyte

transplantation to correct blood Phe in PKU patients, but also illustrate that current methods are sub-therapeutic.

Investigation in the Harding and Grompe Laboratories at OHSU aimed to characterize the corrective cell threshold needed to correct blood Phe levels in *Pah*$^{enu2/enu2}$ mice by hepatocyte transplantation. To this end, a dual Pah-deficient, Fah-deficient mouse was created. This mouse exhibited the elevated Phe levels seen in PKU as well as the hepato-selective effect characteristic of HT1. Hepatocyte transplantation in this mouse demonstrated that replacement of approximately 10% of the native liver with corrected hepatocytes was necessary for blood Phe correction to the therapeutic target of 360 μM [120, 121]. This is well above the threshold that is typically achievable with standard hepatocyte transplantation methodology.

Thus, both gene and cell therapy approaches for PKU are limited by efficiency. Although hepatic deficiency of PAH is causal for PKU, overall liver function is unaffected by elevated Phe levels and PKU does not include a liver phenotype. This means that there is no selective advantage for corrected hepatocytes. Thus, PKU gene and cell therapy could be enhanced by a selective advantage for corrected cells and is an ideal candidate disease for application of acetaminophen selection.

Hemophilia B

Another potential application for the APAP selection system is hemophilia B. Hemophilia B is an X-linked recessive disorder caused by mutations in the human factor 9 (*hF9*) gene. The gene name is typically abbreviated to *F9* while the protein is referred to as FIX, however in this dissertation “F9” will be used for both for clarity. The *F9* gene encodes a clotting factor that participates in the blood coagulation cascade. It is produced and exported by hepatocytes [122]. Hemophilia B patients may experience frequent uncontrollable bleeding events and joint pain [123]. Normal blood F9 concentrations are 4 - 5 μg/mL in people unaffected by hemophilia B [124]. However, 250 ng/mL, roughly 5% of normal levels, is known to be sufficient to prevent a severe clinical phenotype [125]. As previously discussed, an rAAV gene therapy product for hemophilia B has been FDA approved [21]. However, the high cost and vector dose of this product as well as the previously discussed shortcomings of episomal rAAV therapies present an opportunity for improvement. Thus, hemophilia B is still a disorder with unmet therapeutic need. Additionally, it is a highly useful model system for testing gene therapy approaches. Because the corrective level of blood F9 is well characterized and can be measured by a simple ELISA on peripheral blood samples, it is easy to determine when a corrective threshold has been reached. Because antibodies exist that can distinguish human F9 from mouse F9, a curative approach can be ascertained even in wild-type mice using a human-specific ELISA to measure blood hF9, if the gene therapy vector used includes

the human version of the *F9* transgene. There is no inherent selective advantage for transplanted hepatocytes in the hemophilia B liver, making this disorder an ideal test case for the APAP selection system.

Dissertation Aims

The aims of this dissertation were to:

1. Demonstrate proof-of-concept for acetaminophen selection of Cypor-deficient hepatocytes.
2. Demonstrate the applicability of the selection system to both gene and cell therapy approaches.
3. Assess safety concerns associated with acetaminophen toxicity and partial Cypor deficiency.
4. Characterize disease targets amenable to correction with the selection system, specifically its applicability to PKU and hemophilia B.

Chapter 2:

Proof-of-Concept for Acetaminophen Selection and Applications to Lentiviral Gene Therapy for Murine Phenylketonuria and Hemophilia B

Abstract

Gene therapy by integrating vectors is a promising approach for monogenic liver diseases, especially in children where episomal vectors remain transient. However, reaching the therapeutic threshold with genome-integrating vectors is challenging. Therefore, we developed a method to expand hepatocytes bearing therapeutic transgenes. The common fever medicine acetaminophen becomes hepatotoxic via cytochrome p450 metabolism. Knockdown or knockdown of Cypor, the essential cofactor for cytochrome p450 metabolism, is shown to be selectable with acetaminophen administration. Hepatocytes lacking Cypor were selected in vivo by acetaminophen treatment, replacing up to 50% of the hepatic mass. Lentiviral vectors with transgenes linked in cis to a Cypor shRNA were administered to neonatal mice. Acetaminophen treatment of the mice resulted in over 50-fold expansion of transgene-bearing hepatocytes and achieved therapeutic thresholds in hemophilia B and phenylketonuria. Selection was shown to be stable following withdrawal of acetaminophen. We conclude that therapeutically modified hepatocytes can be selected safely and efficiently in preclinical models with a transient regimen of moderately hepatotoxic acetaminophen.

Introduction

Gene therapy is a promising approach to many previously incurable genetic disorders. Recombinant adeno-associated virus (rAAV) is currently the most commonly used vector for in vivo delivery [126, 127]. However, current rAAV gene therapies have some shortcomings. First, large viral doses may be required to transduce a curative threshold of cells. This is associated with a risk of an immune response [128, 129] as well as high cost. Second, due to their episomal nature, rAAVs are ideal for delivery to postmitotic cells as their expression is quickly lost in dividing cells [130], including the developing hepatocytes of children. Problems with loss of episomal gene expression can be overcome by utilizing integrating vectors, including lentiviral or integrating rAAV vectors targeting specific gene loci by homologous recombination [42, 127, 131]. However, the high efficiency of genetic modification required to reach therapeutic thresholds limits broad application of integrating vectors and precise gene editing technologies. Furthermore, it is difficult to produce lentiviral vectors in quantities sufficient for liver gene therapy in human patients. To overcome the efficiency problems, we developed a pharmacological hepatocyte selection regimen applicable to human patients. Here, we demonstrate that the widely used fever medicine acetaminophen (APAP) can be used to select therapeutically modified hepatocytes and attain therapeutic thresholds in preclinical models.

APAP is commonly used to treat mild pain and fever [132]. Although a majority of APAP is metabolized by Cyp-independent pathways, a fraction is metabolized by Cyp1A2, Cyp2E1, and Cyp3A4 in zone 3 hepatocytes [133] to the intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI) which is hepatotoxic [133]. Cypor is a cofactor required by all cytochrome p450 (Cyp) enzymes for electron transfer from NADPH [134]. As Cypor is an obligate co-factor for all Cyp enzymes, we hypothesized that loss of Cypor would abrogate Cyp-mediated metabolism, preventing the conversion of APAP to hepatotoxic NAPQI. Therefore, hepatocytes lacking Cypor would be protected from APAP-induced toxicity and not undergo necrosis. Here, we show that APAP treatment can be used to clonally expand hepatocytes with disrupted expression of *Cypor*. By linking therapeutic transgenes in cis with a CRISPR/Cas9 guide RNA (gRNA) or a short hairpin RNA (shRNA) targeting *Cypor* in an integrating vector, hepatocytes with integration can be selected with APAP. This strategy allows rare populations of hepatocytes harboring a disease-curing transgene to be expanded to therapeutic levels.

Materials and Methods

Animal Husbandry

Wild-type C57BL/6, 129S4 and B6J-Rosa-Cag-Cas9 (Stock #026179) mice were obtained from Jackson Laboratories. *Pah*$^{enu2/enu2}$ mice on a C57BL/6 background were obtained from the Harding Lab [135]. All animal experiments were performed according to the guidelines for animal care at Oregon Health & Science University. All animals were fed tap water and standard mouse chow (LabDiet Picolab Rodent Diet 5LOD) unless otherwise stated. Animals were housed under a standard 12-hour on and off light cycle. APAP diet was created by dissolving APAP in 20 mLs of 100% ethanol and adding 180 g of standard 5LOD diet preheated to 55°C. Diet was mixed until pellets were saturated with ethanol. Diet was then allowed to dry completely before being fed to mice. 1.5%, 1.6%, 1.75% or 1.9% w/w APAP diets were created using this method. All mice were started on 1% w/w APAP diet and ramped up to a higher percentage diet. Mice were given 2 to 4 days on regular diet if weight loss exceeded 20%. Mice were euthanized with CO_2. A cardiac puncture was performed immediately after death to deplete the liver of blood.

Plasmid construction and viral production

pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230; http://n2t.net/addgene:42230 ; RRID:Addgene_42230). gRNAs were cloned into pX330 backbone according to the provided protocol [136].

gRNAs were designed using http://crispor.tefor.net/ [137]. The *Cypor*-targeting gRNA sequence was TCGTGGGGGTCCTGACCTAC. pDG330 was a gift from Paul Thomas (Addgene plasmid # 100898 ; http://n2t.net/addgene:100898 ; RRID:Addgene_100898) and cloned according to the provided protocol. The *Cyp1A2*-targeting gRNA sequence was AAGAATCCACCCGGACCCTG, and the *Cyp2E1*-targeting gRNA sequence was CCACATGGAAGGACGTGCGG.

shRNAs were based on designs by Sigma-Aldrich. The *Cypor*-targeting shRNA sequence was CCTGACCTACTGGTTCATCTTctcgagAAGATGAACCAGTAGG-TCAGG (lower case indicates loop sequence). The transposon plasmid p/T-FAHIG [138] was used for proof-of concept testing of candidate shRNAs. The shRNA driven by a U6 promoter was cloned using the InFusion Cloning Kit (Takara Bio) and standard restriction cloning. A validated shRNA against the mouse *Hpd* gene, which is not involved in APAP metabolism, was used as a non-selectable control. The transposon plasmids were co-delivered with SB100X transposase plasmid.

The lentivirus backbone was received as a gift from Luigi Naldini [139]. cDNA for codon optimized human *F9* and human *PAH* were added by using the InFusion Cloning Kit (Takara Bio). Lentivirus was produced by the OHSU viral production core. Lentiviral titers were determined by quantitative RT-PCR.

Delivery of plasmids and viral vectors

For lentivirus delivery, P1 neonates were injected via the facial vein with 1.4 x 10^6 to 1.9 x 10^7 TU/pup of lentivirus. For delivery of plasmid vectors in 6- to 8-

week old adults, hydrodynamic tail vein injections were performed to deliver plasmid in saline equivalent by volume to 10% of the mouse's body weight.

Acetaminophen preparation and delivery

13 mg/mL acetaminophen (4-Acetamidophenol, 98%, ACROS Organics) was dissolved in saline prewarmed to 50°C, sterilized with a 0.2 μm filter, aliquoted and stored at room temperature for up to one month or until precipitate was observed. Mice were fasted overnight for approximately 16 hours before APAP administration. Mice were weighed and APAP was administered by IP injection twice weekly. The dose of APAP started at 220 mg/kg in males and 250 mg/kg in females and blood was drawn for ALT measurements 6 to 7 hours after APAP treatment. Subsequent APAP doses were increased by 5 to 10 mg/kg until at least 2 consecutive elevated ALT measurements (>800 IU/L) were observed. For most experiments, APAP doses were then kept constant. $Pah^{enu2/enu2}$ mice that received LV-*PAH-Cypor* shRNA lentivirus showed ALT stabilization before the therapeutic threshold was reached and subsequently required continuous dose escalation. After fasting, if greater than 15% body weight was lost, APAP treatment was skipped for one dose.

ALT and blood chemistry

To measure blood ALT, 10 μL of blood was collected from the saphenous vein into 15 μL of heparin-containing saline. Samples were briefly centrifuged and the supernatant was collected and stored at -20°C for up to 1 week. ALT activity was

measured in quadruplicate with the ALT Color Endpoint kit (Teco Diagnostics) with modifications to the manufacturer's protocol. Plasma was obtained by terminal cardiac puncture and submitted to IDEXX Laboratories for a comprehensive blood chemistry panel.

Histology

Liver tissues were sliced into approximately 5 mm sections and fixed in 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 4 hours or at 4°C overnight. Liver slices were then passed through a sucrose gradient consisting of 10%, 20% and 30% sucrose (w/v) in PBS. Tissue slices were embedded in optimal cutting temperature compound and 7 μm sections were cut using a cryostat onto Colorfrost Plus slides (Fisher Scientific). The following primary antibodies were used: Rabbit-anti-Cypor (Abcam, #180597), Goat-anti-human F9 (Affinity biologicals, GAFIX-AP), Rabbit-anti-Cyp2E1 (Abcam, #28146), Goat-anti-Cypor (Abcam, #166800, for Cypor/Cyp2E1 dual stain). Secondary antibodies were Alexa Fluor 555- or 647-conjugated. For visualization of GFP expression, slides were incubated with TrueBlack Lipofuscin Autofluorescence Quencher (Biotium) for 1 minute prior to staining. Sections were permeabilized in 0.1% Triton X-100 in PBS at room temperature for 12 minutes, washed in 3x5 minutes PBS and then blocked in 10% normal goat or donkey serum with 0.3 M glycine for 30 minutes at room temperature. Slides were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. Slides were washed in 3x5 minutes PBS, incubated in secondary antibody for 1 hour at room

temperature, then washed 3x5 minutes. Coverslips were mounted with DAPI Fluoromount-G (SouthernBiotech). Imaging was performed on a Zeiss LSM700, LSM780, or LSM900 confocal microscope.

hF9 ELISA

Blood samples for hF9 measurements were collected from the saphenous vein. hF9 concentrations were measured by utilizing the Asserachrom IX:Ag ELISA kit (Stago) following the manufacturer's protocol with modifications as previously described [131].

Pah Enzymatic Activity and serum phenylalanine

Liver Pah enzyme activity and serum phenylalanine were determined as previously described [135].

TIDE analysis of CRISPR/Cas9 induced indels

Genomic DNA extraction from homogenized liver tissue was performed using the MasterPure Complete DNA and RNA Purification Kit (Lucigen) following the manufacturer's protocol. A 700 base pair region surrounding the Cypor gRNA target locus was amplified (forward primer 5'-GTTTGCGGGTGTTAGCTCTTC-3'; reverse primer 5'-AGTCTACTTCAGTCGCAGCC-3') using MyTaq Red Mix (Bioline). The amplicon was purified using the PCR cleanup and gel extraction kit (Macherey-Nagel) and Sanger sequenced using the forward primer. Indels were analyzed using the TIDE software, https://tide.nki.nl [140]. Percent Cypor-

deficient hepatocytes was estimated assuming that hepatocytes account for 60% of total liver DNA (raw percent indels ÷ 0.6 = percent Cypor-deficient hepatocytes).

Image analysis

Quantification of Cypor-negative areas by image analysis (table 2-1) was conducted manually using ImageJ.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 10.0.0 for Mac (GraphPad Software, La Jolla, California, www.graphpad.com). A Student's unpaired one-tailed or two-tailed *t*-test assuming equal variance was used to analyze differences between selected and unselected mice. One-way ANOVA with a Tukey post-hoc test was used to compare differences between three groups. To analyze differences in blood hF9 or Phe concentrations between selected and unselected mice over a time course, a two-way repeated-measures ANOVA or mixed effects model assuming sphericity with Bonferroni multiple comparisons was used. P values <0.05 were considered statistically significant. For all statistical analysis, *P<0.05, **P<0.01 and ***P≤0.001. All error bars indicate standard deviation.

Results

Selection of Cypor-Knockout Hepatocytes

To demonstrate that hepatocytes deficient in Cypor can be selected by APAP, *Cypor* was knocked out in hepatocytes in vivo. A plasmid expressing *Streptococcus pyogenes* Cas9 (SpCas9) and a U6-driven gRNA targeting *Cypor* (pX330-*Cypor* gRNA, Figure 2-1A) was delivered via hydrodynamic injection [141] into wild-type adult mice. Mice were then treated biweekly with APAP by intraperitoneal (IP) injection at a dose sufficient to produce an ALT elevation >800 IU/L (250 – 450 mg/kg). Plasma alanine aminotransferase (ALT) activity six hours after treatment was monitored as a measure of hepatotoxicity. ALT responses were initially highly elevated following APAP injection but diminished after repeated treatments. This effect was not seen in mice not treated with pX330-*Cypor* gRNA prior to APAP treatment (Figure 2-1B). The decrease in ALT suggested the loss of non-transfected hepatocytes and expansion of APAP-resistant *Cypor* knockout hepatocytes. Liver gDNA was assayed for insertion or deletion mutations (indels) at the *Cypor* gRNA target site using the Tracking of Indels by Decomposition (TIDE) algorithm [142]. Indel analysis showed 2.6% ± 1.3% Cypor-deficient hepatocytes in unselected mice that received the *Cypor* gRNA plasmid without APAP treatment, and a significant ($P < 0.0001$) expansion to 35.5% ± 2.1% Cypor-deficient hepatocytes in mice with APAP selection (Figure 2-1C). This was confirmed by Cypor immunofluorescence (IF) showing large areas of clonally expanded Cypor-negative hepatocytes after selection, as

compared to rare Cypor-negative hepatocytes in unselected mice (Figure 2-1D). These findings demonstrate that a knockout of *Cypor* in hepatocytes in conjunction with APAP treatment is an efficient method to expand a small population of correctly targeted hepatocytes to a substantial portion of the liver.

Selection of Cypor shRNA-expressing hepatocytes

Off-target genomic effects are a concern for clinical use of the CRISPR/Cas9 system, as is the need for codelivery of Cas9 and associated immunogenicity [143]. Therefore, it may prove preferable to target *Cypor* via shRNA-mediated knockdown. Two shRNAs against *Cypor* were screened for selectability by APAP. A transposon plasmid expressing enhanced green fluorescent protein (eGFP) and a U6-driven shRNA was co-delivered with a Sleeping Beauty transposase-expressing plasmid via hydrodynamic injection in adult wild-type mice (Figure 2-2A). Mice were treated with biweekly APAP injections to select for hepatocytes with Cypor knockdown due to shRNA expression. Mice treated with the transposon containing *Cypor*-shRNA2, but not *Cypor*-shRNA1 or a control shRNA, showed large clonally expanded areas of eGFP-positive hepatocytes and decreased Cypor levels by IF staining comprising approximately 50% of the liver area (Figure 2-2B and table 2-1). This indicates that a shRNA can provide sufficient knockdown of *Cypor* expression to allow the expansion of Cypor-negative hepatocytes with APAP selection. *Cypor*-shRNA2 was used for all future experiments utilizing a shRNA.

Selection of Cyp dual-knockout hepatocytes

Cypor knockout prevents APAP hepatotoxicity by preventing the function of the APAP-metabolizing Cyp enzymes Cyp2E1, Cyp1A2, and Cyp3A4. Therefore, an alternative strategy to prevent APAP toxicity is knockout of the Cyp enzymes. A plasmid expressing spCas9 and two gRNAs, one each against *Cyp2E1* and *Cyp1A2*, was created and administered to transgenic SpCas9-expressing mice by hydrodynamic injection (pDG330-*Cyps*; Figure 2-3A). Mice were then treated with APAP injections. ALTs remained elevated over the course of 55 APAP injections. Following terminal harvest, indel analysis at the *Cyp1A2* gRNA locus in liver gDNA showed an increase from 4.5% ± 5.2% *Cyp1A2*-deficient hepatocytes with plasmid treatment alone to 22.9% ± 5.0% with APAP selection (P = 0.0118) (Figure 2-3B) At the *Cyp2E1* gRNA locus, an increase from 8.2% ± 3.4% *Cyp2E1*-deficient hepatocytes without selection to 22.8% ± 8.2% with selection was seen (P = 0.0454) (Figure 2-3C). IF staining for Cyp2E1 showed expansion of negative cells within zone 3 of the hepatic lobule (Figure 2-3D). Due to the less robust affect as compared to Cypor knockout and the technical challenges of dual gene knockout, selection of dual Cyp knockout hepatocytes was not pursued further.

Selectable lentiviral vectors for hemophilia B

Standard rAAV vectors have only transient benefit in neonatal animals as they are rapidly lost by liver growth [26, 130]. To determine whether our selection system could be used to treat a genetic liver disease in neonates, human factor 9

(*hF9*), the deficient gene in hemophilia B, was chosen. 8.5 x 10^6 TU of a lentiviral vector expressing *hF9* from a hepatocyte-specific mouse transthyretin (mTTR) promoter [139] and the *Cypor* gRNA from the U6 promoter (Figure 2-4A) were delivered via facial vein injection to neonatal SpCas9 transgenic mice. To select for hepatocytes that had lentiviral integrations, biweekly APAP IP injections were started at age 8 weeks (n = 4), with half (n = 4) kept as unselected controls. After 27 APAP doses, hF9 concentrations in APAP-selected animals increased to 14,116 ± 2,241 ng/mL (Figure 2-4B), equivalent to a 4- to 57-fold increase (Figure 2-4C). The percentage of Cypor-deficient hepatocytes was significantly higher at 27.2% ± 9.1% in selected mice than in unselected controls at 2.88% ± 2.82% (*P* = 0.001) (Figure 2-4D). This finding was further corroborated by IF staining for Cypor and hF9, revealing clonally expanded regions of Cypor-negative, hF9-positive hepatocytes (Figure 2-4E).

In addition to this Cas9 dependent system, a *hF9* lentiviral vector employing the *Cypor* shRNA (Figure 2-5A) was given to wild-type neonates (6.6 – 8.7 x 10^6 TU/pup). APAP selection was started in half of the mice at age 8 weeks, and blood hF9 concentrations were monitored over time. Prior to APAP administration, the starting blood hF9 concentration in mice treated with the lentivirus was 116 ± 84 ng/mL. After 45 doses, hF9 concentrations ranged from 3,790 to 14,880 ng/mL in APAP selected mice (Figure 2-5B), representing an increase over baseline of 5 to 88-fold (Figure 2-5C). This is well above the blood F9 concentration considered therapeutic for hemophilia B, 250 ng/mL. Cypor IF

staining confirmed the clonal expansion of Cypor-negative, hF9-positive regions in APAP selected mice to approximately 15% of the liver area (Figure 2-5D and table 2-1). Hence, we show that a lentiviral expression of the *Cypor* shRNA allows APAP-mediated selective expansion of hepatocytes expressing *hF9*.

Selectable lentiviral vectors for phenylketonuria

Having shown that APAP selection can achieve therapeutic hF9 levels, we used the same strategy to treat a mouse model of phenylketonuria (PKU). PKU is an inborn error of metabolism caused by a lack of phenylalanine hydroxylase (Pah) resulting in high concentrations of circulating phenylalanine (Phe) and neurotoxicity [144]. Patients with PKU are treated with a Phe-restricted diet. Although treated patients ideally have Phe concentrations less than 360 μM, concentrations below 600 μM are considered mild hyperphenyalanemia and have a less severe clinical phenotype [145]. In the $Pah^{enu2/enu2}$ PKU mouse model, Phe concentrations in untreated animals are typically over 1,500 μM on a standard diet. Unlike hemophilia B, which can be corrected by transgene expression from a small percentage of hepatocytes, PKU is expected to require at least of 10% of hepatocytes to be corrected in order to have an effect on disease phenotype [146, 147].

A lentivirus containing the *Cypor* shRNA and human *PAH* cDNA (Figure 2-6A) was delivered via facial vein in $Pah^{enu2/enu2}$ neonates (1.9×10^7 TU/pup). At 6-8 weeks of age, APAP selection was started and serum Phe concentration was

measured once every 6 APAP doses. At baseline, serum Phe concentrations were 2,080 ± 372 μM in males, and 1,904 ± 283 μM in females. After 42-48 doses of APAP, male mice showed an average serum Phe of 366 ± 166 μM, significantly lower (P = 0.002) than in unselected controls (Figure 2-6B). Two of four selected male mice showed Phe concentrations below the therapeutic threshold of 360 μM, and all four had Phe concentrations less than 600 μM. APAP-selected female mice also showed a significant (P = 0.0006) decrease as compared to unselected controls. However, selected female mice had a final Phe concentration of 981 ± 70 μM, still above the therapeutic threshold. Selected mice showed a darkened coat color associated with restored melanin synthesis (Figure 2-6C). Pah enzymatic activity in liver homogenate indicated a significant increase in both female and male selected mice compared to unselected controls (Figure 2-6D). Cypor staining showed that approximately 40-55% of hepatocytes were Cypor negative (Figure 2-6E and table 2-1). The percentage of Cypor-negative liver area as estimated by IF staining was not notably different between male and female mice (table 2-1). H&E staining of a selected liver revealed healthy hepatocytes (Figure 2-6F). These results show a therapeutic correction of PKU using APAP selection of a *Cypor* shRNA in male mice and demonstrate that this method can be utilized in gene therapy for diseases that require a higher therapeutic threshold.

Partial selection

The APAP-metabolizing enzymes Cyp2E1, Cyp1A2, and Cyp3A4 are expressed in zone 3 of the hepatic lobule. Cypor is an obligate cofactor for all Cyp enzymes and functions in the metabolism of many drugs. Therefore, one potential barrier to the clinical applicability of APAP selection of Cypor-deficient hepatocytes is the theoretical loss of all Cypor-dependent zone 3 metabolic activity. To address this safety concern, we sought to demonstrate that therapeutic levels of selection can be achieved while retaining Cypor functionality in the majority of zone 3 hepatocytes. Neonatal SpCas9 transgenic mice were treated with a lentivirus expressing *hF9* and the *Cypor* gRNA via facial vein injection ($1.4 – 4.0 \times 10^6$ TU/pup). Upon weaning, baseline hF9 concentrations ranged from 9 to 82 ng/mL. Mice were APAP-selected with frequent monitoring of blood hF9 concentration. Selection was halted when blood hF9 concentrations exceeded 3,000 ng/mL, well above the therapeutic threshold for hemophilia B but still well below the maximum level achievable with extensive APAP selection. Indel analysis showed 6.7% to 16.7% Cypor-deficient hepatocytes in these partially selected mice (Figure 2-7A). Compared to frequencies as high as 42% in fully selected mice, this suggests that more than half of APAP-susceptible zone 3 hepatocytes retained their Cypor activity. IF staining for Cypor and Cyp2E1, a zone 3-specific gene, confirmed this result, showing that the majority of Cyp2E1-expressing zone 3 hepatocytes retained *Cypor* expression (Figure 2-7B - D). These data establish that therapeutic levels of gene expression can be attained with only partial loss of zone 3 *Cypor*-expressing hepatocytes.

Long-term stability of Cypor-deficient hepatocytes

An additional concern for the clinical applicability of this system is the stability of Cypor-deficient hepatocytes in the absence of continuous selective pressure from APAP. To address this, mice were treated with pX330-*Cypor* gRNA via hydrodynamic injection and selected with biweekly APAP injections until a lack of ALT response to APAP injection was observed. APAP was then stopped in half of the mice (n = 4). The others (n = 4) received once weekly APAP injections. Mice were harvested 42 weeks after halting APAP treatment in the first group (Figure 2-8A). Indel analysis showed no significant differences between the percentage of Cypor-deficient hepatocytes between the two groups ($P = 0.095$) (Figure 2-8B). Cypor IF staining of livers from mice that received no continued APAP treatment over the 42-week period showed that large areas of Cypor-deficient hepatocytes were still present (Figure 2-8C). Liver function and lipid panels revealed no significant differences between the selected mice that received no continued APAP treatment and untreated controls (Figure 2-9). In addition, H&E staining of liver tissue from mice in both the continued and discontinued APAP groups revealed mild lipid accumulation with no signs of hepatocellular injury, inflammation, or necrosis (Figure 2-8D).

Selection with modest liver damage

High doses of injected APAP were used in all experiments described above. In mice receiving IP APAP injections, ALT responses upwards of 10,000 IU/L were observed, representing a degree of liver injury unacceptable in a clinical setting.

To assess whether selection could be accomplished without extreme ALT elevation, an APAP-containing diet was formulated. Adult mice that had received hydrodynamic injections of pX330-*Cypor* gRNA were fed the diet for 6 to 12 weeks, with brief breaks. ALTs were monitored, and no ALTs exceeding 3,000 IU/L were observed (Figure 2-10A). None of the treated animals died. Upon euthanasia, indel analysis showed an average of 20.7% ± 9.4% Cypor-deficient hepatocytes, a significant increase compared to mice receiving no selection ($P < 0.0001$) (Figure 2-10B). Additionally, Cypor staining in mice that received APAP diet (Figure 2-10C) showed clonally expanded Cypor-deficient areas consistent with APAP selection. Hence, it is possible to select for Cypor-deficient hepatocytes gradually without periods of highly acute liver damage, suggesting this system could be applied clinically while minimizing the risk of dangerous levels of hepatotoxicity.

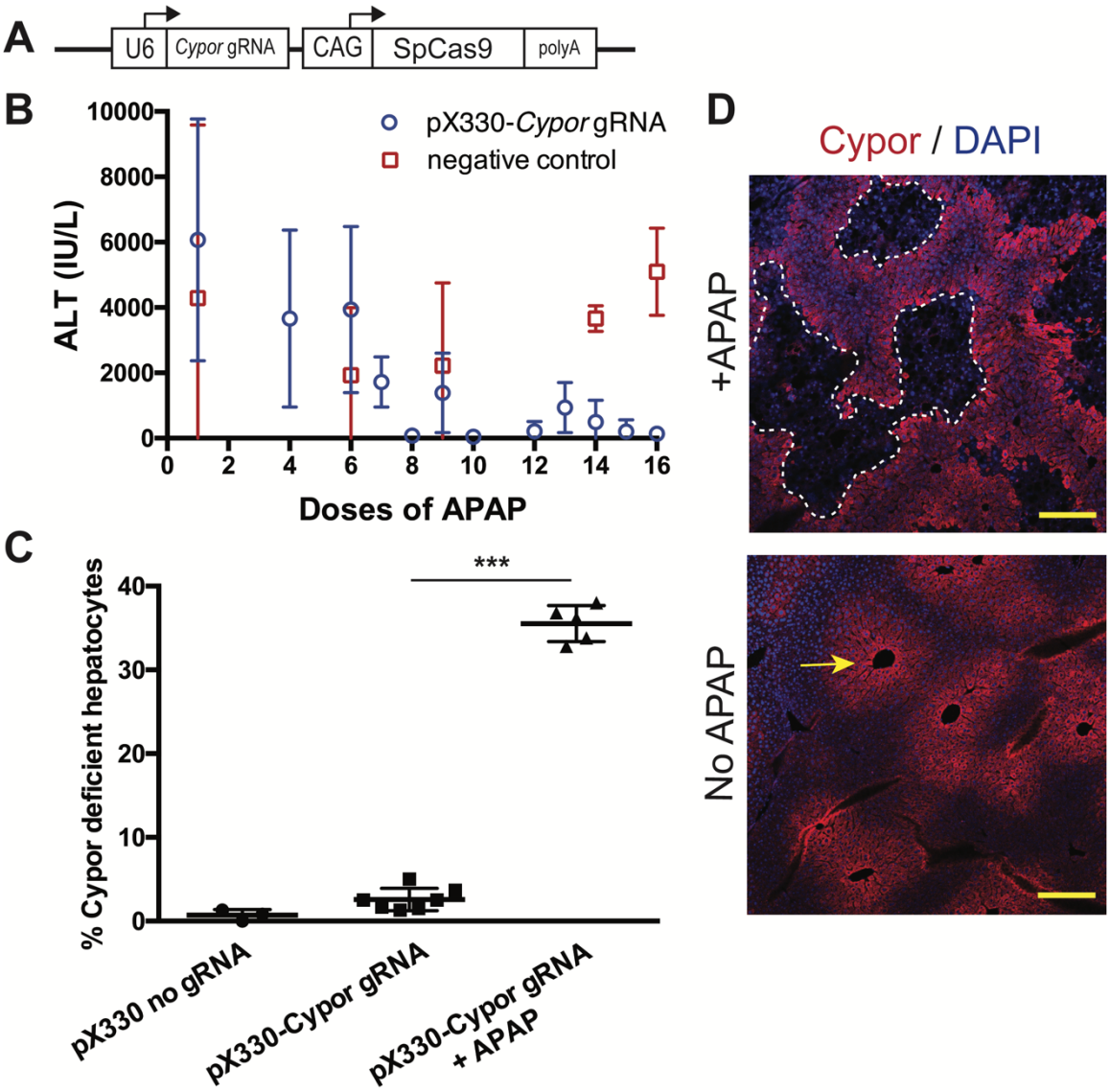


Figure 2-1. APAP selection of Cypor-knockout hepatocytes. (**A**) Schematic of the pX330-*Cypor* gRNA vector. (**B**) ALT in APAP-treated mice 6 hours after APAP injection for mice treated with pX330-*Cypor* gRNA (n = 10) or negative control (n = 8). (**C**) Percent Cypor-deficient hepatocytes measured by indel analysis of whole-liver homogenate gDNA in mice that received a negative control pX330 vector lacking a gRNA (n = 3), pX330-*Cypor* gRNA and no APAP (n = 7), or pX330-*Cypor* gRNA with APAP treatment (n = 5). ***P<0.001 by one-way ANOVA. (**D**) Cypor immunofluorescent staining (red) in liver sections from

mice that received pX330-*Cypor* gRNA with or without APAP treatment. Arrow indicates zone 3 expression of Cypor. Scale bars, 200 μm. Data are means ± SD.

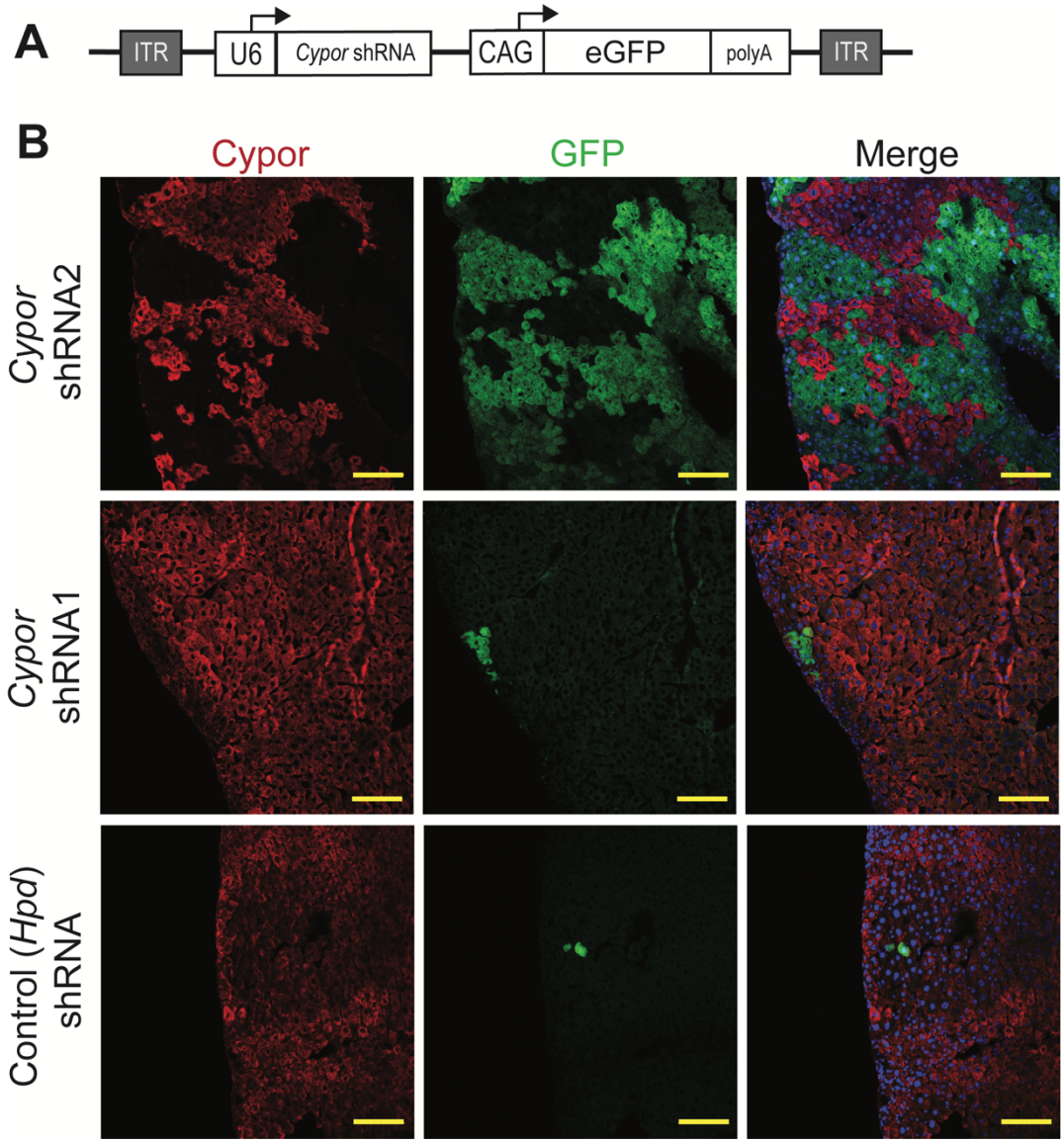


Figure 2-2. APAP selection of Cypor-knockdown hepatocytes. (**A**) Schematic of the transposon vector expressing GFP and a shRNA. (**B**) Top panels: Cypor immunofluorescent staining and GFP expression in liver sections from mice treated with a *Cypor* shRNA2-expressing transposon and APAP selection (representative; n = 3). Middle panels: Cypor immunofluorescent staining and GFP expression in liver sections from mice treated with a *Cypor* shRNA1-expressing transposon and APAP selection (representative; n = 3). Bottom

panels: Negative control: Mouse treated with a transposon expressing a shRNA against an unrelated gene (*Hpd*) and APAP selection (representative; n = 3). Scale bars, 100 μm.

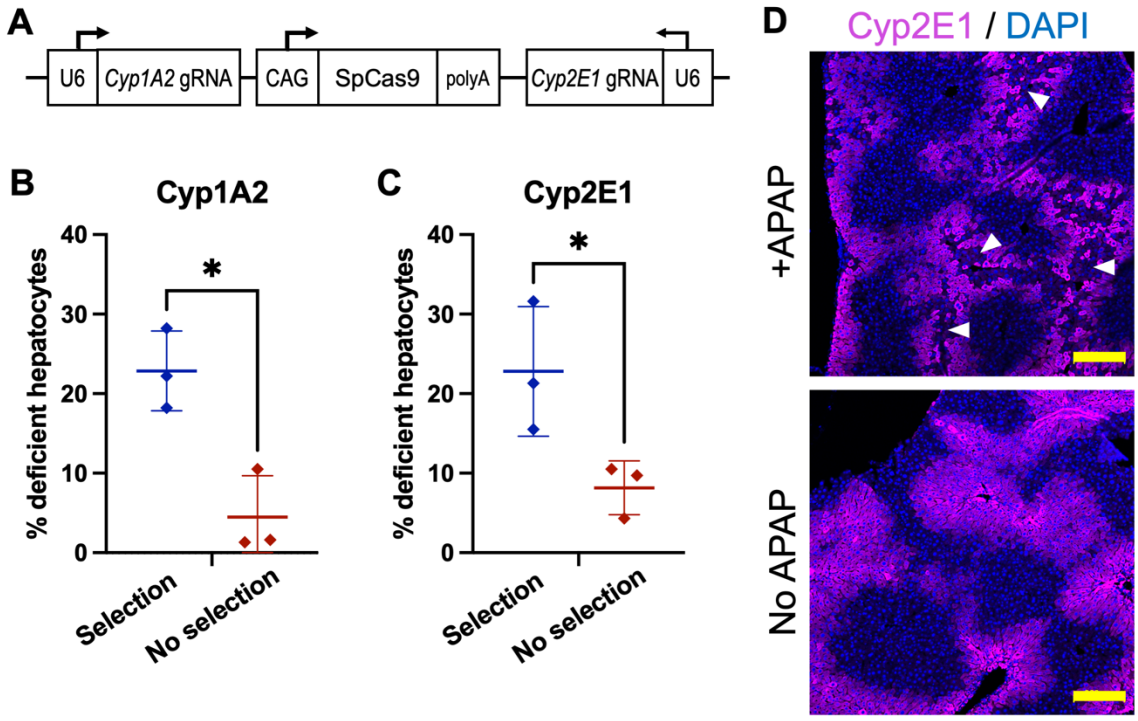


Figure 2-3. APAP selection of Cyp1A2 / Cyp2E1 dual knockout hepatocytes. (**A**) Schematic of the pDG330-*Cyps* plasmid. (**B**) Percent Cyp1A2-deficient hepatocytes measured by indel analysis of whole-liver homogenate gDNA in mice that received pDG330-*Cyps* and no APAP (n = 3), or pDG330-*Cyps* with APAP treatment (n = 3). (**C**) Percent Cyp2E1-deficient hepatocytes measured by indel analysis of whole-liver homogenate gDNA in mice that received pDG330-*Cyps* and no APAP (n = 3), or pDG330-*Cyps* with APAP treatment (n = 3). *P<0.05 by Student's two-tailed t-test. (**D**) Cyp2E1 immunofluorescent staining (magenta) in liver sections from mice that received pDG330-*Cyps* with or without APAP treatment. White arrowheads highlight pericentral areas where Cyp2E1 is normally expressed but is absent due to expansion of knockout hepatocytes. Scale bars = 200 μm. Data are means ± SD. Unpublished.

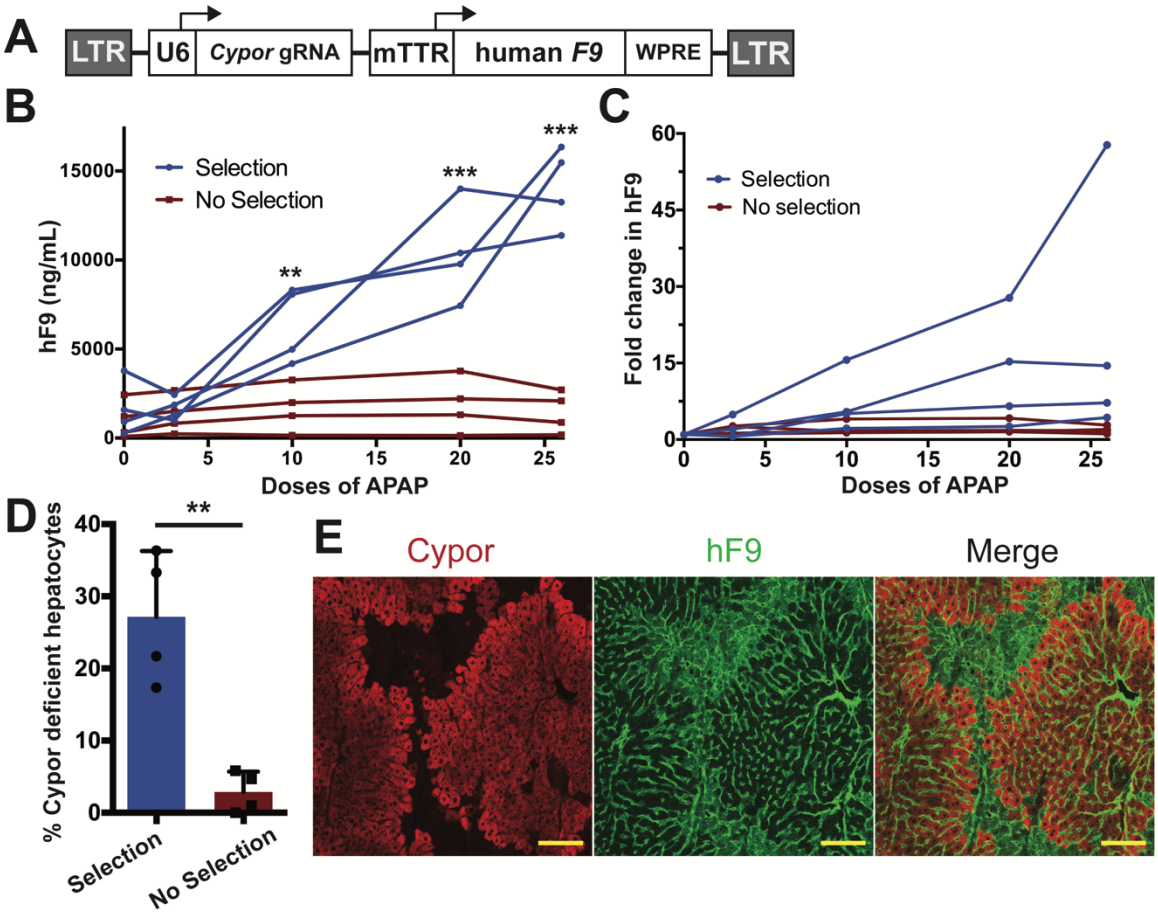


Figure 2-4. Selection of *Cypor* gRNA and *hF9*-expressing lentiviral vectors. (**A**) Schematic of the lentiviral vector containing a U6-driven *Cypor* gRNA and *hF9* driven by the liver specific mTTR promoter (LV-*hF9*-*Cypor* gRNA) (**B**) Blood hF9 concentrations in transgenic SpCas9 mice treated with LV-*hF9*-*Cypor* gRNA over the course of APAP selection. **P<0.01 and ***P≤0.001 by two-way ANOVA. (**C**) Fold change over baseline in blood hF9 concentration in mice treated with LV-*hF9*-*Cypor* gRNA and repeated APAP injection (n = 4) and unselected mice (n = 4). (**D**) Quantification of Cypor-deficient hepatocytes by indel analysis in APAP-selected (n = 4) and unselected (n = 4) SpCas9 mice treated with LV-*hF9*-*Cypor* gRNA. **P<0.01 by Student's one-tailed t-test. (**E**)

Cypor and hF9 liver immunofluorescence in mice treated with LV-*hF9-Cypor* gRNA followed by APAP selection. Scale bars, 100 μm.

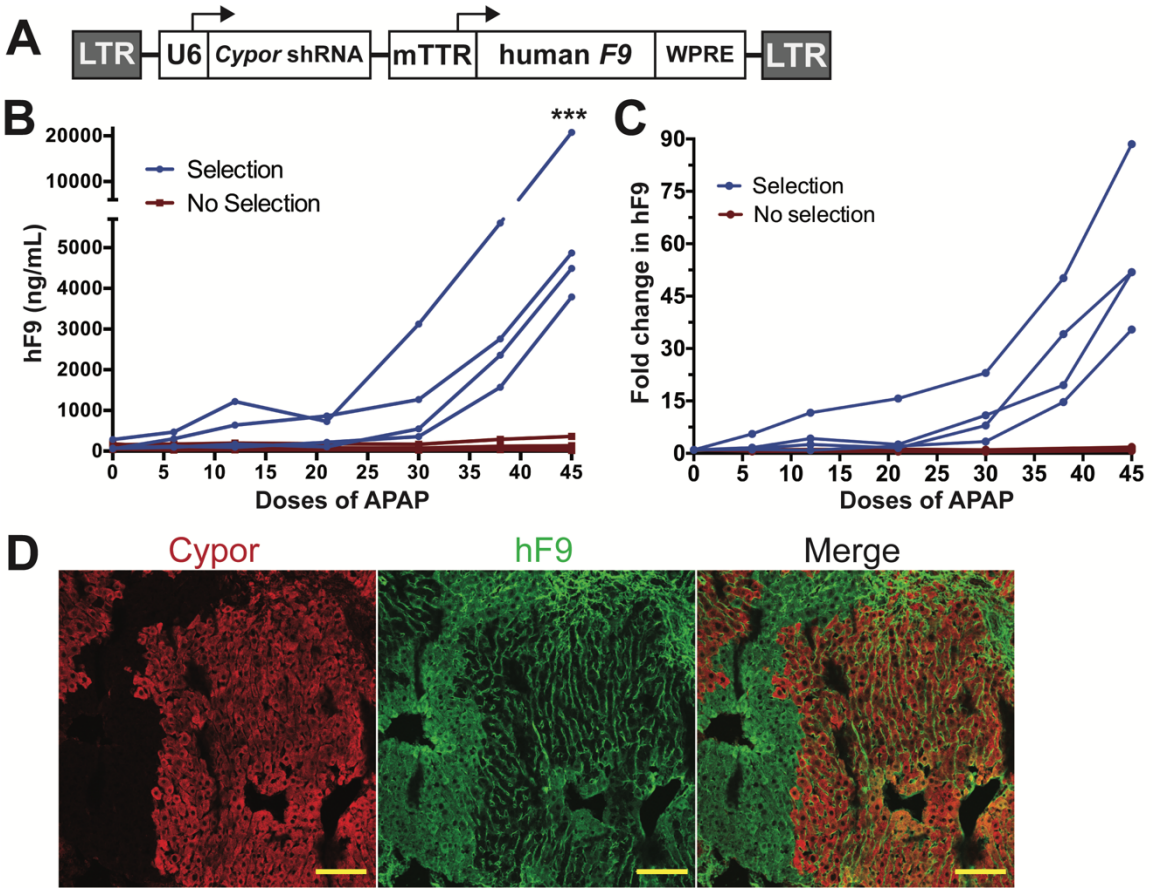


Figure 2-5. Selection of *Cypor* shRNA and *hF9*-expressing lentiviral vectors. (**A**) Schematic of the lentiviral vector expressing *Cypor* shRNA and *hF9* (LV-*hF9*-*Cypor* shRNA). (**B**) Blood hF9 concentrations over the course of treatment in wild-type mice treated with LV-*hF9*-*Cypor* shRNA and APAP selection (n = 4) and unselected controls (n = 4). ***P≤0.001 by two-way ANOVA. (**C**) Fold change over baseline in blood hF9 concentration in mice treated with LV-*hF9*-*Cypor* shRNA and repeated APAP injection (n = 4) and unselected mice (n = 4). (**D**) Cypor and hF9 liver immunofluorescence in wild-type mice treated with LV-*hF9*-*Cypor* shRNA followed by APAP selection. Scale bars, 100 μm.

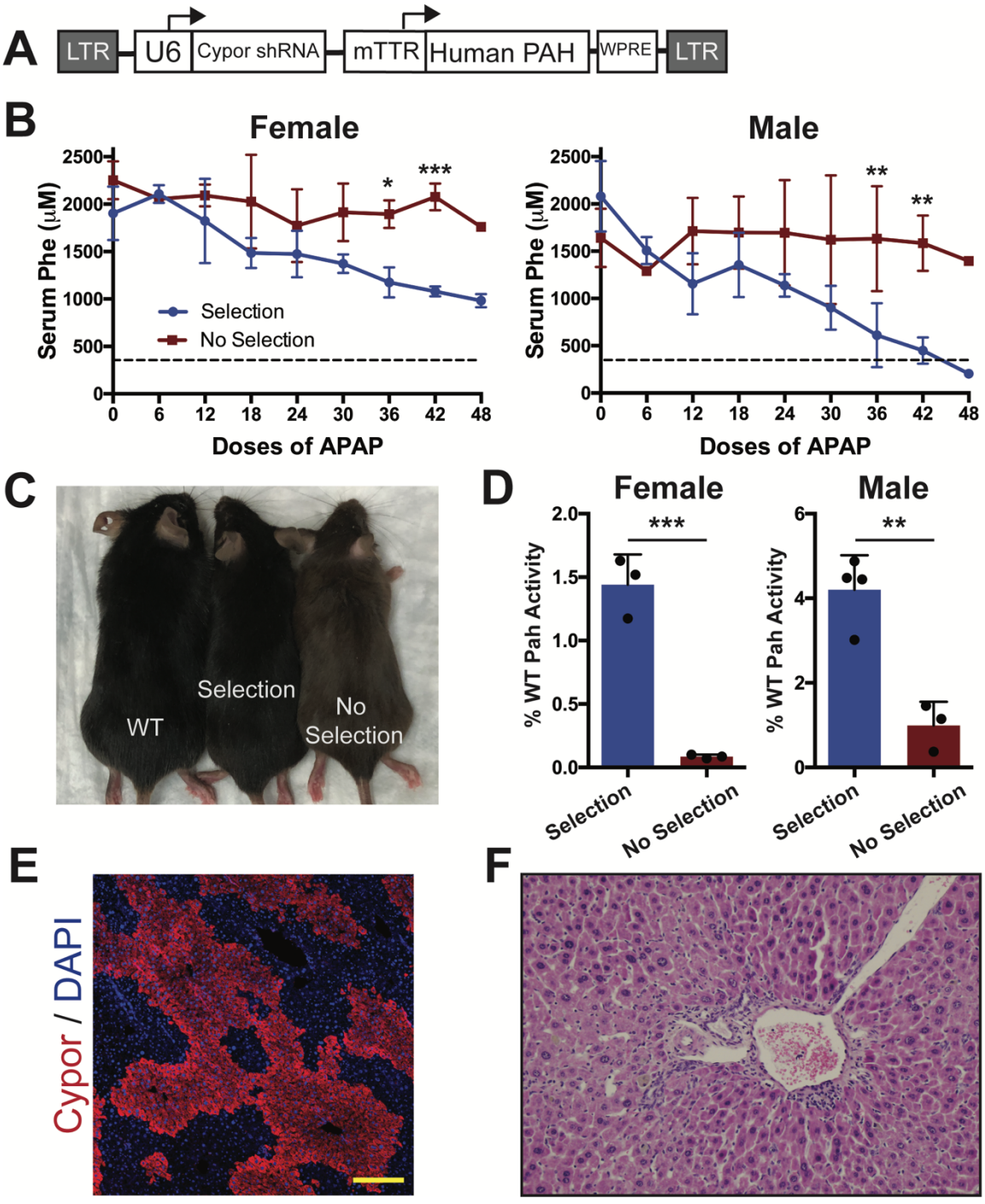
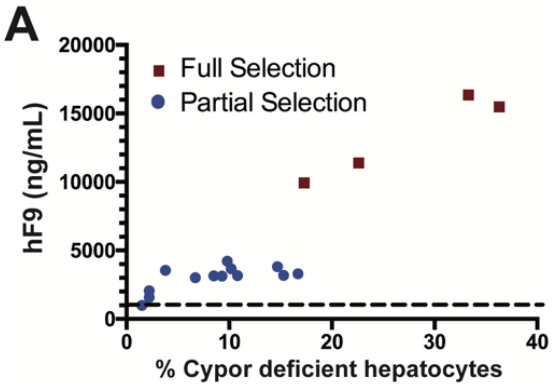
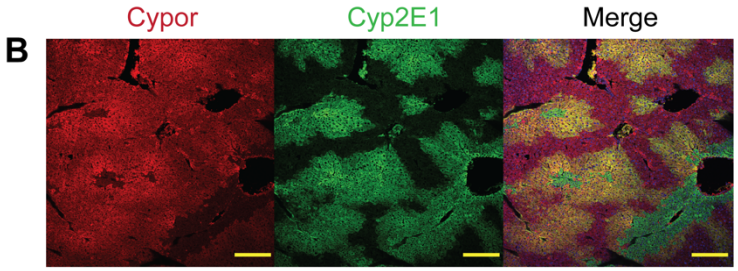


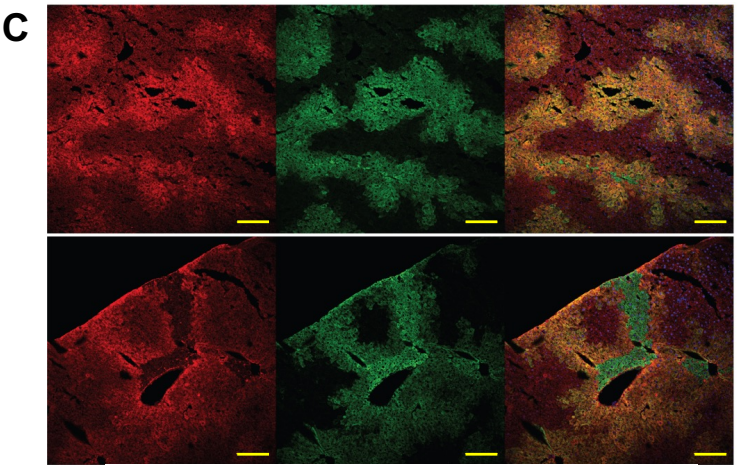
Figure 2-6. Selection of *PAH*-expressing lentiviral vectors in a PKU mouse model. (**A**) Schematic of selectable lentiviral vector expressing the *Cypor* shRNA and human *PAH* (LV-*PAH*-*Cypor* shRNA). (**B**) Serum phenylalanine concentrations in female and male *Pah*$^{enu2/enu2}$ mice. All mice received LV-*PAH*-

Cypor shRNA as neonates, followed by either APAP selection (female n = 3, male n = 4) or no further treatment (female n = 3, male n = 3). Dashed line represents the therapeutic threshold of 360 μM. *P<0.05, **P<0.01 and ***P≤0.001 by two-way ANOVA. (**C**) Coat color comparison of wild-type (WT), APAP-selected *Pah*$^{enu2/enu2}$ and unselected *Pah*$^{enu2/enu2}$ male mice. (**D**) Pah enzyme activity in liver homogenate as percentage of wild-type. **P<0.01 and ***P<0.001 by Student's one-tailed t test. (**E**) Cypor immunofluorescent staining in liver from a selected *Pah*$^{enu2/enu2}$ mouse. Scale bar, 200 μm. (**F**) Representative H&E staining of a liver section from an APAP-selected *Pah*$^{enu2/enu2}$ mouse. Data are means ± SD.

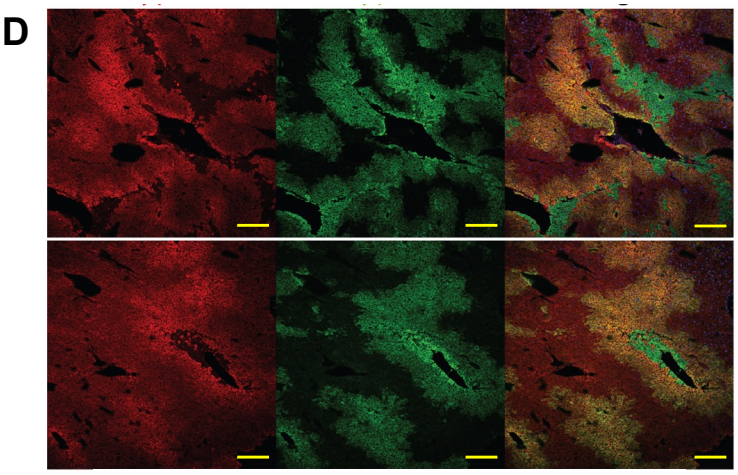




15.3% Cypor-deficient hepatocytes, hF9 = 3181 ng/mL



9.3% Cypor-deficient hepatocytes, hF9 = 3136 ng/mL



8.5% Cypor-deficient hepatocytes, hF9 = 3144 ng/mL

Figure 2-7. Partial selection of Cypor-deficient hepatocytes. (**A**) Final blood hF9 concentrations and estimated percent Cypor-deficient hepatocytes in fully (n = 4) and partially (n = 13) APAP-selected mice. Dashed line represents the therapeutic hF9 threshold. (**B - D**) Representative Cypor and Cyp2E1 immunofluorescence in three partially selected mice. Cypor/Cyp2E1 dual-positive regions appear as yellow in the merged images. The percentage of Cypor deficient hepatocytes determined by indel analysis and blood concentration of hF9 at time of harvest for each mouse are indicated below each set of images. Scale bars, 200 μm.

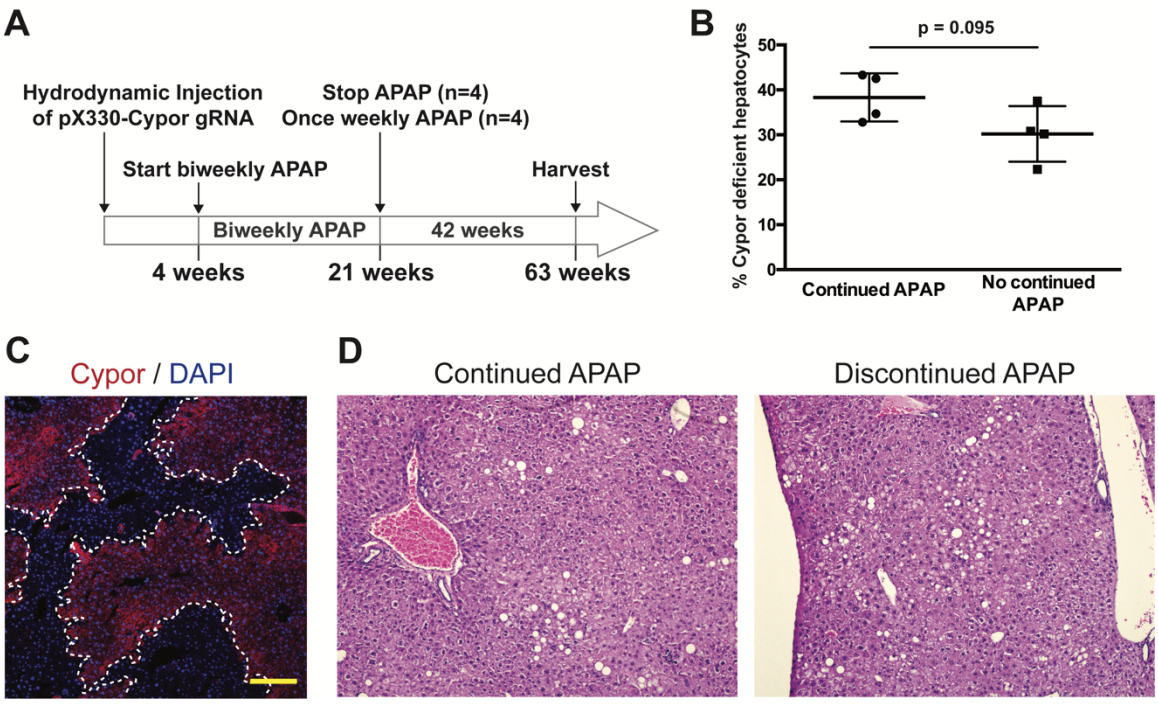


Figure 2-8. Long-term stability of Cypor-deficient hepatocytes. (**A**) Experimental timeline to test long term stability of Cypor-deficient hepatocytes. (**B**) Estimated percent Cypor-deficient hepatocytes in mice that received continued weekly APAP (n = 4) or no continued APAP (n = 4) over a 42-week period. P-value by Student's two-tailed t-test is indicated. (**C**) Representative Cypor immunofluorescence 42 weeks after the last APAP dose. Dotted lines surround Cypor-negative regions. Scale bar, 200 μm. **(D)** H&E staining of liver sections in APAP selected mice receiving either continued weekly APAP or no further APAP for 42 weeks.

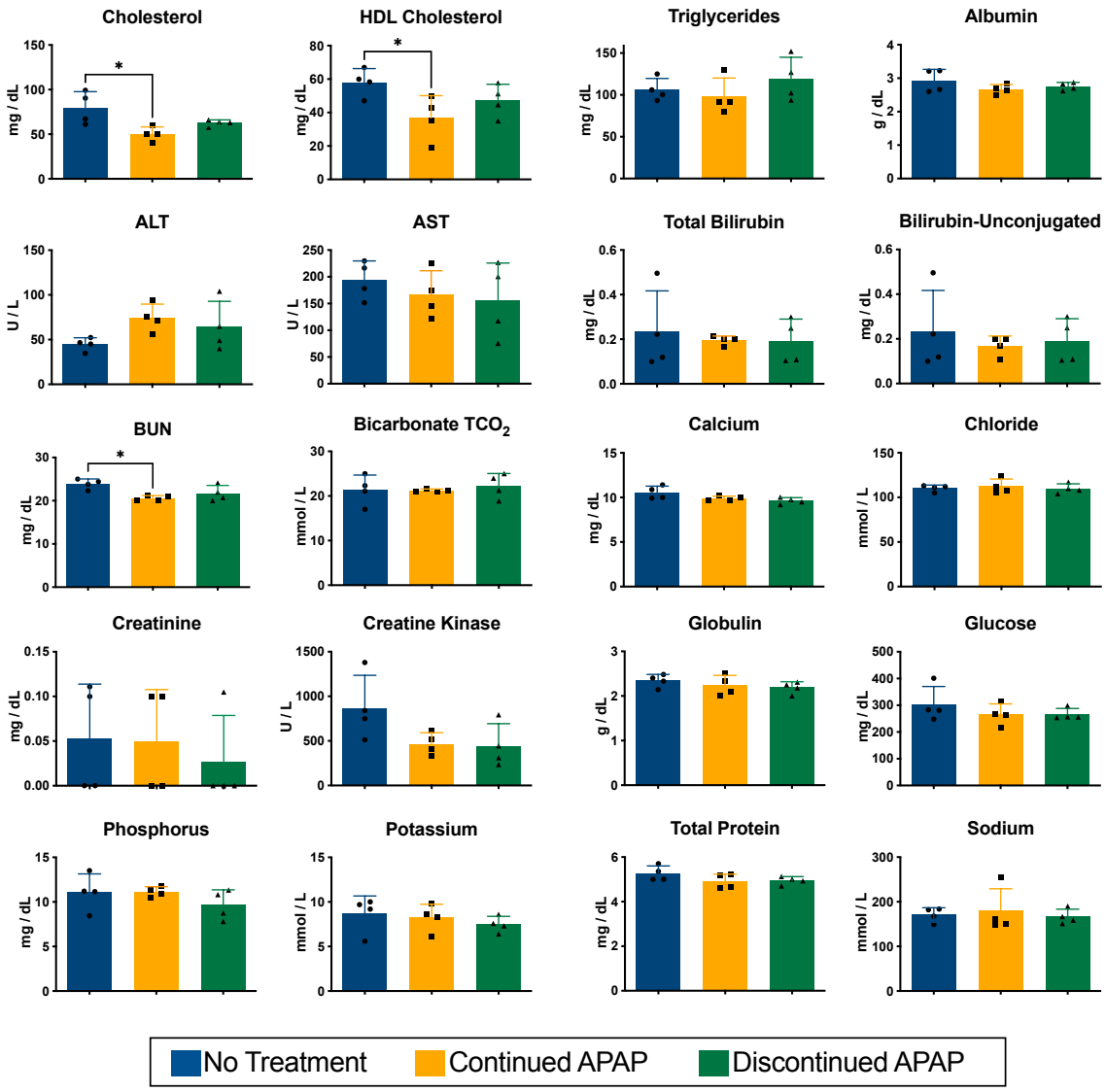


Figure 2-9. Liver and lipid panel in long-term selected mice. Results of a full liver function test and lipid panel on blood taken at terminal harvest in mice receiving continued weekly APAP or no further APAP for 42 weeks after selection and untreated controls. n = 4 for all groups. Differences are not significant unless indicated. Data are means ± SD. *P<0.05 by one-way ANOVA.

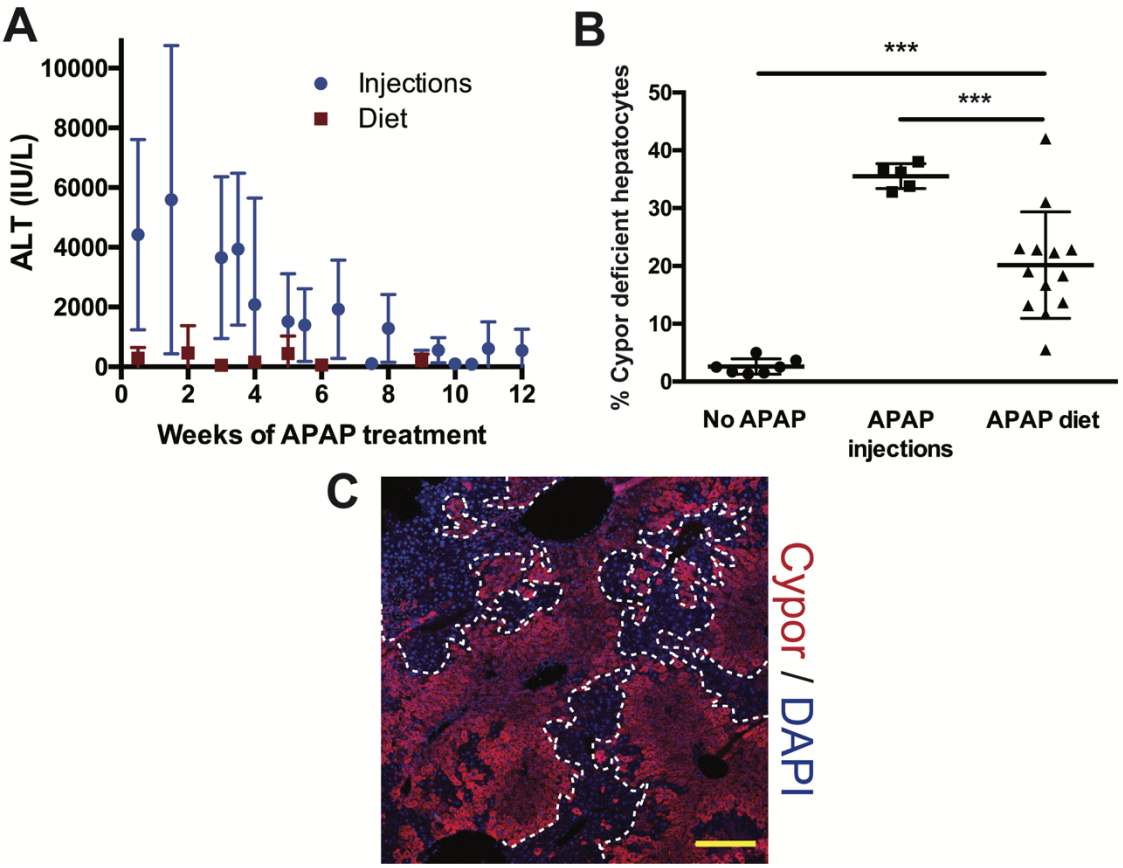


Figure 2-10. APAP selection by dietary administration. (**A**) ALT concentrations in mice receiving pX330-*Cypor* gRNA and either IP APAP (6 hours after injection) (n = 19) or on an APAP-containing diet (measured 4 hours into the light cycle) (n = 10). (**B**) Percent Cypor-deficient hepatocytes in mice treated with pX330-*Cypor* gRNA followed by no further treatment (n = 7), IP APAP injections (n = 5), or APAP diet (n = 13). ***P ≤ 0.001 by one-way ANOVA. (**C**) Representative Cypor immunofluorescence in a mouse treated with APAP diet for 6 weeks. Dotted lines surround Cypor-negative regions. Scale bar, 200 μm. Data are means ± SD.

***Cypor* shRNA transposon**	
Mouse ID	% Cypor-negative hepatocytes
A	62% (Figure 2-2B)
B	38%
C	50%
LV-*hF9-Cypor* shRNA	
Mouse ID	% Cypor-negative hepatocytes
A	14%
B	6%
C	11%
D	11%
LV-*PAH-Cypor* shRNA - Females	
Mouse ID	% Cypor-negative hepatocytes
A	45%
B	40%
C	51%
LV-*PAH-Cypor* shRNA - Males	
Mouse ID	% Cypor-negative hepatocytes
A	56%
B	43% (Figure 2-6E)
C	45%
D	50%

Table 2-1. Quantification of Cypor-negative hepatocytes by image analysis. Approximate quantification of Cypor-negative liver area by measurement of representative IF images for mice treated with *Cypor* shRNA-containing vectors and APAP selection. No APAP control animals were not quantifiable by this method.

Discussion

Here, we report robust pharmacological selection of Cypor-deficient hepatocytes in vivo by APAP administration. APAP has several properties that make it ideal as an *in vivo* hepatocyte selection drug: it is cheap and orally available; it has been used in millions of adult and pediatric patients; its mechanism of hepatoxicity is well understood; an antidote is available; toxicity is limited to only zone 3 of the hepatic lobule; and no long-term sequelae of even severe poisoning have been reported in humans [133]. Here we show that the delivery of a therapeutic gene in *cis* with knockout or knockdown of *Cypor* is an efficient method for the selection and expansion of a small population of correctly targeted hepatocytes using moderately toxic doses of APAP. This strategy allowed us to overcome some of the current major limitations of rAAV and lentiviral liver-directed gene therapy. By permanently modifying a small starting population of hepatocytes and allowing their expansion, only one viral administration at a much lower vector dose is required and problems with the low efficiency of initial transduction are eliminated. Lower vector doses will reduce immune responses and considerably reduce the cost of vector production. Although we focused on lentiviral vectors in the work reported here, APAP mediated selection of hepatocytes can be applied to any integrating vector, including transposons or rAAV vectors targeted at safe-harbor loci [42, 131]. The only requirement is that the therapeutic transgene and the *Cypor* knockdown cassette are linked in *cis*.

Selection of gene targeted hepatocytes was found to be successful in animals treated as neonates, allowing the treatment of inherited genetic disorders from birth. In contrast, rAAV remains largely episomal and is quickly lost during rapid cell division as shown in developing mouse and non-human primate livers [26, 130]. Adult animals that undergo partial hepatectomy [148] also lose over 90% of transgene expression with just 1-2 cell divisions. Hence, standard rAAV is inadequate for long term treatment of genetic liver diseases in pediatric patients. Our method enables the selection of permanently integrated therapeutic transgenes that will persist for the life of the cell. Normal liver homeostasis and injury responses are largely driven by mature hepatocytes [149] and therefore the effect of gene therapy is expected to be life-long.

Severe APAP overdoses can cause liver failure and death [132], and this type of acute liver injury is therefore a concern for the clinical applicability of APAP selection in patients. However, we show here that selection was also successful using an only moderately hepatotoxic APAP diet, and not associated with morbidity or mortality. A clinical protocol would likely involve a gradual dose-escalation with careful monitoring of the degree of liver injury and rapid intervention with the APAP antidote N-acetylcysteine [133] if transaminase concentrations exceeded a defined threshold.

An additional concern associated with our protocol is the partial deficiency of hepatic Cypor itself. Several considerations indicate that this is not likely to cause

clinical problems. First, human patients with germline mutations in *CYPOR* have the developmental disorder Antley-Bixler Syndrome [150, 151]; liver disease has not been reported as part of this condition. Second, liver-specific *Cypor* knockout mice show normal development and reproduction, although they show impaired drug metabolism and hepatic lipid accumulation [152, 153]. Third, selection of Cypor-deficient hepatocytes with APAP is strictly limited to zone 3 hepatocytes as the Cyp enzymes required for APAP metabolism are expressed only in this zone. Therefore, zone 1 and 2 hepatocytes retain normal Cypor activity. Last, we demonstrated here that partial selection of only a fraction of zone 3 hepatocytes is possible (Fig. 5B). Human F9 concentrations in the fully therapeutic range were achieved while retaining intact Cyp metabolism in >50% of zone 3 hepatocytes.

If partial Cypor deficiency is found to be poorly tolerated, a possible alternative strategy is to target the specific the specific Cyp enzymes responsible for APAP metabolism. We demonstrated that dual knockout of *Cyp2E1* and *Cyp1A2* is sufficient for selection up to 20% of the liver. Additional knockout of the third APAP metabolizing Cyp enzyme, *Cyp3A4*, may allow this strategy to be improved. As loss of Cypor prevents the functionality of all Cyp enzymes, dual or triple Cyp-targeting would be expected to have less of an effect on normal liver function, despite the increased number of genetic modifications needed.

Long-term stability is another important, positive feature of this system. Cypor-deficient hepatocytes persisted for the entire duration of our long-term follow up, up to 42 weeks, without requiring selective pressure. This indicates that Cypor deficiency does not cause hepatocyte injury or cell loss. Additionally, liver function tests and blood lipid tests were normal in animals that had undergone APAP selection and allowed to recover. Although additional investigations into the effects of partial Cypor deficiency in larger animals are necessary, together these findings are promising for the safe utilization of this selection strategy in human gene therapy.

In addition to demonstrating proof-of-principle for the secreted protein F9, we were also successful in treating PKU in male mice. The serum concentration of toxic phenylalanine was reduced from ~2,000 μM to as low as 204 μM, below the therapeutic target of 360 μM. Although the lowest Phe concentrations in two of four male mice treated with APAP selection were ~500 μM, this is within the range considered acceptable for PKU patients treated with dietary therapy. Despite evidence of extensive selection, we were not able to achieve a therapeutic correction in female PKU mice. This sexual dimorphism in treating *Pah*$^{enu2/enu2}$ mice has previously been described [154]. The threshold for therapeutic correction in male mice was higher than expected at around 40-50% of hepatocytes, likely due to the dominant negative effect of mutant Pah subunits in the *Pah*$^{enu2/enu2}$ mouse model [116, 155]. Pah is a homo-tetrameric enzyme and thus the wild-type protein generated by gene therapy needs to “crowd out”

the endogenous mutant Pah protein, if present, to generate sufficient amounts of non-mutant homo-tetramers to provide enzyme activity. Much higher doses of rAAV than are typical for liver directed gene transfer were needed to achieve correction of hyperphenylalaninemia in the *Pah*$^{enu2/enu2}$ model, particularly in female mice [154]. Hence, our results are those expected for this model. In a recently reported PKU null mouse [116] obtained by a CRISPR-induced deletion of exon 1, substantially lower doses of rAAV corrected blood phenylalanine concentrations when compared to the *Pah*$^{enu2/enu2}$ model.

Here, two distinct approaches to rendering hepatocytes Cypor deficient were used. The CRISPR/Cas9 gene knockout approach has the advantage of creating a complete enzyme deficiency and entirely preventing APAP metabolism by Cyp enzymes. However, this method requires co-administration of Cas9, i.e., use of a second agent, be it a Cas9 rAAV or nanoparticle. This need for two drugs complicates regulatory approval. In addition, Cas9 is immunogenic [143] and can cause off-target genome cutting [156]. shRNA mediated knockdown of Cypor avoids these issues. However, it is inherently less efficient in reducing enzyme activity because gene expression is only knocked down, not knocked out.

Liver-directed lentiviral gene therapy has yet to be applied in human patients, even in severe disorders that require liver transplantation. One of the main reasons is the difficulty in producing clinical scale vector doses at reasonable cost. The in vivo selection methodology described here could potentially

overcome this barrier and allow gene therapy to replace orthotopic liver transplantation in liver disorders not amenable to standard rAAV therapy. Examples of such conditions includes disorders that require intervention in early childhood such as Crigler-Najjar syndrome, urea cycle disorders, genetic cholestasis diseases, and severe organic acidemias. Liver gene therapy with lentiviral vectors could also enable the treatment of hemophilia in pediatric patients [139] without concerns about transgene loss by normal liver growth. Risk/benefit considerations will be important in deciding whether transient APAP toxicity and potentially permanent changes in Cyp-mediated drug metabolism are justifiable vis-à-vis the high unmet therapeutic need in these disorders and the risks inherent to orthotopic liver transplantation.

Chapter 3:

Complete Correction of Murine Phenylketonuria by Acetaminophen Selection of Transplanted Hepatocytes

Abstract

Hepatocyte transplantation for genetic liver diseases has several potential advantages over gene therapy. However, low efficiency of cell engraftment has limited its clinical implementation. This problem could be overcome by selectively expanding transplanted donor cells until they replace enough of the liver mass to achieve therapeutic benefit. We previously described a gene therapy method to selectively expand hepatocytes deficient in cytochrome p450 reductase (Cypor) using acetaminophen (APAP). Because Cypor is required for the transformation of APAP to a hepatotoxic metabolite, Cypor deficient cells are protected from toxicity and are able to expand following APAP-induced liver injury. Here, we apply this selection system to correct a mouse model of phenylketonuria (PKU) by cell transplantation. Hepatocytes from a wild-type donor animal were edited in vitro to create Cypor deficiency and then transplanted into PKU mice. Following selection with APAP, blood phenylalanine concentrations were fully normalized and remained stable following APAP withdrawal. Cypor-deficient hepatocytes expanded from <1% to ~14% in corrected animals, and they showed no abnormalities in blood chemistries, liver histology, or drug metabolism. We conclude that APAP-mediated selection of transplanted hepatocytes is a potential therapeutic for PKU with long-term efficacy and a favorable safety profile.

Introduction

Hepatocyte transplantation is a strategy that represents a potential therapeutic approach for any genetic disorder affecting the liver [44]. Hepatocytes are delivered into the portal circulation, usually by catheterization of the portal or umbilical veins [44]. Hepatocytes then engraft in the liver and support liver function long-term. Hepatocyte transplantation has been applied clinically in over 100 human patients with a variety of inborn and acquired liver disorders, in both adult and pediatric patients [157, 158]. Although partial phenotypic correction has been described in numerous disorders [44, 159, 160], complete and sustained correction of inborn errors of metabolism has not yet been reported. This lack of complete efficacy is likely due to the low efficiency of engraftment of the delivered cells. Only approximately 1% of the recipient's liver mass can be replaced through standard transplantation methodology, which is insufficient for complete phenotypic correction for the majority of disorders [161]. This limitation could be overcome by providing a selective advantage to allow cell division of the transplanted donor cells until they make up a sufficient percentage of the hepatic mass to allow disease correction. This threshold is estimated to be about 10% for several genetic liver conditions [44].

We have previously reported a robust system for the selective expansion of hepatocytes that were genetically modified in vivo using gene therapy methods [162]. This system relies upon the use of the common fever and pain medication

acetaminophen (APAP) as a selection agent. APAP is metabolized in hepatocytes through several metabolic pathways. Although a majority of APAP is directly converted into non-toxic metabolites, a minority is acted upon by cytochrome p450 enzymes (Cyps) in hepatocytes in zone 3 of the hepatic lobule to form an electrophilic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [163]. NAPQI is hepatotoxic at high concentrations, depleting hepatocytes of glutathione. The Cyps involved in this process require a cofactor, cytochrome p450 reductase (Cypor) [90]. Thus, Cypor knockout prevents Cyp-mediated metabolism and renders targeted cells resistant to APAP-induced hepatotoxicity. We showed in prior work that hepatocytes made Cypor deficient by CRISPR gene editing can be expanded over 100-fold to > 40% of the hepatic mass in vivo by APAP treatment [162, 164].

Here, we apply the APAP selection approach to the selective expansion of transplanted hepatocytes in mouse models of phenylketonuria (PKU). PKU is caused by a deficiency in phenylalanine hydroxylase (Pah). *Pah* is normally expressed in hepatocytes, and it functions to convert dietary phenylalanine (Phe) into tyrosine. In the absence of Pah, high circulating blood Phe levels lead to a severe neurologic phenotype. PKU patients must maintain a severely protein-restricted diet throughout their lifetime to avoid intellectual disability [110]. If blood Phe levels are maintained at ≤360 μM by dietary restriction or alternate therapy, neurologic symptoms are largely avoided [110]. Several human hepatocyte transplantation trials have shown partial correction of PKU, but complete

correction has not yet been achieved [71, 119]. Studies in mouse models have demonstrated that approximately 10% replacement of the liver with donor hepatocytes is sufficient for correction of blood Phe levels [120, 121]. Here, we demonstrate that APAP selection of transplanted hepatocytes can achieve the therapeutic cell replacement threshold for PKU and provide complete and long-term correction of hyperphenylalaninemia.

Materials and Methods

Cypor knockout in vitro and cell transplantation

Hepatocytes were isolated from 6 -12-week-old female mTmG mice on a C57BL/6 background by collagenase perfusion as previously described [164]. A chemically modified synthetic single guide RNA (sgRNA) (sequence: 5'-UCGUGGGGGUCCUGACCUAC-3') targeting the mouse Cypor gene was obtained (Synthego). Along with SpCas9 protein (Integrated DNA Technologies), sgRNA was delivered to primary hepatocytes using the CRISPRMAX reagent (Thermo Fisher Scientific) with modifications to manufacturer protocol. Briefly, the assembled ribonucleoprotein complexes were added to non-attached primary hepatocytes in suspension at $1x10^6$ cells/mL in Hepatocyte Culture Medium (Lonza) in suspension culture 6 well plates. Cells were incubated with rocking at 37°C for 2 hours before transplantation. Hepatocyte transplantation was performed by injecting $5x10^5$ hepatocytes into the spleen. An aliquot of transfected cells was plated on a collagen-coated plate in Hepatocyte Culture Medium and harvested on day 5 post-transfection for genomic DNA (gDNA) extraction and insertion/deletion mutation (indel) assessment.

Animal husbandry

mTmG mice on the C57BL/6 background [165] (stock no. 007576) were obtained from The Jackson Laboratory. $Pah^{enu2/enu2}$ mice[115] and $Pah^{\Delta exon1/\Delta exon1}$ mice [116] on a C57BL/6 background were obtained from the Harding Lab at Oregon

Health & Science University. All animal experiments were performed according to the guidelines for animal care at Oregon Health & Science University (protocol TR02-IP00000445). Mice were housed in a Thoren caging system with ¼" pelleted cellulose bedding (BioFresh) in an animal room maintained at 70°F. All animals were fed tap water and standard mouse chow (LabDiet PicoLab Rodent Diet 5LOD; ≥23% protein) ad libitum unless otherwise stated. Animals were housed under a standard 12-hour light-dark cycle. All interventions and animal handling were conducted during the light cycle. The APAP diet was prepared by dissolving APAP (Sigma-Aldrich) in 20 ml of 100% ethanol and adding 180 g of standard 5LOD diet. The chow was stirred until pellets were saturated with ethanol and then allowed to air-dry at room temperature completely before being fed to mice. All mice were started on 1.2% (w/w) APAP diet at first and transitioned to a 1.5% diet after one week. Mice were given 2 to 4 days on regular diet if weight loss exceeded 20%. Mice were fasted for 4 hours prior to harvest and were euthanized with CO_2. A cardiac puncture was performed immediately after death.

TIDE analysis of CRISPR/Cas9-induced indels

Genomic DNA extraction from homogenized liver tissue was performed using the MasterPure Complete DNA and RNA Purification Kit (Lucigen) following the manufacturer's protocol. An 800–base pair region surrounding the target site of the Cypor guide RNA (gRNA) was amplified (forward primer 5′-GTTTGCGGGTGTTAGCTCTTC-3′; reverse primer 5′-

TTGGTGGGTAAATCACACCGT-3′) using MyTaq Red Mix (Bioline). The amplicon was purified using a PCR clean-up and gel extraction kit (Macherey-Nagel) and Sanger sequenced using the forward primer. Indels were analyzed using TIDE analysis (https://tide.nki.nl) [166]. The percentage of Cypor-deficient hepatocytes was estimated assuming that hepatocytes account for 60% of total liver DNA (percent indels ÷ 0.6 = percent Cypor-deficient hepatocytes).

Histology

Approximately 5 mm-thick cross-sections of liver tissue were fixed in 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 4 hours or at 4°C overnight. Liver slices were then passed through a sucrose gradient consisting of 10%, 20%, and 30% sucrose (w/v) in phosphate-buffered saline (PBS). Tissues were embedded in optimal cutting temperature compound, and 7 μm sections were cut using a cryostat onto Colorfrost Plus slides (Thermo Fisher Scientific). Sections were permeabilized in 0.25% Triton X-100 in PBS at room temperature for 12 minutes, washed 3× 5 minutes in PBS, and then blocked in 10% normal donkey serum in PBS for 30 minutes at room temperature. Slides were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. The following primary antibodies were used: rabbit anti-Cypor (Abcam, no. 180597), rabbit anti-Cyp2E1 (Abcam, no. 28146), and rabbit anti-PAH (Boster Bio, no. A00761-1). Slides were washed 3× 5 minutes in PBS, incubated with secondary antibody (Alexa Fluor® 647-conjugated donkey anti-rabbit; Jackson Immunoresearch, no. 711-606-152) for 1 hour at room temperature, incubated in

1 μg/mL Hoechst 33342 (Invitrogen) in PBS for 3 minutes, and then washed 3× 5 minutes. Coverslips were mounted with Fluoromount-G (SouthernBiotech). Imaging was performed on a Zeiss LSM 700 confocal microscope.
H&E-stained slides were imaged on an Axioskop 2 plus microscope (Zeiss) and were assessed by an investigator blinded to the treatment groups.

Pah enzymatic activity and serum Phe

Liver Pah enzyme activity and serum Phe concentrations were determined as previously described [167]. Mice were fasted for 4 hours prior to blood collection for serum Phe assessment.

Blood Chemistry

Serum was obtained by terminal cardiac puncture and submitted to IDEXX Laboratories (www.idexx.com) for liver and lipid blood chemistry panels.

Blood Caffeine Analysis

Caffeine (Sigma-Aldrich) in saline was administered to mice intraperitoneally at a dose of 1 mg/kg body weight. Mice were unfasted and 3.5 hours into the daily light cycle at the time of caffeine administration. Blood was collected from the saphenous vein at 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours after injection. Serum was analyzed for caffeine by ELISA (Abcam, no. 285229) with modifications to the manufacturer's protocol. At the time of caffeine administration, mice had been off of APAP diet for 200-250 days.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 10.0.0 for Mac (GraphPad Software, La Jolla, California, www.graphpad.com). A Student's unpaired one-tailed *t* test assuming equal variance was used to analyze differences in percent Cypor-deficient hepatocytes, Pah activity, and body weight between selected and unselected mice. A Student's unpaired two-tailed *t* test assuming equal variance was used to analyze differences in blood chemistry and liver-to-body weight ratio between selected and unselected mice. To analyze differences in blood Phe levels between selected and unselected mice over a time course, a two-way repeated-measures ANOVA or mixed effects model assuming sphericity with Bonferroni multiple comparisons was used. *P* values <0.05 were considered statistically significant. Area under the curve (AUC) values for caffeine metabolism (assuming baseline = 0) were computed using GraphPad Prism, followed by a Student's unpaired two-tailed *t* test assuming equal variance. For all statistical analyses, $*P < 0.05$, $**P < 0.01$, and $***P \leq 0.001$. All error bars indicate SD.

Results

In vivo selection of mouse hepatocytes for PKU

To prepare donor cells, primary mouse hepatocytes were isolated by collagenase perfusion of the liver from a healthy adult donor mouse [164]. The donor animal was wild-type for *Pah*, hence transplanted hepatocytes were capable of Phe metabolism. They also expressed a membranous *tdTomato* marker transgene to allow for in vivo visualization of transplanted cells [165]. Hepatocytes were treated in vitro with ribonucleoproteins containing SpCas9 protein and a *Cypor*-targeting sgRNA delivered by Lipofectamine. This procedure yielded indels in 84-90% of alleles in the total population of transfected cells (Figure 3-1), as assessed by analysis of gDNA from hepatocytes that were plated after transfection and analyzed using the TIDE algorithm [166].

The treated hepatocytes were delivered to adult hyperphenylalaninemic male and female mice homozygous for the Pah^{enu2} mutation via intrasplenic injection [48]. This mouse model has a missense point mutation in the *Pah* gene that results in the production of a non-functional protein [115]. Mice were assessed for baseline blood Phe levels 2-4 weeks after transplantation and continued to have elevated blood Phe levels ≥1500 μM, indicating that the transplanted cell dose was subtherapeutic (Figure 3-2A). Mice were then placed on a selection diet containing 1.5% APAP w/w. Mice were maintained on this diet for 50 days, at which time mice were placed on standard chow for 10 days prior to being

assessed for blood Phe concentration. At this time, a subset of animals showed corrected blood Phe (≤360 μM) and APAP was discontinued in these animals. APAP-treated animals that still showed elevated blood Phe levels after 50 days of selection were placed on APAP diet for an additional 25 days before being analyzed again. At this timepoint, APAP diet was discontinued because biochemical correction had been achieved in all animals. Following APAP treatment, blood Phe concentrations decreased to within the corrected range in both male and female mice to an average of 207 μM ± 78 μM and as low as 130 μM. Blood Phe levels remained within the corrected range for more than 250 days after APAP diet was stopped (Figure 3-2A). Control animals that received cell transplantation but remained on standard diet without APAP showed highly elevated Phe levels throughout the course of the experiment. Corrected mice of both sexes also showed the characteristic darkening of coat color associated with a decrease in blood Phe (Figure 3-2B). A significant increase in body weight was also seen in corrected male mice as compared to unselected controls (Figure 3-2C).

Analysis of indels at the Cypor gRNA target locus in liver gDNA from corrected mice indicated an average of 13.6% ± 6.4% Cypor deficient hepatocytes, compared to 1.6% ± 1.0% in mice that received transplantation without APAP selection ($P < 0.0001$). Complete correction of blood Phe levels was seen in animals with a replacement index of as low as 8% (Figure 3-3A). Clonal expansion of Cypor-negative cells was observed in livers of APAP-treated

animals by immunofluorescence (IF) staining (Figure 3-3B). As expected, Cypor-negative cells were positive for membranous tdTomato, indicating that they originated from the donor mouse. Pah enzyme activity assessment showed 15.4% ± 6.7% of wild-type Pah activity in corrected *Pah*$^{enu2/enu2}$ animals compared to 0% in unselected controls ($P < 0.0001$) (Figure 3-3C).

Mice of a newly described Pah-null strain, *Pah*$^{\Delta exon1/\Delta exon1}$ [116], were also treated by hepatocyte transplantation and APAP selection with the same protocol. *Pah*$^{\Delta exon1/\Delta exon1}$ animals showed similar correction, with average post-treatment blood Phe levels of 156 μM following 50-75 days of dietary treatment (Figure 3-4A). Coat color darkening (Figure 3-4B) was also observed in these mice. Indel analysis on livers of treated animals showed an average of 15.0% ± 2.8% Cypor-deficient hepatocytes, compared to 1.2% ± 1.0% in unselected animals ($P = 0.0037$) (Figure 3-4C), and clonal expansion of Cypor-negative cells was evident by IF staining (Figure 3-4D). IF staining for Pah in the livers of corrected *Pah*$^{\Delta exon1/\Delta exon1}$ animals revealed that Pah expression co-localized with tdTomato-positive transplanted hepatocytes (Figure 3-4E). IF staining for Pah is not possible in the *Pah*$^{enu2/enu2}$ model due to presence of mutant protein. Pah enzyme activity in corrected *Pah*$^{\Delta exon1/\Delta exon1}$ mice showed an average of 17.5% ± 1.2% of wild-type Pah activity compared to an average of 0.06% in unselected mice ($P = 0.0001$) (Figure 3-4F).

Safety Outcomes

A potential safety concern for the APAP selection system is whether partial Cypor deficiency in the liver will lead to a metabolic phenotype. A mouse model of complete hepatic Cypor deficiency has been described [93, 94]. Circulating levels of cholesterol and triglycerides are decreased in this mouse, and accumulation of lipids in hepatocytes corresponds to a significant increase in liver-to-body weight ratio. Here, corrected *Pah*$^{enu2/enu2}$ mice with only ~14% Cypor-null hepatocytes (partial Cypor deficiency) showed no significant difference in blood levels of cholesterol (P = 0.41) or triglycerides (P = 0.49) compared to unselected controls (Figure 3-5A and 3-5B). Liver function markers were also measured and showed no significant differences between selected and unselected *Pah*$^{enu2/enu2}$ mice (Figure 3-6). Liver weight to body weight ratio also showed no significant difference between selected and unselected *Pah*$^{enu2/enu2}$ animals (P = 0.18) (Figure 3-5C). H&E staining of the livers of APAP selected mice revealed no distinct differences between APAP-treated and unselected animals (Figure 3-5D). Notably, while hepatic Cypor-null mice have hepatic accumulation of lipids, hepatic lipid accumulation was not seen in any cells in selected animals with partial Cypor deficiency of either the *Pah*$^{enu2/enu2}$ or the *Pah*$^{\Delta exon1/\Delta exon1}$ strain (Figure 3-5D).

Drug metabolism

Cypor deficiency in APAP selected animals is limited to cells in zone 3 of the hepatic lobule, constituting approximately one-third to one-half of all hepatocytes,

because these are the only hepatocytes that express those Cyp enzymes (Cyp2E1, Cyp1A2, and Cyp3A4) necessary for the conversion of APAP to NAPQI [163]. As zone 3 Cyp activity is important for metabolism of some xenobiotic substrates [168], it is desirable to achieve the therapeutic threshold while still retaining a population of hepatocytes in zone 3 with Cypor activity. We previously performed proof of concept experiments showing that up to 40% of the liver can be made Cypor-deficient with extensive APAP selection [162]. By comparison, corrected PKU animals with ~14% Cypor deficient hepatocytes are expected to retain more than half of residual zone 3 hepatocytes with Cypor activity. Immunofluorescent staining for Cyp2E1, one of the zone 3-expressed Cyps involved in APAP metabolism [163], confirmed that the majority of Cyp2E1-expressing cells did not overlap with the tdTomato-positive transplanted cells, indicating that most zone 3 hepatocytes retain Cypor activity (Figure 3-7A).

As a functional assay of Cypor activity, the metabolism of a Cyp-metabolized drug was assessed in selected mice. Caffeine is a well-characterized probe drug [169] for the activity of Cyp1A2, a zone 3-specific Cyp enzyme that also plays a role in the metabolism of APAP [170]. It is estimated that Cyp1A2 is responsible for >95% of caffeine metabolism [171], and mice completely lacking Cypor expression in the liver have been reported to have highly deficient caffeine elimination [95, 172]. As an assessment of zone 3 Cyp activity, caffeine was administered to a subset of corrected *Pah*$^{enu2/enu2}$ male mice (average 17% Cypor-deficient hepatocytes) and unselected *Pah*$^{enu2/enu2}$ male control animals.

Following an intraperitoneal administration of 1 mg/kg caffeine, blood was collected at 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours for assessment of caffeine levels. No significant difference was seen in AUC between selected and unselected animals (P = 0.34) (Figure 3-7B), indicating retention of adequate zone 3 Cypor activity despite partial genetic deficiency.

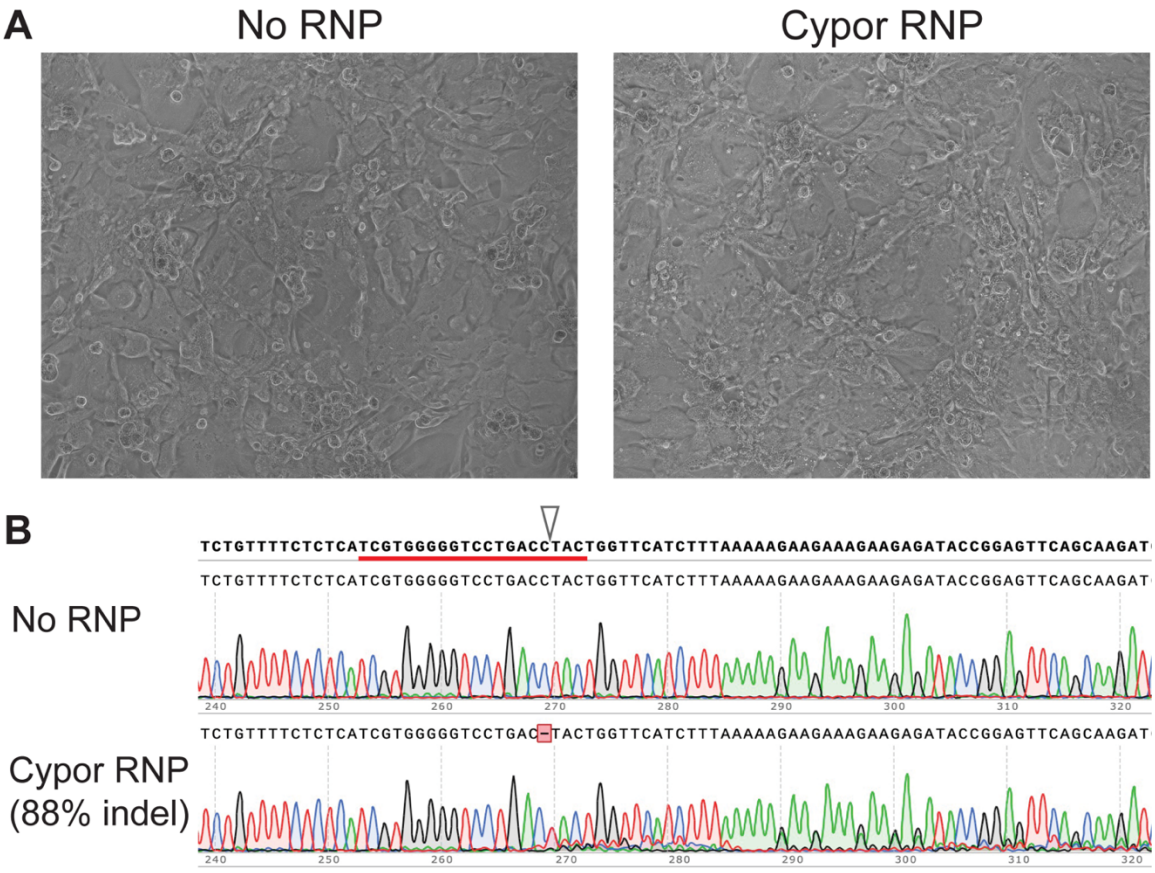


Figure 3-1: In vitro manipulation of primary mouse hepatocytes. (A) Plated untreated control and Cypor-targeting CRISPR/Cas9 ribonucleoprotein (RNP)-treated primary mouse hepatocytes on day 5 after perfusion and transfection. **(B)** Representative Sanger sequencing trace from amplicon sequencing from plated cells harvested on day 5 after transfection. gRNA target sequence is underlined, and arrow indicates expected cut site. Indel percentage, as estimated from the trace using the TIDE algorithm, is indicated**.**

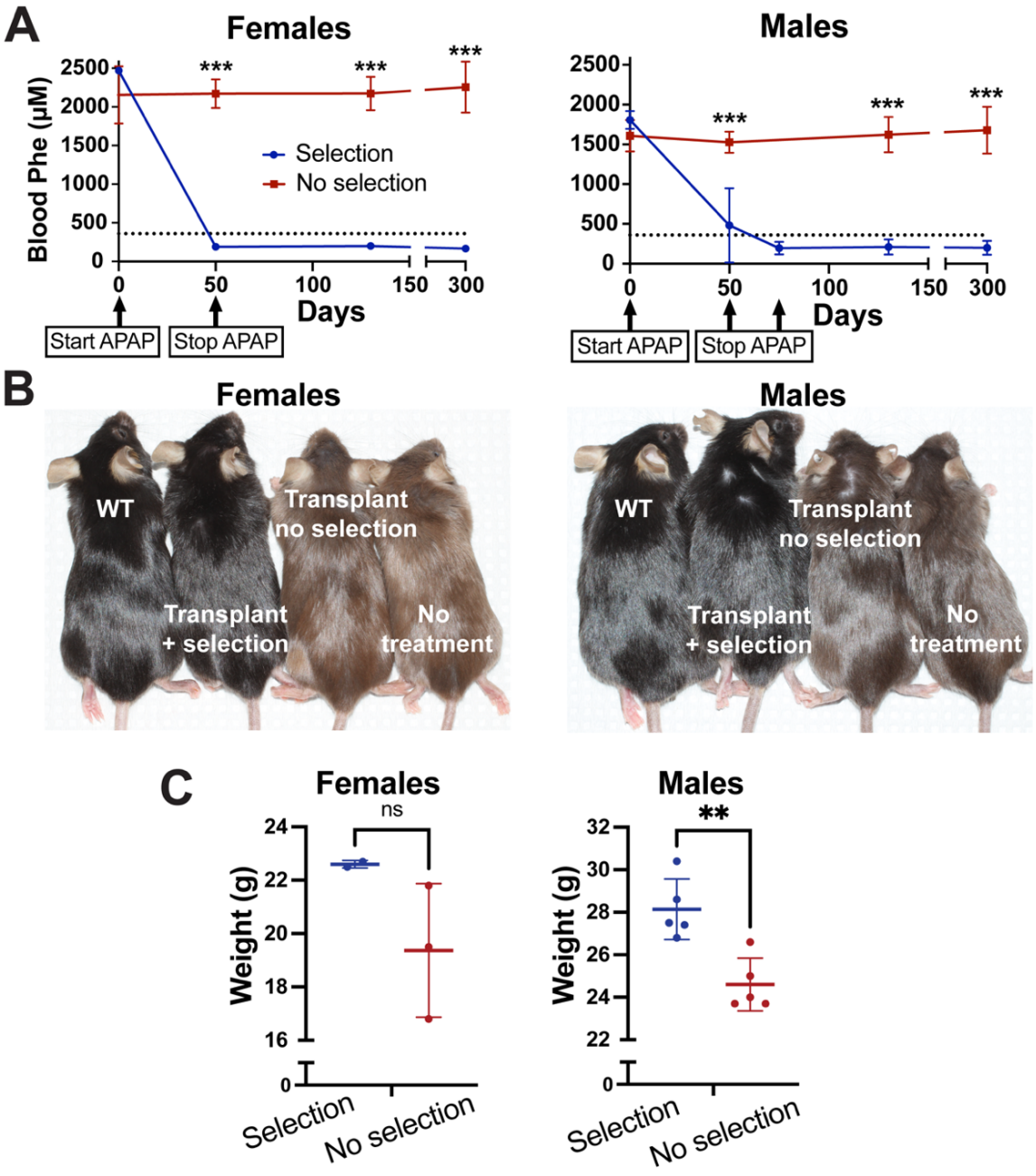


Figure 3-2: APAP selection of transplanted hepatocytes in *Pah*$^{enu2/enu2}$ mice: phenotypic analysis. (**A**) Blood Phe concentrations in male (selection n = 5; no selection n = 6) and female mice (selection n = 3; no selection n = 3) of the *Pah*$^{enu2/enu2}$ strain treated with hepatocyte transplantation. Arrows indicate when APAP diet treatment was commenced and discontinued. Dashed line indicates

therapeutic threshold of 360 μM. ***P ≤ 0.001 by two-way ANOVA. (**B**) Coat color: wild-type C57BL/6 (WT), *Pah*$^{enu2/enu2}$ treated with cell transplant and APAP selection, *Pah*$^{enu2/enu2}$ treated with cell transplant without APAP selection, and untreated *Pah*$^{enu2/enu2}$. (**C**) Body weight at time of harvest in corrected mice and unselected controls. **P<0.01, ns= not significant by Student's one-tailed t-test. Error bars indicate SD.

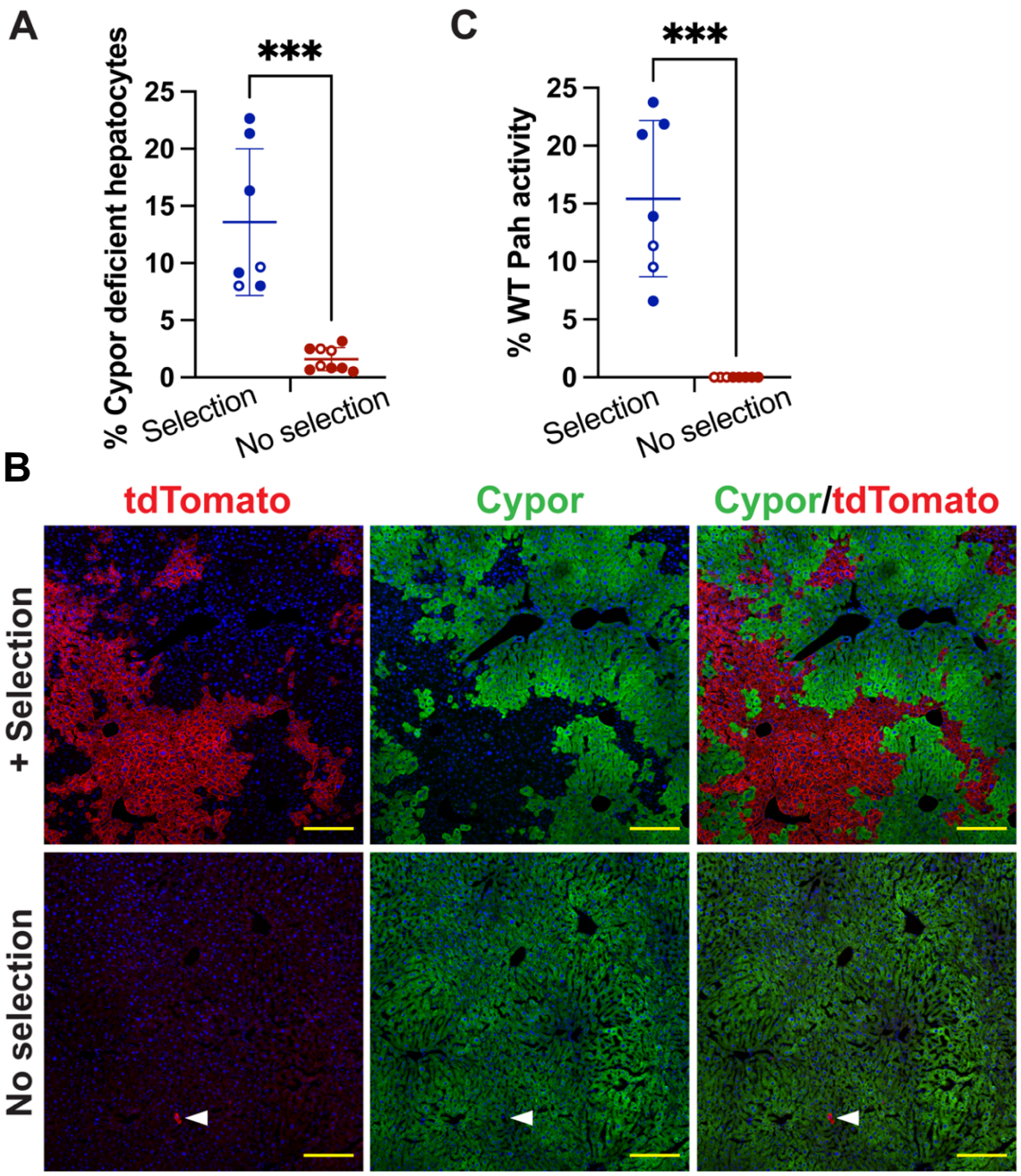


Figure 3-3: APAP selection of transplanted hepatocytes in *Pah*$^{enu2/enu2}$ mice: liver analysis. (**A**) Percent Cypor-deficient hepatocytes estimated based on indel analysis from whole liver homogenate gDNA from Phe-corrected, APAP selected *Pah*$^{enu2/enu2}$ mice (n = 7) and unselected controls (n = 9). Filled circles indicate males, unfilled circles indicate females. ***P ≤ 0.001 by Student's one-

tailed t-test. (**B**) tdTomato fluorescence (red), Cypor immunofluorescence (green), and nuclear Hoechst (blue) in liver from an APAP-selected, Phe-corrected *Pah*$^{enu2/enu2}$ mouse (top), and an unselected control (bottom). Arrowhead indicates an individual donor hepatocyte in the unselected liver. Scale bars = 200 μm. (**C**) Pah enzyme activity in liver homogenate from treated animals as a percentage of wild-type Pah activity. Filled circles indicate males, unfilled circles indicate females. ***P ≤ 0.001 by Student's one-tailed t-test. Data are reported as means ± SD.

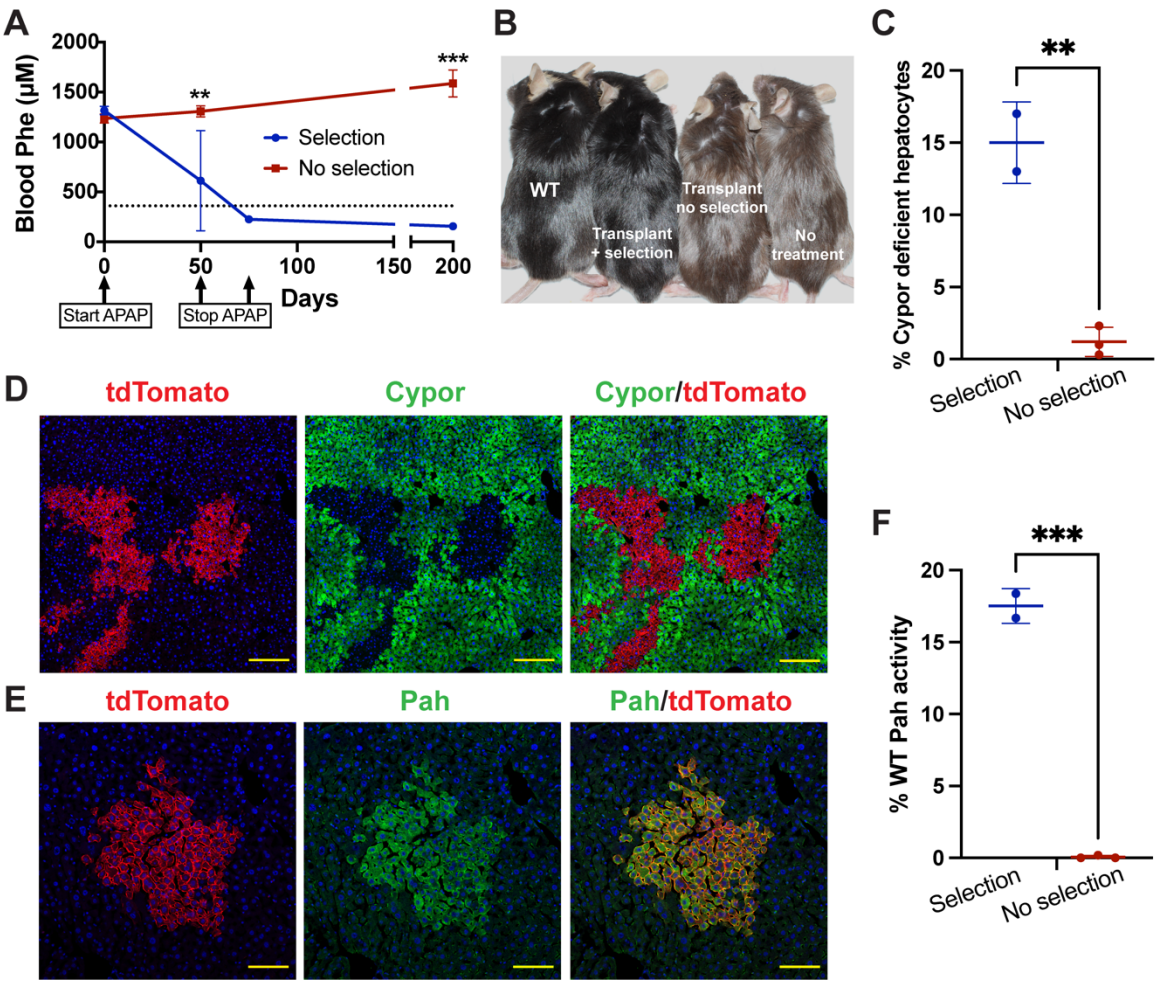


Figure 3-4: APAP selection of transplanted hepatocytes in *Pah*$^{\Delta exon1/\Delta exon1}$ mice. (**A**) Blood Phe levels in treated male mice (n = 2) and unselected controls (n = 3) of the *Pah*$^{\Delta exon1/\Delta exon1}$ strain. Dashed line indicates therapeutic threshold of 360 μM. **P<0.01 and ***P≤0.001 by two-way ANOVA. (**B**) Coat color: wild-type C57BL/6, *Pah*$^{\Delta exon1/\Delta exon1}$ treated with cell transplant and APAP selection, *Pah*$^{\Delta exon1/\Delta exon1}$ treated with cell transplant without APAP selection, and untreated *Pah*$^{\Delta exon1/\Delta exon1}$. (**C**) Indel analysis of corrected and unselected control livers. **P<0.01 by Student's one-tailed t-test. (**D**) tdTomato fluorescence (red), Cypor immunofluorescence (green), and nuclear Hoechst (blue) in liver from an APAP-

selected, Phe-corrected *Pah*$^{\Delta exon1/\Delta exon1}$ mouse. Scale bars = 200 μm. (**E**) Pah immunofluorescence (green), tdTomato fluorescence (red), and nuclear Hoechst (blue) in liver from an APAP-selected, Phe- corrected *Pah*$^{\Delta exon1/\Delta exon1}$ mouse. Scale bars = 100 μm. (**F**) Pah activity in liver homogenate from treated *Pah*$^{\Delta exon1/\Delta exon1}$ animals as a percentage of wild-type Pah activity. ***P ≤ 0.001 by Student's one-tailed t-test. All error bars indicate SD.

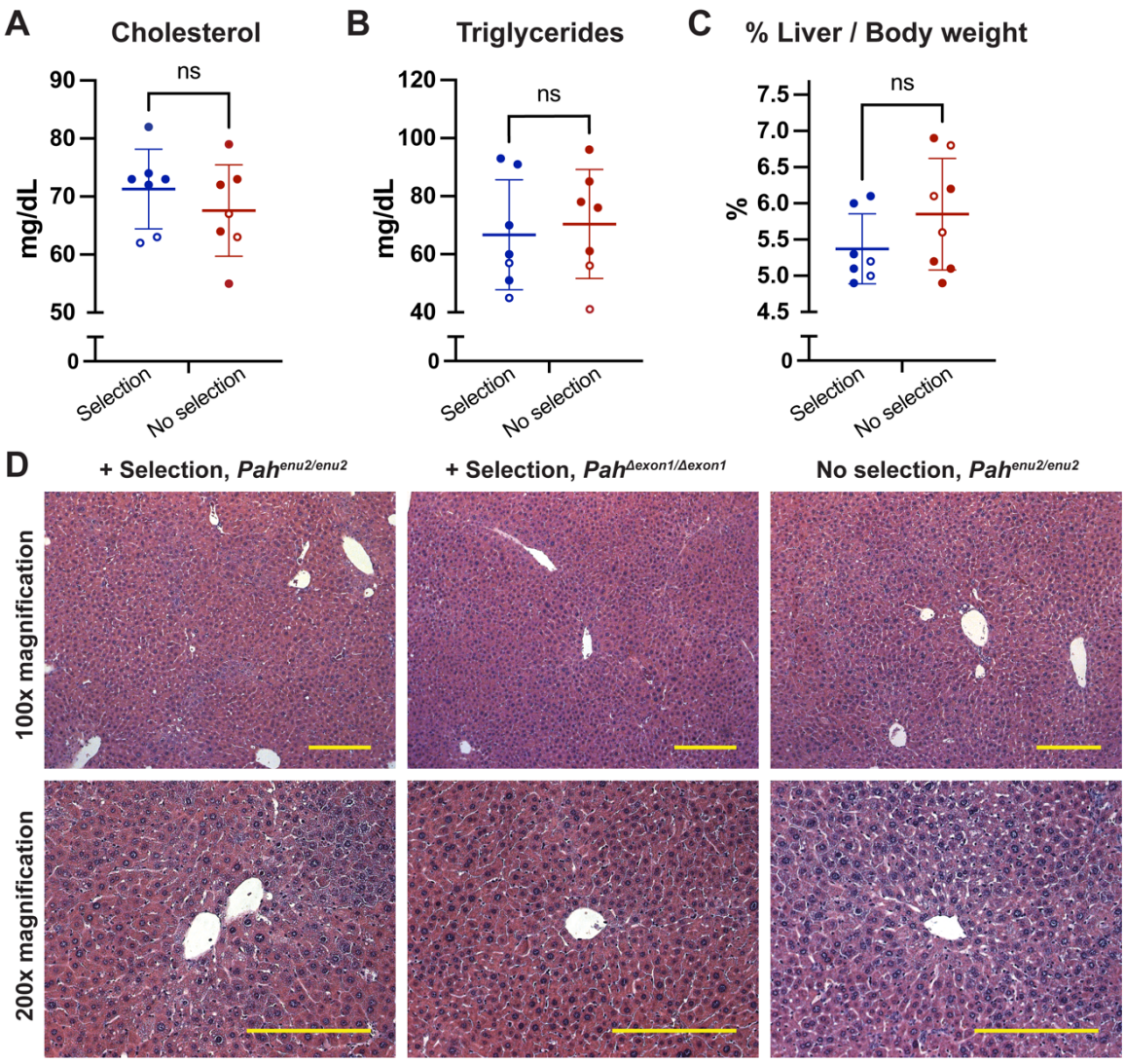


Figure 3-5: Safety assessments in corrected *Pah*$^{enu2/enu2}$ mice. (**A-B**) Blood chemistry analysis upon terminal harvest in corrected vs. unselected *Pah*$^{enu2/enu2}$ animals: (**A**) Cholesterol (**B**) Triglycerides. (**C**) Liver weight as percent of body weight in corrected vs. unselected *Pah*$^{enu2/enu2}$ animals. Filled circles represent males, unfilled circles represent females. ns= not significant by Student's two-tailed t-test. Data are means ± SD. (**D**) Representative H&E staining in a corrected *Pah*$^{enu2/enu2}$ animal, a corrected *Pah* $^{\Delta exon1/\Delta exon1}$ animal, and an unselected control *Pah*$^{enu2/enu2}$ animal. Scale bar = 200 μm.

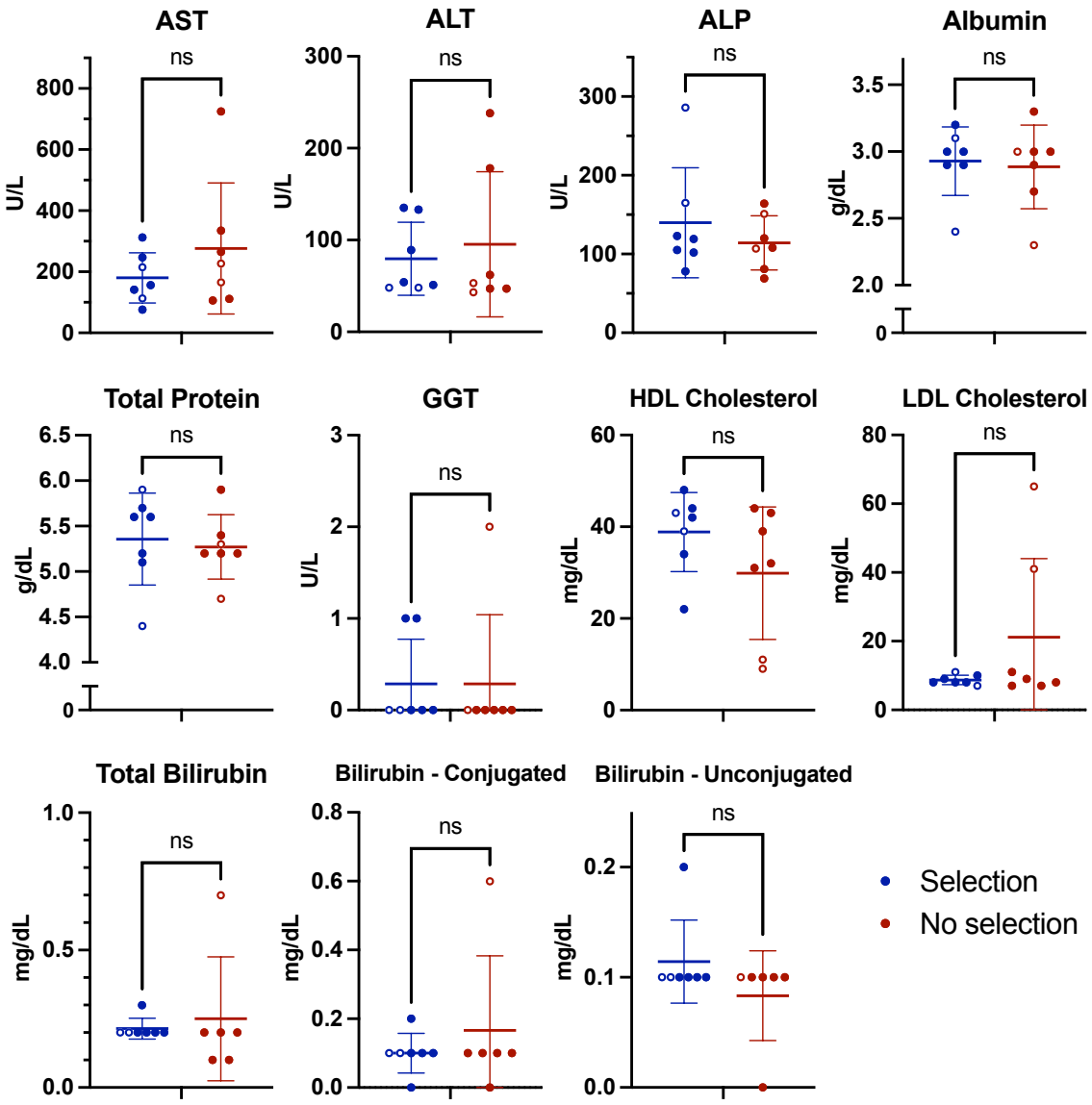


Figure 3-6: Blood Chemistry of Corrected *Pah*$^{enu2/enu2}$ mice. Liver function and lipid analysis on blood taken at terminal harvest from corrected *Pah*$^{enu2/enu2}$ animals and unselected controls. Filled circles indicate males, unfilled circles indicate females. $P > 0.15$ for all analyses by Student's two-tailed t-test. All error bars indicate SD.

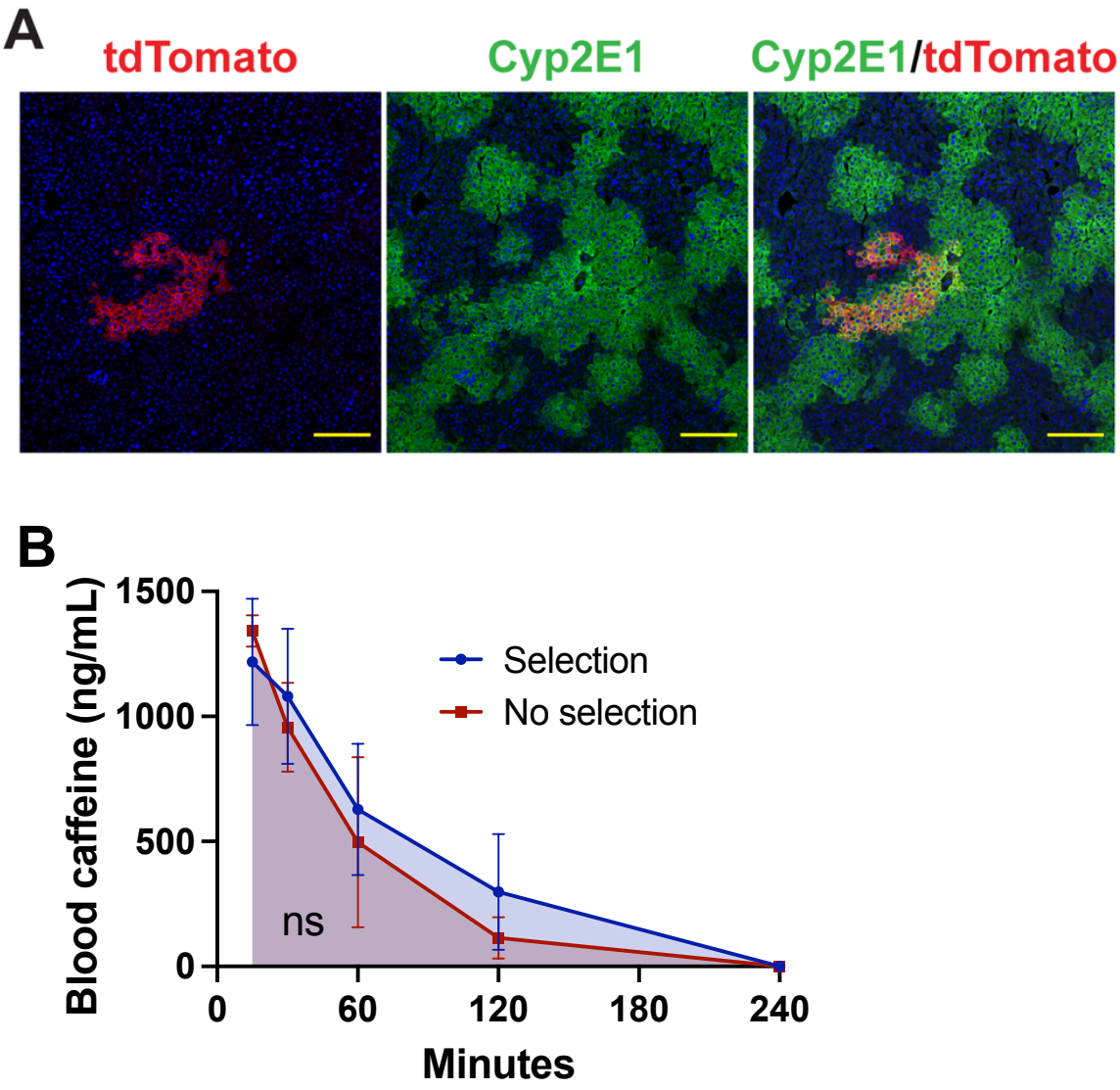


Figure 3-7: Drug metabolism in corrected *Pah*$^{enu2/enu2}$ mice. (**A**) tdTomato fluorescence (red), immunofluorescent staining for Cyp2E1 (green), and nuclear Hoechst (blue) in a corrected *Pah*$^{enu2/enu2}$ animal. Scale bars = 200 μm. (**B**) Blood caffeine concentration over time in corrected male *Pah*$^{enu2/enu2}$ mice (n = 4) and unselected control animals (n = 3). Caffeine was administered at a dose of 1 mg/kg at timepoint 0. ns = not significant by Student's two-tailed t-test (AUC). Data are reported as means ± SD.

Discussion

Here, we report complete, long-lasting correction of hyperphenylalaninemia in two different mouse models of PKU via selective expansion of transplanted donor hepatocytes. Hepatocyte transplantation carries several potential advantages as compared to viral gene therapy methods, which are already used clinically. First and foremost, wild-type hepatocytes represent a single “drug” for many different genetically distinct hepatic disorders. The donor cell expansion strategy presented here is likely to be applicable to any genetic disorder that can be corrected by expression of the wild-type gene in approximately 40% or less of hepatocytes. In contrast, viral gene therapy requires customization of genetic cargo to each specific disorder, and each viral vector must go through clinical trials and be approved as a novel therapeutic. The versatility of this single therapeutic strategy may be of particular benefit for very rare disorders for which development of a novel drug for very few patients may not be economically feasible. In addition, concerns related to activity of the viral vectors in off-target tissue types are avoided, as are potentially life-threatening immune responses to high doses of viral vectors [173]. While immune responses to viral vectors typically prevents redosing due to the formation of high levels of neutralizing antibodies to the viral capsid [174], repeat administration of hepatocytes is feasible and has been done successfully in a clinical setting [175]. Applications of hepatocyte transplantation in humans have shown a favorable safety profile, including in very young pediatric patients [44, 161]. While episomal rAAV gene

therapies have only transient efficacy in the pediatric liver due to episome loss with cell division [26, 176], transplanted hepatocytes are expected to divide with the growing liver and provide life-long benefit.

Despite its potential advantages, clinical application of hepatocyte transplantation has lagged behind gene therapy approaches due to three major obstacles: low cell engraftment levels, lack of donor material, and allogeneic rejection. The selection strategy presented here represents a way to overcome the problem of the low cell replacement index. Our technique may also help to overcome challenges with availability of donor material because in vivo expansion allows a therapeutic effect to be achieved with lower amounts of starting material. Additionally, animal bioreactors are being developed to massively expand primary hepatocytes for transplantation [177, 178]. To overcome immunological barriers to allogeneic cell transplantation, approaches have already been developed to create universal donor T cells and induced pluripotent stem cells (iPSCs) by genome engineering of major histocompatibility complex (MHC) genes [179-181]. It is likely that a similar method will be applicable to hepatocytes to allow creation of off-the-shelf universal donor cells. Combined with our selection strategy, we envision that these approaches will allow clinical implementation of hepatocyte transplantation for a variety of disease indications.

In the previous chapter, we achieved near-complete correction of blood Phe in male *Pah*$^{enu2/enu2}$ animals and incomplete correction in female animals by

expanding hepatocytes harboring a PAH-expressing lentivirus to >40% of the liver by APAP selection [162]. In contrast, we were able to obtain complete correction of Phe levels here in both male and female *Pah*$^{enu2/enu2}$ mice following selective expansion of Pah-expressing transplanted hepatocytes with a replacement index as low as 8% of total hepatocytes. The difference in the percentage of Pah-expressing hepatocytes needed to achieve correction is likely to be due to the known dominant negative effect of mutant Pah monomers. In our lentiviral gene therapy work [162], Pah enzyme activity was less than 5% of wild-type although >40% of hepatocytes expressed Pah. In contrast, much higher Pah activity was achieved here, and the enzyme activity correlated very well with the cell replacement index (~15%) in our cell transplantation experiments. Pah functions as a homotetramer, and the *Pah*$^{enu2/enu2}$ mouse expresses a mutant protein product that can assemble with the wild-type protein and inhibit the activity of the tetramer [182]. It is currently unknown how many human *PAH* mutations may be similarly problematic for gene therapy. This dominant interference mechanism does not occur in cell therapy, because mutant and wild-type Pah reside in separate cells. The similar cell replacement threshold required to correct two different PKU mouse models highlights the mutation-agnostic nature of the hepatocyte transplantation strategy.

A concern for the safety of our APAP selection system is that the partial CYPOR deficiency could result in unintended consequences. Humans with germline CYPOR deficiency are affected by Antley-Bixler syndrome. The primary

phenotype of this condition results from disordered skeletal development and steroid synthesis in extrahepatic tissues, and affected patients do not have a reported liver phenotype [183]. Although decreased rates of metabolism of Cyp-metabolized drugs have been reported in affected patients [184], our experiments indicate that the limited Cypor deficiency created by APAP selection will not have a significant effect on drug metabolism. As human Cyp metabolism is already highly polymorphic [185], slight fluctuations are not likely to create significant medical risk.

Mice with a complete hepatic deficiency of Cypor [93, 94] have been shown to develop and reproduce normally. None of the physiologic changes that have been reported in these mice, including alterations to hepatic and circulating lipid levels, were seen in corrected PKU mice with partial Cypor deficiency. Of the Cyp-metabolized drugs that have been studied in this mouse model, caffeine is among the most affected [95]. Here, AUC values for caffeine metabolism were not significantly altered as compared to unselected controls, demonstrating the metabolic safety of a limited number of hepatocytes being Cypor deficient.

As zone 1 and 2 hepatocytes are not susceptible to APAP-induced liver injury, completely ablating hepatic Cyp metabolism is not possible with our selection system. Furthermore, most major drug-metabolizing Cyps have intestinal as well as hepatic expression [186], representing another reservoir of Cyp activity that is not susceptible to depletion by APAP selection.

APAP selection was accomplished using gradual APAP administration via dietary consumption. This protocol is advantageous as it accomplishes selective expansion of hepatocytes while avoiding acute liver injury associated with APAP administration by injection [162]. We envision that a clinical protocol would involve a similarly gradual APAP administration regimen. If ALT responses exceed an acceptable threshold, intervention with the antidote N-acetyl cystine would be available.

Taken together, the data presented here, indicate that APAP selection of transplanted hepatocytes is efficacious and safe in mice and that the technology has significant potential for clinical development.

CHAPTER 4

Conclusions and Further Directions

Use of the APAP selection system for precise gene editing

In order for APAP selection to be used with viral gene therapy, an integrating viral vector is needed. Selective expansion necessitates cell division, meaning that episomal vectors would be rapidly lost. The viral gene therapy experiments described in Chapter 2 used a randomly integrating lentiviral vector. Despite decreased concerns of insertional mutagenesis with lentiviral vectors compared to many other technologies, any integrating viral vector carrying a promoter can theoretically cause oncogene activation. Another line of investigation in the Grompe Laboratory, led by Dr. Amita Tiyaboonchai, expanded the applications of the APAP selection system to include promoterless rAAV vectors with targeted integration into the mouse albumin (*Alb*) locus [164]. An rAAV vector was created with arms of homology targeting integration to immediately downstream of the *Alb* protein coding sequence. A P2A ribosomal skipping sequence allowed expression of a *hF9* transgene from the endogenous *Alb* protomer, making the vector promoterless. In addition, the Cypor gRNA was present in the vector flanked by ribozyme sequences to allow a "self-cleaving" version of the gRNA to be expressed from the same cellular promoter (Figure 4-1A). Importantly, this approach ensures that hepatocytes become APAP resistant only after precise homologous recombination as proper integration is required for the transcription of the gRNA. This vector was delivered to neonatal wild-type mice along with a second (non-integrating) rAAV vector encoding SpCas9. Acetaminophen selection allowed up to 15% liver repopulation by precisely edited hepatocytes, and a 300-fold increase in hF9 levels to within the therapeutic range (Figure 4-1B

- D). As compared to lentiviral gene therapy, this integrating rAAV approach has decreased potential for insertional mutagenesis but increased challenges with efficiency. The need for co-administration of Cas9 also is a downside of this method. Nonetheless, this approach expands the gene therapy “tool box” for applications of the APAP selection system.

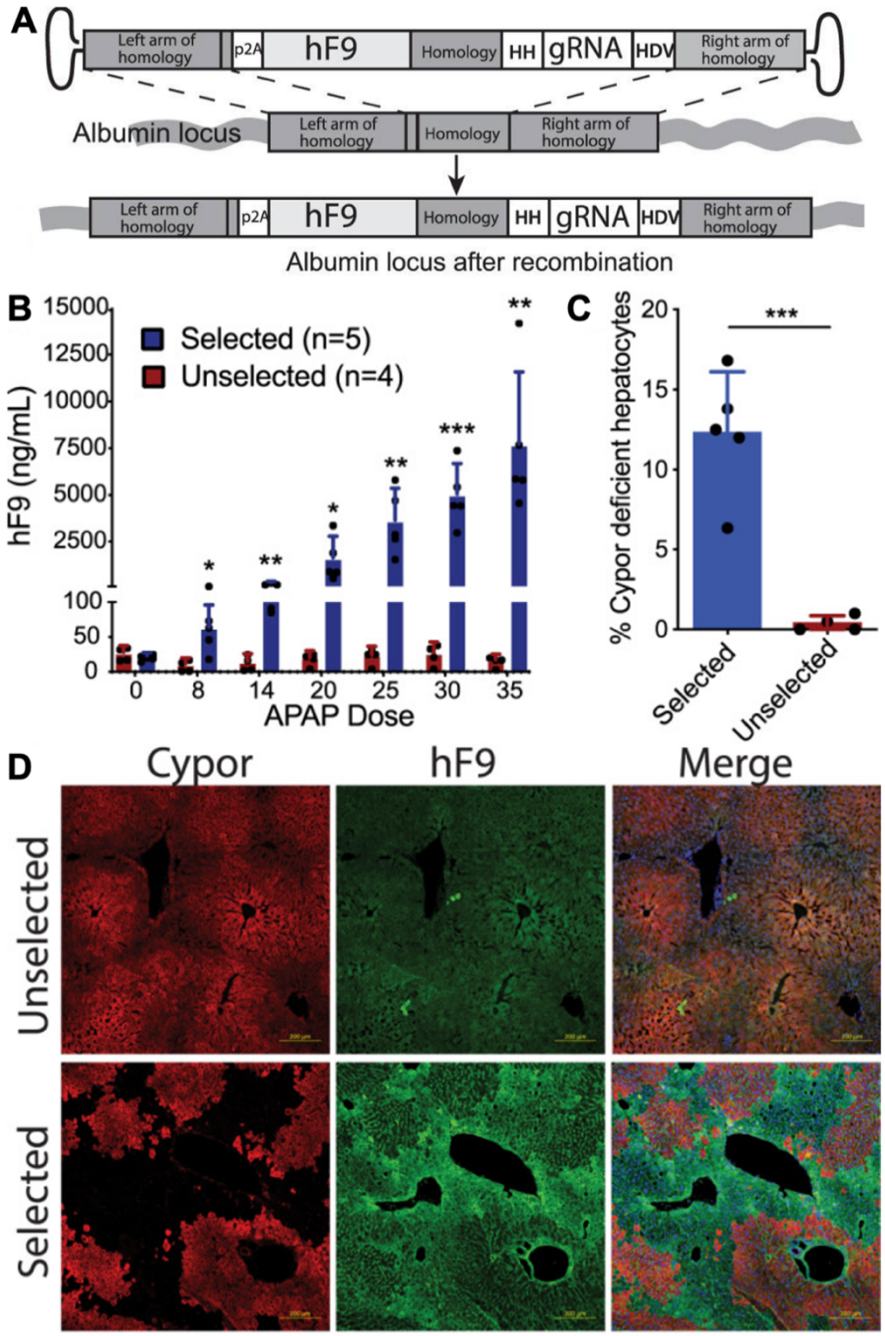


Figure 4-1: APAP selection of a precisely integrating rAAV vector. (A) Schematic of targeted vector integration into the *Alb* locus. HH = hammerhead ribozyme, HDV = hepatitis delta virus ribozyme. (**B**) Blood hF9 concentrations over the course of treatment in mice treated with the integrating rAAV vector and APAP selection (n = 5) compared to unselected mice (n = 4). (**C**) Quantification of Cypor-deficient hepatocytes by indel analysis in APAP-selected mice (n = 5) and unselected controls (n = 4). (**D**) Cypor and hF9 liver immunofluorescence in unselected controls (top) and APAP-selected mice (bottom). Scale bars, 200 μm. Adapted from [164].

Gene therapy vs. cell transplantation therapy

In this dissertation, I described two modalities for the application of the acetaminophen selection system: gene therapy with integrating viral vectors [162, 164] or hepatocyte transplantation [187]. Both showed positive proof-of-concept efficacy and safety data. As will be discussed further below, we believe hepatocyte transplantation to be the more exciting candidate for clinical translation. However, if its current limitations cannot be overcome, gene therapy approaches are also a viable alternative.

Hepatocyte transplantation carries several advantages relative to viral gene therapy. Wild-type hepatocytes represent a single therapeutic agent that can be applied to a plethora of disease indications. By comparison, gene therapy requires customization of cargo for each genetic disorder and sometimes for each mutant allele. To become a drug approved for clinical use, each cargo must go through the regulatory process separately as its own “drug”. Furthermore, severe systemic immune reactions have been seen in response to viral vector administration [25]. In comparison, no severe adverse events have been seen in any patient dosed with transplanted hepatocytes to date. While neutralizing antibody responses prohibit re-dosing with rAAV viral vectors [174], repeat infusion of transplanted hepatocytes is standard practice. Integrating viral vectors are also associated with risk of insertional mutagenesis [188]. Because wild-type hepatocytes are therapeutic without the insertion of a transgene expression cassette, these concerns are avoided in hepatocyte transplantation.

Despite all these advantages, clinical translation of hepatocyte transplantation is significantly less advanced than viral gene therapy. The most significant barrier to clinical hepatocyte transplantation is immune rejection of allogenic donor cells [44]. Gene therapy is not affected by this limitation as viral vectors are delivered to the patient's own cells, preventing an allogeneic immune response. Sourcing of materials is another area where gene therapy has the advantage: although viral vectors are expensive and laborious to produce, they can be grown in a laboratory. Thus far, hepatocyte transplantation requires primary donor hepatocytes and must compete with orthotopic liver transplantation for donor material. Hepatocytes do not readily proliferate in vitro and therefore cannot be easily manufactured in a lab setting. Approaches are being developed with hopes of overcoming these challenges, which shall be discussed further below. If successful in overcoming these challenges, hepatocyte transplantation could become a very exciting therapeutic option for a wide variety of disorders. As of now, however, its clinical implementation remains limited.

Other considerations for the benefits of cell vs. gene therapy are disease specific. The experiments presented here demonstrate a benefit of hepatocyte transplantation for the treatment of PKU in the *Pah*$^{enu2/enu2}$ mouse model: sequestering the wild-type gene from the native mutant gene prevents dominant negative interference by mutant proteins and allows correction with a lower cell threshold. Another case where hepatocyte transplantation may be particularly advantageous is in disorders wherein normal physiologic gene regulation is

required for the proper gene function. Gene therapy typically involves expression of transgenes from constitutive promoters that do not respond to endogenous gene regulation signals. An example of this is ornithine aminotransferase deficiency (gyrate atrophy of the choroid and retina), where gene expression must be confined to pericentral hepatocyte to avoid opposing metabolic processes in periportal hepatocytes [189]. An example of the opposite case, where gene therapy may have the edge over cell therapy, is hemophilia B. Because F9 is excreted into the blood, it does not matter if it is expressed strongly by a small number of cells or weakly by a large number of cells. Expression of a hyperactive variant of the F9 transgene from a strong gene therapy promoter may allow therapeutic levels to be achieved by targeting only a small population of hepatocytes [190].

A possible hybrid approach is ex vivo viral gene delivery to explanted hepatocytes from the patient's own liver, followed by hepatocyte transplantation and APAP selection [191]. This has the advantages of avoiding several of the main challenges of both approaches: the cells are congenic (immune matched) to the patient, and immune reactions to the viral vector as is seen with systemic administration are avoided. Concerns of insertional mutagenesis are not avoided. However, explantation of the quantity of liver tissue that would be needed for this approach is an invasive and laborious process, and this would present a significant barrier to clinical implementation for such a strategy.

The future of hepatocyte transplantation

Although often overshadowed by gene therapy, development of hepatocyte transplantation as a therapeutic is very much ongoing. Three major challenges have limited the applications of hepatocyte transplantation: cell sourcing, low engraftment levels, and immunological rejection of allogenic cells [51]. The method proposed herein can overcome problems associated with low engraftment levels by expanding a rare population of engrafted cells. It may also help to overcome problems with cell sourcing by allowing lower starting thresholds, although increased availability of donor material is still needed. Immunological rejection represents the main remaining major hurdle that must be overcome before hepatocyte transplantation can achieve long-term efficacy.

To improve cell availability, alternative strategies are being developed that would allow cell sourcing from something other than a whole liver. These include animal bioreactors for expanding human hepatocytes and creation of induced hepatocytes from alternative cellular sources. Fah-deficient animals that also carry immunodeficiency mutations preventing rejection of xenotransplanted cells can be used as animal bioreactors for human hepatocyte expansion. Because of the selective effect seen in the $Fah^{-/-}$ liver, xenotransplanted human hepatocytes selectively repopulate the liver. A mouse model of this effect is widely used as an experimental tool [192], and rat [178] and pig [177] models are being developed that would allow in vivo production of human hepatocytes at an increased scale. In addition to animal bioreactors, efforts are ongoing to produce hepatocytes in

vitro from induced pluripotent stem cells (iPSCs) [193] or amnion epithelial cells [194]. However, thus far induced hepatocytes approaches have not fully overcome problems of scalability and phenotypic immaturity [195].

Although many attempts at hepatocyte transplantation in humans have achieved partial efficacy, the effect is consistently lost in long-term follow up due to allorejection of transplanted cells. Standard immunosuppression regimens similar to those given after whole organ transplantation are typically applied after hepatocyte transplantation. However, this approach is insufficient to prevent allorejection in the long run, with the beneficial effects of hepatocyte transplant typically being lost by around 1 year [196]. As discussed above, this problem could be overcome by ex vivo engineering of the patients' own cells (autologous cell source), although this approach is invasive and low-throughput. Alternatively, approaches to engineering "universal donor" cells have been reported for T-cells [180] and iPSCs [179] by genetic manipulation of major histocompatibility complex (MHC) presentation. Similar methods are expected to be applicable to hepatocytes and could enable creation of an off-the-shelf cell therapy product for hepatocyte transplantation. Whether immunological barriers can be overcome will be a major determinant of the therapeutic potential of hepatocyte transplantation.

Disease targets for hepatocyte selection

This dissertation focused on application of the selection system to phenylketonuria and hemophilia B, but many different diseases are expected to be amenable to this selection strategy. Future work is needed in animal models of various disorders to characterize other disease targets. Theoretically, any disorder where the phenotype can be treated by correction of up to ~40% of hepatocytes within zone 3 of the hepatic lobule can likely be treated with this strategy. This likely includes other amino acid metabolic disorders such as maple syrup urine disease, Alkaptonuria, gyrate atrophy, homocystinuria, urea cycle disorders, methylmalonic acidemia and propionic acidemia, as well as glycogen storage diseases, lysosomal enzyme deficiencies, and secreted protein disorders.

Alternate methods of hepatocyte selection

Recently, an alternative approach for universal hepatocyte selection has been proposed. Similar to the previously described strategy utilizing CEHPOBA, this hepatocyte selection strategy aims to transiently mimic the pathology of HT1 [197][1]. An siRNA against mouse *Fah* was administered to create hepatoxicity. An integrating rAAV vector encoding a therapeutic transgene linked in cis to a human *FAH* cDNA— functional on the protein level but distinct enough from its mouse homolog on the nucleic acid level to evade the siRNA— is thus selected. This approach allowed a 5-fold increase in hepatocytes harboring therapeutic

[1] This study is available as a pre-print ahead of peer review.

integrations up to 25% of the liver and achieved therapeutic levels of hF9. This system has the advantage of avoiding any permanent genetic changes in the donor cells. However, it relies on a hepatotoxin that is not FDA approved and is expensive to produce. It is also likely not applicable to hepatocyte transplant without an integrating viral vector as it requires a synthetic copy of FAH to be introduced as the selectable edit.

As a selective agent, acetaminophen carries the benefits of being cheap and readily available, with a well-described mechanism of toxicity and available antidote. It is not only FDA approved, it is one of the most commonly taken drugs in the world. By contrast, the alternate selection system proposed relies on a hepatotoxin that is much less well characterized. The selectability of a simple knockout edit also broadens the applications of our selection system. Further characterization of all potential selection systems will reveal their merits and shortcomings. We propose that our selection system has sufficient merit to warrant further clinical development.

Final thoughts on partial Cypor deficiency

Addressing concerns associated with a genetic CYPOR deficiency in targeted cells is an essential part of ensuring that this selection system is safe for human uses. We made efforts to address these concerns in both gene and cell therapy applications.

Fortunately for the potential of our selection system, the main effects of germline CYPOR deficiency are seen in extrahepatic tissues. Mouse models with complete germline Cypor deficiency are embryonic lethal [198], and human homozygous null mutations are presumed to have the same effect [199]. Humans with reduction-of-function CYPOR mutations show the primary phenotypes of congenital adrenal hyperplasia (disordered steroidogenesis) and Antley-Bixler skeletal malformation syndrome (ABS) [183]. A mouse model of bone-specific Cypor knockout recapitulates the ABS phenotype [200], indicating that loss of hepatic Cypor is not causal for this phenotype. The specific CYPs that are implicated in the steroidogenesis phenotype (CYP17A1, CYP21A2) [199] are expressed predominantly in the adrenal gland, again suggesting no phenotypic involvement of the liver. Liver disease has not been reported in humans with CYPOR-deficiency [85], although reduced metabolic rates of CYP-metabolized drugs have been reported [184]. The lack of liver involvement in human presentation of CYPOR deficiency suggests that partial hepatic CYPOR deficiency would be well tolerated.

Mouse models of complete hepatic Cypor deficiency have been created by use of a floxed Cypor gene and liver-specific Cre recombinase expression [93, 94]. This transgenic mouse is a useful benchmark for the “worst case scenario” for the effects of complete hepatic Cypor deficiency caused by APAP selection. Notably, because APAP toxicity is limited to pericentral hepatocytes, complete hepatic Cypor deficiency is not achievable with the APAP selection system. Mice

with complete hepatic Cypor deficiency develop and reproduce normally, and do not show any of the primary phenotypes that have been described in human CYPOR deficiency. However, severe hepatic lipidosis has been reported in this mouse. This has been reported to correspond with elevations in ALT and decreases in cholesterol and triglycerides in the blood [93, 94]. None of these changes to blood chemistry were seen in APAP-selected mice that had been sustained for a long period of time (>30 weeks) without APAP, including fully-selected wild-type animals and partially-selected PKU animals (Figure 2-9, Figure 3-5, Figure 3-6). A mild decrease in cholesterol was seen in wild-type mice that received continuous APAP for a >1-year period (Figure 2-9), although this is not likely to be of clinical concern. Some hepatic lipidosis, although much milder than that reported in hepatic Cypor-null mice, was seen in APAP-selected wild-type mice treated with either lentiviral gene therapy or a plasmid vector. Even with long term follow-up of 42 weeks, this did not progress to fibrosis or cancer (Figure 2-8D), and no adverse health outcomes were seen. Interestingly, no evidence of any hepatic lipidosis was seen in PKU mice treated with either lentiviral vectors or hepatocyte transplantation (Figure 2-6, Figure 3-5). Similar assessments of liver function should be conducted in large animal models prior to clinical translation, but the mouse data shown here suggest that partial hepatic CYPOR deficiency is unlikely to cause a significant clinical phenotype.

Assessment of a Cyp-metabolized probe drug, caffeine, showed adequate retention of Cyp metabolic activity in selected PKU mice fully cured of their

hyperphenylalaninemia. Unlike in inbred mouse strains, human CYP metabolism is highly variable [201]. Individual genes are highly polymorphic [202], and induction or inhibition of Cyp enzymes by many common compounds adds further fluctuation [201]. Thus, a possible slight reduction in Cyp activity as a result of partial CYPOR deficiency is likely to be well tolerated and possibly indistinguishable from normal genetic variability. Protocols for non-invasive in vivo Cyp phenotyping are established [203] and could be included as part of follow-up to inform modified dosing regimens of CYP-metabolized drugs if this strategy is applied clinically. Specifically, if the common CYP3A-metabolized anti-rejection drug tacrolimus is given to promote survival of allogenic transplanted hepatocytes, CYP phenotyping should be applied to achieve an appropriate dose. A similar modified dosing strategy has been successfully applied for organ transplant recipients with CYP3A gene polymorphisms [204].

Final thoughts on acetaminophen toxicity

Successful clinical translation of this approach will depend upon development of a protocol that allows sufficient hepatotoxicity to allow selection without acute liver failure, a potentially fatal consequence of APAP overdose. This will likely involve slow administration of APAP with intervention with NAC if ALTs exceed a designated threshold. In the mouse model, this gradual administration was successfully accomplished with addition of APAP to the diet (Figure 2-10). This approach was associated with very little mortality when mice were carefully monitored and withdrawn from APAP diet if excessive weight loss was seen.

Asymptomatic ALT elevations have been documented in response to therapeutic APAP doses (4 g/day) in humans [205, 206]. These elevations resolved without incident after APAP was discontinued. As ALT levels in blood directly correspond to hepatocyte cell death, this suggests that it may be possible to accomplish hepatocyte selection at therapeutic doses of APAP. Future work in large animal models is needed to establish a selection protocol with applicability to humans. Although mice are widely used as a model of APAP toxicity due to similarity in metabolic pathways, the rates of cellular injury progression differ significantly between rodents and humans [207]. Cynomolgus macaques have been established as an animal model of APAP hepatotoxicity with phenotypic similarity to humans [208], and could be used to establish a protocol for safe APAP administration prior to clinical use. Whether a protocol can be developed to allow hepatocyte selection without acute liver failure in a large animal model will be a major determinant of whether this selection system can be applied clinically.

Risk of genotoxicity

Knockout of Cypor as described in these experiments was accomplished by creating a CRISPR/Cas9 induced double stranded break, which is followed by imperfect repair by non-homologous end joining to create indel mutations. Recently, concerns have been raised about large-scale genomic rearrangements that can occur following double-strand break creation by Cas9 nuclease [209, 210]. Fortunately, our system is amenable to use with any genome editing method that creates a knockout mutation. Possible alternatives include base

editing [211] and prime editing [212], both of which do not involve the creation of a double strand break. Analysis of off target mutations was not conducted here as the gRNA used is mouse-specific and does not have direct clinical applicability. Thorough off-target mutation analysis in human cells should be conducted for any future human genome editing reagents developed prior to clinical application.

What would a human clinical trial look like?

If safety studies in large animal models and efforts to generate immune stealthy hepatocytes are successful, we believe these data may warrant a clinical trial. A clinical trial of APAP selection with gene therapy or hepatocyte transplantation could look as follows: For hepatocyte transplantation, the cell infusion would be done, likely several consecutive infusions within a day or two to maximize starting the frequency of engrafted cells. For gene therapy, a single vector administration would be done at a low dose. After a predefined recovery period (~1-2 weeks, after hepatic remodeling is done or the transgene is expressed), patients would be started on a once daily oral dose of acetaminophen. As 4 g/day, the maximum recommended daily dose for standard use as an analgesic, has been reported to cause ALT elevations to the range of 300-600 U/L in some cases when taken consecutively over a period of 7 days [205], this would likely be a good starting dose. Non-human primate trials will also be useful in informing the proper starting dose. If after ~7 days on the starting dose no ALT elevation is observed, the daily dose would then be slowly escalated until an ALT elevation in

the range of 300-400 U/L is observed. This dose would then be held constant and repeated daily. This dosing should be done at the same time each day, likely in an outpatient setting with medical/nursing supervision to ensure good compliance. At the same time, blood would be drawn daily for ALT measurement. If the ALT exceeds a predefined threshold (~1000 U/L), the patient would be admitted to a hospital with a supervising gastroenterologist and treated with NAC. Being willing to live near such a hospital would be a condition of participation. Disease phenotype indicators (e.g. blood Phe concentration for PKU) should be monitored periodically, and APAP treatment halted when the desired phenotypic correction is achieved (e.g. blood Phe of 360 μM). ALTs should be monitored periodically over the recovery period to ensure the liver damage resolves successfully. Long term follow-up visits should include the following: blood tests for assessment of disease phenotype (e.g. Phe), markers of liver injury (ALT, AST, etc.), lipids (cholesterol and triglycerides), and HCC biomarkers (e.g. alpha-fetoprotein); administration of a CYP phenotyping cocktail and assessment based on blood levels of CYP metabolized drugs; ultrasound of the liver to check for normal morphology; and assessment of immune cells in the blood to check for an immune response to transplanted cells.

Final thoughts

My thesis characterized a novel method for selective expansion of gene-edited hepatocytes in vivo and its application to gene therapy and hepatocyte transplantation. Compelling preclinical data was achieved for treatment of murine phenylketonuria. Several next steps are needed to determine the clinically applicability of the selection system. Studies in large animal models will be needed to definitively assess the risks of partial Cypor deficiency and acetaminophen toxicity associated with this system. For application to hepatocyte transplantation, new methods of cell sourcing and immune evasion are needed. If these lines of investigation are successful, we believe this work will merit a clinical trial.

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BIOGRAPHY

Education

Oregon Health and Science University, Portland, OR (2019 – 2023)

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Whitman College, Walla Walla, WA (2013- 2017)

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Research Experience

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Research Assistant, Laboratory of Arielle Cooley, Department of Biology, Whitman College, Walla Walla, WA

September 2015- May 2017

Publications

Vonada A, Wakefield L, Martinez M, Harding CO, Grompe M, Tiyaboonchai A (2023). Complete correction of murine phenylketonuria by selection-enhanced hepatocyte transplantation. Hepatology. 2023 Oct 12.

Tiyaboonchai A, Wakefield L, **Vonada A**, May CL, Dorrell C, Enicks D, Sairavi A, Kaestner KH, Grompe M (2023). In vivo tracing of the Cytokeratin 14 lineages using self-cleaving guide RNAs and CRISPR/Cas9. Dev Biol. 2023 Dec;504:120-127.

Tiyaboonchai A, **Vonada A**, Posey J, Wakefield LA, Pelz C, Grompe M (2022). Self-cleaving guide RNAs enable pharmacological selection of precise gene editing events in vivo. Nat Commun. 2022 Nov 30;13(1):7391.

Vonada A, Tiyaboonchai A, Nygaard S, Posey J, Peters AM, Winn SR, Cantore A, Naldini L, Harding CO, Grompe M (2021). Therapeutic liver repopulation by transient acetaminophen selection of gene-modified hepatocytes. *Sci Transl Med*. 2021 Jun 9;13(597):eabg3047.

Presentations and Posters

Vonada, Anne; Tiyaboonchai, Amita; Harding, Cary; Grompe, Markus (2023). Selective Expansion of Transplanted Hepatocytes Corrects a Mouse Model of

Phenylketonuria. Poster presentation at the 26th annual meeting of the American Society of Gene and Cell Therapy, Los Angeles, CA, May 16 – 20, 2023.

Vonada, Anne; Tiyaboonchai, Amita; Harding, Cary; Grompe, Markus (2022). Drug-mediated selection of transplanted hepatocytes for PKU. Oral presentation at the biannual meeting of the National Phenylketonuria Alliance. Vancouver, WA, July 7 -10, 2022.

Vonada, Anne; Tiyaboonchai, Amita; Harding, Cary; Grompe, Markus (2022). Correction of Phenylketonuria by Drug-Mediated Expansion of Transplanted Hepatocytes In Vivo. Poster presentation at the 25th annual meeting of the American Society of Gene and Cell Therapy, Washington, DC, May 16 – 19, 2022.

Vonada, Anne; Tiyaboonchai, Amita; Grompe, Markus (2021). Drug-Mediated Expansion of Transplanted Hepatocytes In Vivo. Digital (poster) presentation at the 24th annual meeting of the American Society of Gene and Cell Therapy, Virtual format, May 11 - 14, 2021.

Vonada, Anne; Nygaard, Sean; Tiyaboonchai, Amita; Posey, Jeffrey; Grompe, Markus (2020). Selective Expansion of Gene-Targeted Hepatocytes In Vivo Leads to Therapeutic Levels of Transgene Expression. Oral presentation at the

23rd annual meeting of the American Society of Gene and Cell Therapy, Virtual meeting, May 12 – 15, 2020

Vonada, Anne; Nygaard, Sean; Grompe, Markus (2019). Selective Expansion of Gene-Targeted Hepatocytes Using Acetaminophen Leads to Reproducible Long-Term Liver Repopulation. Oral presentation at the 22nd annual meeting of the American Society of Gene and Cell Therapy, Washington, DC, April 29 - May 2, 2019

Awards

Outstanding Poster Presentation Award, American Society of Gene & Cell Therapy	2022
Travel Award, American Society of Gene & Cell Therapy	2022
OHSU School of Medicine Outstanding Journal Article Award	2022
Ruth L Kirschstein T32 PBMS Training Grant	2021
Ruth L Kirschstein T32 PERT Training Grant	2020
Travel Award, American Society of Gene & Cell Therapy	2020
Excellence in Research Award, American Society of Gene & Cell Therapy	2019
Travel Award, American Society of Gene & Cell Therapy	2019