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A universal system to select gene-modified hepatocytes in vivo

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Abstract

Many genetic and acquired liver disorders are amenable to gene and/or cell therapy. However, the efficiencies of cell engraftment and stable genetic modification are low and often sub-therapeutic. In particular, targeted gene modifications from homologous recombination are rare events. These obstacles could be overcome if hepatocytes that have undergone genetic modification were to be selectively amplified or expanded. Here we describe a universally applicable system for in vivo selection and expansion of gene-modified hepatocytes in any genetic background. In this system, the therapeutic transgene is co-expressed with a short hairpin RNA (shRNA) that confers modified hepatocytes with resistance to drug-induced toxicity. An shRNA against the tyrosine catabolic enzyme 4-OH-phenylpyruvate dioxygenase (*Hpd*) protected hepatocytes from 4-[(2-carboxyethyl)-hydroxyphosphinyl]-3-oxobutyrate (CEHPOBA), a small molecule inhibitor of fumarylacetoacetate hydrolase (FAH). In order to select for specific gene targeting events, the protective shRNA was embedded in a miRNA and inserted into a recombinant AAV vector designed to integrate site-specifically into the highly active albumin locus. After selection of the gene-targeted cells, transgene expression increased 10- to 1,000-fold, reaching supra-physiological levels of 50,000 ng/mL of human factor 9 protein in mice. This drug resistance system can be used to achieve therapeutically relevant transgene levels in hepatocytes in any setting.

Introduction

Liver-directed gene therapy has the potential to produce permanent cures for many hepatic disorders, in particular inborn errors of metabolism (1). Recently, some clinical success has

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Data and materials availability: All data and materials are available from the corresponding author upon request. Plasmids containing viral vectors and *Fah*^{-/-} mutant mice require the completion of a standard academic MTA.

been achieved with hemophilia B, using recombinant adeno-associated viral (rAAV) vectors (2). Despite this success, there are limitations to this method. rAAV is an episomal vector, and transgene expression is rapidly lost during cell division, as is the case in the growing liver of young children (3). High vector doses are required and can result in anti-capsid immune responses (4-6). In addition, concerns about insertional mutagenesis and cancer remain (7-9). In order to achieve both transgene persistence with replication and to minimize insertional mutagenesis, several groups, including ours, have reported methods to achieve site-specific gene targeting in hepatocytes (10-12). Proof-of-principle experiments have been performed; however, very high vector doses and/or targeted nucleases (zinc fingers, TALENs, CRISPR/Cas9) are needed to achieve reasonable efficiencies of gene repair (13-15). The clinical potential of targeted nucleases is currently unknown, and it is unlikely that the reported rAAV vector doses could be tolerated in humans.

Many of these obstacles could be overcome if it was possible to amplify and selectively expand hepatocytes that have already undergone the desirable genetic modification. Lower vector doses could be used and it may be possible to achieve therapeutic levels of gene targeting without nucleases. It is known that hepatocytes can be massively selected *in vivo* in both mice and humans, in settings where the host liver has a genetic disadvantage (16). Gene-corrected hepatocytes have a selective advantage in Wilson's disease (17), α 1-antitrypsin deficiency (18), progressive familial intrahepatic cholestasis (19), and hereditary tyrosinemia (16, 20). These rare examples highlight the inherent regenerative capacity of mature hepatocytes. However, there is no selective advantage for transplanted or genetically corrected hepatocytes in most liver diseases, including phenylketonuria, the hemophilias, or urea cycle disorders.

Genetic hepatocyte selection is strongest in fumarylacetoacetate hydrolase (FAH) deficiency, and is known to occur in the human liver (16). We therefore set out to develop an *in vivo* selection system that takes advantage of the tyrosine catabolic pathway, which converts tyrosine to fumarylacetoacetate (FAA), to be hydrolyzed by the enzyme FAH into fumarate and acetoacetate (Fig. 1A). Hepatotoxicity in *Fah* deficiency is caused by accumulation of FAA. We previously developed small-molecule inhibitors of FAH and showed that treatment with these drugs faithfully mimic human genetic *FAH* deficiency in mice (21-23). In addition, it has been shown that mutations in the tyrosine catabolic pathway upstream of FAH can prevent the accumulation of FAA, completely protect *Fah*^{-/-} hepatocytes, and prevent liver disease in mice (24,25). In fact, pharmacological blockage of 4-hydroxy (OH)-phenylpyruvate dioxygenase (HPD) with the small molecule 2-[2-nitro-4-(trifluoromethyl)benzoyl] cyclohexane-1,3-dione (NTBC) is the standard treatment for human FAH deficiency (Fig. 1A) (26, 27).

Taking advantage of this principle, we recently demonstrated that hepatocytes genetically deficient in homogentisic acid dioxygenase (HGD) (Fig. 1A) could be strongly selected in wild-type mice treated with an FAH-inhibitor 4-[(2-carboxyethyl)-hydroxyphosphinyl]-3-oxobutyrate (CEHPOBA) (23). Based on this finding we reasoned that shRNA-mediated knockdown of enzymes upstream of FAH would make hepatocytes resistant to CEHPOBA and achieve their *in vivo* selection (Fig. 1A). Here, we report the development of a versatile

system that provides potent hepatocyte selection *in vivo* in mice, independent of genetic background, and can be used to amplify therapeutic cells in multiple settings.

Results

Selection of a protective shRNA

In order to determine whether knockdown of the gene encoding tyrosine aminotransferase (*Tat*), 4-OH-phenylpyruvate dioxygenase (*Hpd*), or homogentisic acid dioxygenase (*Hgd*) would result in hepatocyte selection (Fig. 1A), a small lentiviral library was tested in *Fah*-deficient mice. Four shRNAs (table S1) were designed against each candidate gene and cloned into a lentiviral backbone under a U6 promoter (Fig. 1B). Neonatal *Fah*^{-/-} mice were injected with sh*Tat*, sh*Hpd*, or sh*Hgd* lentiviruses via the facial vein and kept on NTBC until weaning (Fig. 1C). NTBC was then stopped to permit liver injury and selection of resistant hepatocytes. Only mice injected with the *Hpd* shRNA library gained weight after complete NTBC withdrawal, indicating the emergence of FAA-resistant hepatocytes (Fig. 1D). Animals injected with an *Hgd* shRNA, *Tat* shRNA or a control lentivirus devoid of an shRNA required reintroduction of intermittent NTBC therapy to maintain weight (interrupted grey bars in Fig. 1D).

After several weeks of selection, the livers were harvested and analyzed histologically. Mice injected with the sh*Hpd* library showed clear evidence of regenerative nodules (Fig. 1E). These nodules consisted of healthy appearing hepatocytes staining positive for the GFP-transgene and were negative for the damage marker α -fetoprotein. Next, the shRNA sequences were rescued by PCR and sequenced (Fig. 1F). Only a single sh*Hpd* sequence, 5'-

CCGGGCCTCAGAATGGTACCTGAACTCGAGTTTCAGGTACCATTCTGAGGCTTTT TG-3'', was retrieved from multiple weight-stabilized *Fah*^{-/-} mice and used for all future experiments.

In vivo selection of an integrating rAAV vector

We have previously shown that AAV vectors harboring homology to ribosomal DNA have increased integration frequency in hepatocytes (28, 29), but their absolute efficiency of chromosomal integration is still very low. We therefore constructed rDNA vectors containing a human factor 9 (*hF9*) transgene as well as the selectable *Hpd* shRNA (Fig. 2A). Twenty-five-day-old post-weaning *Fah*^{-/-} mice were injected with 1×10^{11} vg each of the vector, kept on NTBC for two weeks after injection, and then subjected to selective pressure (Fig. 2B). All mice injected with the rDNA-*F9*-sh*Hpd* vector gained weight after complete NTBC withdrawal whereas control vector injected animals required continued NTBC therapy to maintain their weight (Fig. 2C). Similarly, hF9 levels rose significantly and continuously in response to NTBC withdrawal (Fig. 2D), indicating expansion of FAA-resistant, transgene-expressing hepatocytes. This result demonstrates that a transgene linked in *cis* to the selectable shRNA was amplified, leading to therapeutic levels of transgene expression unachievable without selection.

In vivo selection of gene-targeted hepatocytes

Recently, rAAV-mediated targeted homologous recombination into the highly expressed albumin gene was used to achieve therapeutic levels of transgene expression in the liver (30). These “generide” constructs are promoterless and hence are predicted to have reduced cancer risk from insertional mutagenesis upon random integration (8, 31). However, the efficiency of targeted integration was <1% even when high vector doses were used in neonatal animals. In order to determine whether these rare gene-targeting events could be amplified in vivo, two different vector designs were tested. First, we inserted a cassette containing the selectable sh*Hpd* driven by a U6 promoter into an intron of the albumin gene (Fig. 3A). Second, a promoterless construct was generated. Here, the sh*Hpd* was embedded within a microRNA and inserted in an albumin intron downstream of the therapeutic F9 cassette (Fig. 3A). The intent was to have the shRNA expression driven by the chromosomal albumin promoter and, hence, induce *Hpd* gene knockdown only after homologous recombination.

Neonatal *Fah*^{-/-} mice were injected with 1×10^{11} vg each, kept on NTBC until weaning, and then subjected to selection by withdrawing NTBC (Fig. 3B). Human F9 levels were monitored during NTBC withdrawal. Interestingly, increases in F9 level were only observed with the promoterless vector design in which the endogenous albumin promoter drives the sh*Hpd* after gene targeting. Starting from a low baseline, selective pressure resulted in greater than 100-fold increase in hF9 levels and produced very high, even supraphysiologic levels of human F9, exceeding 40,000 ng/mL (Fig. 3C). This result indicated that gene-targeted hepatocytes could be selectively expanded using a miR-embedded protective shRNA.

In order to confirm that selection had occurred, histology was performed in animals with hF9 levels ranging from high (38,000 ng/ml) to low (800 ng/ml) serum (Fig. 3E). As expected, a high percentage of their liver mass (up to 50%, estimated by eye) had been replaced by hF9-producing hepatocytes, whereas less than 0.1% of cells were positive in the controls. In addition to histology, molecular analysis was performed to detect properly targeted albumin alleles. PCR amplification demonstrated the amplification products expected from homologous integration, which were confirmed by sequencing (fig. S1). In aggregate, these data prove that gene-targeted hepatocytes expanded over 100-fold in response to FAA-mediated selection producing very high levels of therapeutic transgene expression.

High levels of shRNA expression can be toxic in the liver (32). In order to test whether continuous expression of the protective shRNA was toxic to gene-targeted hepatocytes, animals underwent NTBC withdrawal until therapeutic levels of hF9 (250 ng/ml, or 5%) were reached (dashed line in Fig. 3E) and then put back on NTBC to halt selective pressure. We hypothesized that the hF9 plasma concentration would drop with time if gene-targeted hepatocytes were damaged by the shRNA expression. Importantly, hF9 levels remained stable on NTBC, indicating that selective sh*Hpd* did not cause hepatocyte death (Fig. 3E). To further demonstrate that the expanded hF9-expressing nodules consisted of healthy cells, markers characteristic of normal hepatic zonation and function were evaluated and found to

be normal (Fig. 4A). Additionally, hepatocyte morphology and cell cycle status were normal in selected nodules (Fig. 4A).

In order to establish that this approach could also work not only after neonatal injection, but also in adult animals, four mice 8 weeks of age were injected with 8×10^{11} vg of the selectable generide vector each. After NTBC withdrawal hF9 levels increased more than 20-fold (Fig. 4B).

Pharmacologic selection in wild-type animals

Transgene selection in *Fah*^{-/-} mice was only used as proof-of-principle to develop a gene therapy module that could be selected by resistance to high levels of FAA. In order to apply this system in any genetic liver disease, selection must work in animals that not deficient in FAH. We previously described a small-molecule inhibitor of FAH that was capable of inducing FAA accumulation and exerting selective pressure in vivo in mice (22, 23). In order to demonstrate that the *Hpd* shRNA could be used in any genetic background, neonatal wild-type C57Bl6 mice were injected with 2×10^{11} vg of the promoterless F9 generide construct. Starting at age 4 weeks, three treated animals were injected daily with CEHPOBA; littermate controls that had also been given vector were given saline instead (Fig. 5A).

CEHPOBA administration was well-tolerated and hF9 levels in plasma rose steadily during the injection period (Fig. 5B), reaching therapeutic levels of >250 ng/ml in all cases. Histological analysis demonstrated clusters of hepatocytes expressing hF9, as expected from clonal expansion of gene-targeted hepatocytes in CEHPOBA-treated animals (Fig. 5C). In contrast, only rare single F9 positive hepatocytes were seen in saline controls. After 4 weeks of injections and at therapeutic levels of hF9, selection was stopped and hF9 levels were monitored. hF9 levels were stable in two animals, but declined in the 3rd (Fig. 5B). A second round of CEHPOBA injections was initiated in this animal, resulting in an increase of transgene expression back into the therapeutic range.

All mice were followed for another month and then harvested to assess renal and hepatic function and histology (Table 1, Fig. 5D). No differences in kidney or liver function in treated and normal C57Bl6 mice were detected, demonstrating that the selective regimen had no permanent negative effects on these organs. Hepatocytes of CEHPOBA-treated livers displayed minimal variation in size of the cell and the nuclei (Fig. 6A). The patterns of the CEHPOBA treated animal are completely normal with rare Ki67⁺ hepatocytes (no proliferation), normal homogeneous hepatocyte nuclear and cellular size, normal Hnf4a staining (all hepatocyte nuclei positive) and normal zone 3 distribution of Cyp2E1 (brown cytoplasmic staining next to vessels). Other cell types, including bile ducts, endothelial, Kupffer and stellate cells, were also unremarkable. In contrast, *Fah* deficient animals have severe liver damage. H&E staining shows hepatocyte macrocytosis, nuclear ballooning, as well as pleiocytosis (Fig. 6A).

Finally, molecular analysis was performed using quantitative RT-PCR to detect and measure mRNA molecules that were the product of gene targeting. The relative abundance of mouse albumin mRNA from untargeted chromosomes was compared to that of hybrid mRNAs. PCR primer pairs specific to the hybrid mouse albumin-human F9 transcripts were used. As

expected from the hF9 levels in blood, CEHPOBA selection increased the hybrid mRNA levels by ~1,000-fold compared to vector-injected wild-type animals that received no selection (Fig. 6B). Similarly, high levels of hybrid albumin mRNA derived from targeted hepatocytes were found after genetic selection in vector injected *Fah*^{-/-} mice (Fig. 6B). Together these results demonstrate that CEHPOBA treatment can be used to amplify gene-targeted hepatocytes and significantly enhance the therapeutic transgene expression regardless of genetic background.

Discussion

The need for cytotoxic conditioning is necessary in hematopoietic stem cell gene therapy and bone marrow transplantation to achieve therapeutic levels of healthy stem cells (33). Typically, the conditioning regimen is applied before transplantation of stem cells, such as the enzyme O(6)-methylguanine-DNA methyltransferase (MGMT), which renders the cells resistant to the chemotherapy drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (34). Clinical trials using this combination of cytotoxic drug and resistance gene have been successful in protecting the bone marrow during intense chemotherapy of glioblastoma (35). Although hepatocytes are known to harbor stem cell-like regenerative capacity and can be extensively selected in certain genetic conditions, a clinically relevant drug/resistance gene combination for the liver—analogue to BCNU/MTMG—has not been developed to date. Overexpression of the oncogene *Bcl2* can protect hepatocytes from Fas-mediated apoptosis (36, 37) and produce extensive hepatocyte selection. However, this approach has little clinical potential because the protective gene renders cells permanently apoptosis-resistant and, hence, cancer-prone.

We have previously shown that transplanted hepatocytes can be selected with CEHPOBA in vivo if they carry a genetic mutation in tyrosine catabolic enzyme (23). However, patients with such mutations are rare leaving the vast majority of clinically relevant metabolic liver diseases unamenable to this powerful selection. Here, we demonstrated that shRNA-mediated knockdown of 4-OH-phenylpyruvate dioxygenase (*Hpd*) can protect hepatocytes of any genotype against the effects of the potent FAH inhibitor CEHPOBA. Hepatocytes expressing this shRNA were strongly selected in mice, resulting in a 10-30-fold expansion in only 4 weeks. Importantly, this method worked in wild-type mice with no genetic liver disease. Any transgene connected in cis to the protective shRNA may be co-selected, making this system applicable to any liver-directed gene therapy producing stably modified cells. Potential target diseases include hemophilia B and metabolic liver diseases with neonatal onset, such as the urea cycle disorders. Normal liver growth in infants leads to rapid loss of episomal transgene expression (3), but the ability to select for chromosomally integrated genes may overcome this problem. Although the expansion achieved with the pharmacological agent CEHPOBA was at maximum ~30-fold over 4 weeks, the experiments performed in mice with genetic *Fah*^{-/-} deficiency demonstrated that expansion of >100-fold is feasible with longer selection periods.

The *Hpd* shRNA system is versatile and not limited to any particular delivery vector. We showed equally potent selection with both lentiviral vectors and integrating rAAV vectors. Any gene delivery system that produces chromosomal integration of a transgene cassette can

be selected. Therefore, potential gene delivery platforms include nonviral systems, such as Sleeping Beauty and PiggyBac transposons, as well as typical viral vectors, such as oncoretroviral, lentiviral, and rAAV vectors capable of integration. Lentiviruses in particular may be of interest because of their large packaging limit, and could therefore be used for transgenes too big to fit into rAAV vectors. In order to apply our approach to cell therapy the donor hepatocytes would have to be genetically modified to express *Hpd* shRNA prior to transplantation. This could be achieved with a variety of ex vivo gene delivery systems.

Although our initial constructs expressed the protective shRNA from the strong, nonspecific U6 promoter, we also showed that selection could be achieved when a tissue-specific promoter drove expression. To make this possible, the *Hpd* shRNA was embedded in a microRNA such that it could be processed from a *Pol2* transcript. Importantly, we were able to incorporate this approach into a promoterless rAAV construct designed to produce transgene expression only after homologous recombination into the hepatocyte-specific albumin locus (38). Although the initial targeting frequency with this system has been estimated to be only <1%, gene-targeted, transgene-expressing hepatocytes constituted ~50% of the liver after selection. Our vector design permitted in vivo pharmacological selection of a specific gene-targeting event in hepatocytes, which has not been possible before.

Given recent concerns about the oncogenic potential of random integration of rAAV vectors containing liver-specific promoters (7-9, 31, 39), the ability to use promoterless constructs provides an important safety feature. Although the frequency of random rAAV integrations is not affected by concurrent gene-targeting (11), randomly integrated vectors without promoters cannot trigger oncogene activation. Efficient gene therapy using specific genome modifications as opposed to random gene addition should now be feasible, even without the use of site-specific nucleases. No adverse side effects of CEHPOBA were seen in our gene-selected mice several weeks after the drug was stopped. Liver function reverted back to normal within four weeks of stopping selection. However, CEHPOBA is not an FDA-approved drug and no clinical experience with transient FAH blockage exists in humans. Conversely, much is known about the natural history of FAH deficiency in humans, which is informative regarding the predicted risks of transient FAH inhibition in humans. Several studies have shown that the effects of FAH deficiency in humans are completely reversible as long as NTBC therapy is started before age 6 months (27, 40). In fact, most patients treated before age 2 years have excellent long-term outcomes. These studies suggest that transient inhibition of FAH is likely to be tolerated in humans and not lead to long-term sequelae, including liver cancer. Given the high efficiency of our selection system shown herein, large animal experiments to further evaluate the safety and clinical potential of this approach now appear warranted.

The impact of our results is not narrowly confined to only the tyrosine catabolic pathway or the liver. There are many drugs that are specifically hepatotoxic, and the toxin/protective-shRNA selection approach described herein could apply to many of these. Some drugs have to be metabolically activated to be hepatotoxic (41); others require specific transporters to enter the hepatocyte (42). Each of these activation-dependent liver toxins, many of which are FDA-approved medicines, represents an opportunity to develop a clinically applicable

hepatocyte selection system. Furthermore, the overall approach should apply to any tissue or cell type capable of proliferation after injury, including the bone marrow, intestine, renal tubules, or skin.

Materials and Methods

Study design

Our study involved comparisons between cohorts of mice. In some experiments different gene therapy vectors were compared to each other. All mice were exposed to selective pressure, but received vectors to determine which vector design permitted in vivo selection. Presence or absence of selection was determined by measurement of blood level of human factor 9 or measurement of vector copy number in liver DNA. Laboratory personnel performing the hF9 ELISAs were blinded to the treatment regimen. In other experiments, mice were treated with the same vector, but exposed to different selection regimens. Again, both hF9 measurements and vector copy number were measured to determine whether selection had occurred. All measurements were performed at least in duplicate and all in vivo experiments were repeated multiple times. The precise number of experimental animals and replicates are given in the figure legends. The number of experimental animals was chosen to permit the detection of at least 10-fold differences in either hF9 levels or DNA copy number with a p-value of < 0.05 determined by an unpaired, two-tailed t-test. Animals were assigned to experimental cohorts randomly.

Mouse strains and animal husbandry

All animal experiments were performed according to the guidelines for animal care at Oregon Health & Science University. Male and female *Fah*^{-/-} mice were of the *Fah* exon5 strain on the 129s4 background as described previously (43). *Fah*^{-/-} mice were maintained with drinking water containing NTBC (Yecuris Corp) at a concentration of 8 mg/L (44).

Plasmid vectors and rAAV production

Lentiviral vectors encoding U6-driven shRNAs against *Tat*, *Hgd*, and *Hpd* were produced using the backbone plasmid pLKO.1-hPGK-Puro-CMV-tGFP (Sigma) (table S1). The selected U6-HPD shRNA cassette was subcloned into an AAV vector with 500 bp ribosomal DNA homology arms (27). From the pLKO.1 template a 579-bp PCR product was amplified using the following primers: forward, 5'-AGTCGAATTCGCCTATTTCCCATGATTCCTTC-3', and reverse, 5'-GACTACTTTTCCTCGCCTGTTC-3'. The PCR product was cut with *EcoRI* and ligated to the recipient rDNA AAV vector linearized with *EcoRI*. The selectable generide vector was constructed by first incorporating the HPD shRNA into a micro RNA. An optimized miR-30, "miR-E," was chosen as a template (45). The HPD-targeted miR-E sequence was:

TCGACTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAG
CGCGC
CTCAGAATGGTACCTGAAATAGTGAAGCCACAGATGTATTTTCAGGTACCATTCT
GAG GCATGCCTACTGCCTCGGACTTCAAGGGGCTAGAATTCGA (target and guide
sequence are in bold). To incorporate this HPD shRNA mir sequence into the generide
vector, pAB269 was cut with *PflMI* to excise an 810-bp fragment spanning the hF9

transgene and 298 bp of an albumin intron sequence. We generated a synthetic sequence identical to this 810 bp fragment, but replaced 157 bp of albumin intron sequence with the HPD shRNAmir. The HPD shRNA was inserted 55 bp downstream of the hF9 stop codon, with the following flanking albumin intron sequence: 5'-AACCGGT-HPD shRNAmir-CCAACTT-3'. This synthetic fragment was cut with *Pf1* and ligated to the backbone of pAB269, cut with the same enzyme. Standard transfection and viral isolation protocols were described previously (46). AAV titering was performed by quantitative PCR. Lentiviral vector titers were determined using puromycin resistance in 3T3 cells.

Vector administration, NTBC cycling, and bleeding

Adult *Fah*^{-/-} mice (age 5 weeks) were administered 2×10^{10} vector genomes (vg) per mouse of rAAV (in 100- μ L volume) by i.v. tail vein injection. NTBC treatment was continued for 2-weeks following vector administration. NTBC cycling consisted of 2-3-week withdrawal periods interspersed with 4 days of NTBC administration. For neonatal AAV administration, P1 *Fah*^{-/-} neonates were injected with 2×10^{11} vg per mouse (in 10- μ L volume) of rAAV8 or 4×10^3 transducing units of lentivirus by intravenous (IV) injection into the superficial temporal vein. NTBC administration was continued for 5-weeks following vector administration, and then cycling was performed as described above. For vectors expressing hF9, blood samples were collected by saphenous vein puncture at the onset of NTBC cycling and every week thereafter.

CEHPOBA administration

Wild-type P1 neonates were injected with 2×10^{11} vg per mouse of rAAV8. At 3 weeks of age, blood samples were collected by saphenous vein puncture and analyzed for hF9 levels. Two cohorts of three mice each with similar baseline hF9 levels were chosen. Beginning at 4 weeks of age, each cohort was given daily injections of saline or CEHPOBA (1- μ mol/g) as described previously (23). During CEHPOBA/saline treatment, the mice received drinking water supplemented with 20 mg/ml L-phenylalanine (Acros) and 30 mg/ml dextrose. After 30 days of CEHPOBA/saline treatment, injections were terminated and the mice were given regular drinking water for another 30 days, then sacrificed for tissue and blood collection. Blood samples were drawn weekly throughout the experiment to monitor hF9 levels.

Liver Immunohistochemistry

Immunohistochemical fixation and staining protocols for FAH and H&E were completed as described (47). For analysis of turboGFO (tGFP), methods for FAH staining were used except the primary antibody was rabbit anti-tGFP (Axxora) used at 1:500 overnight at 4 °C. Fixation and staining methods for hF9 immunohistochemistry were as described (30).

For serial sections the primary antibodies used were in order: glutamine synthetase (1:200), Ki67 (1:200), Cyp2E1 (1:100, Abcam ab28146), Hnf4 α (1:150, Santa Cruz Bio sc-8987) and hFIX (1:400). Vector IMPress conjugated to HRP detection kit was used as the secondary antibody. Positive staining was visualized with AEC (3-amino-9-ethylcarbazole) and DAB (3,3-diaminobenzidine substrate. Microscopy was performed on a DM IL LED microscope (Leica) using Leica LAS Image Analysis Software.

Assessment of F9-containing albumin mRNAs by qPCR

The method was described in detail in our previous generide work (30). Briefly, cDNA produced from reverse transcription with a poly-dT primer served as a template for two different TaqMan qPCR assays. We quantified the abundance of wild-type albumin mRNA by qPCR with primers: 5'-CTGACAAGGACACCTGCTTC-3' and 5'-TGAGTCCTGAGTCTTCATGTCTT-3', and TaqMan probe: 5'-CCACAACCTTCTCAGGCTACCCTGA-3'. We quantified the abundance of fused mRNAs by a TaqMan qPCR with primers: 5'-CCAAGGTGTCCAGATACGTG-3' and 5'-TGAGTCCTGAGTCTTCATGTCTT-3', and TaqMan probe: 5'-CCACAACCTTCTCAGGCTACCCTGA-3'. For non-injected controls, no qPCR signal was detected with primers specific for the fused mRNA. The rate of F9-containing albumin mRNAs was calculated as the ratio between the abundance of template for the wild-type albumin primer pair to the abundance of template for the fusion mRNA specific primer pair.

PCR to identify selected shRNA

To identify shRNAs that were clonally expanded during NTBC withdrawal, genomic liver DNA from weight stabilized mice was amplified using two primer sets: (i) forward, 5'-TGGAATCACACGACCTGGATGG-3', and reverse, 5'-CCCGTCCTAAATGTCCTTCTGC-3'; (ii) forward, 5'-ATCGTTTCAGACCCACCTCCCAAC-3', and reverse, 5'-CCCAAGTCCCGTCCTAAATGTC-3'. PCR products were gel purified, subcloned using a TOPO TA cloning kit (Life Technologies), and sequenced using M13 forward and reverse primers.

Integration site analysis following selection in gene-targeted mouse liver DNA

Primers used to amplify the 5-prime gene-ride integration site: Primer 1, 5'-CAACGTCATGGGTGTGACTTTTG-3', and primer 2, 5'-TACTGCTTCCAGAACTCGGTGG-3'. Sequencing primers were: 5'-GGTGAACAACATTATGAGC-3' and 5'-AGAGACATAGTGCTGTGTAGGG-3'. Primers used to amplify the 3-prime gene-ride integration site: primer 3, 5'-CACCAACATCTTTCTGAAGTTCGG-3', and primer 4, 5'-TTCCACACCTGGCTCGTTATTAG-3'. Sequencing primers were: 5'-CTGTCTACACCAGTGAAAATCG-3' and 5'-TGACCCTTTGACCACGCAAC-3'.

Human Factor 9 ELISA

hF9 levels were measured using the Asserachrom IX:Ag ELISA kit (Stago). The manufacturers directions were followed with the following modifications. Blood samples (5 µl) were collected from saphenous vein puncture and diluted into 245 µl R4 buffer from the ELISA kit supplemented with 11 mM sodium citrate as an anticoagulant. Following brief centrifugation to pellet blood cells, supernatants were stored at -80° C for up to 3 weeks. Two hundred µl of each sample were used for the hF9 ELISA. The limit of detection was approximately 20 ng/ml hF9 in whole blood. Specificity for human FIX was confirmed by diluting purified hF9 in mouse plasma or saline with no significant difference in measured hF9 levels.

Liver function and blood chemistry tests

Mouse serum samples were obtained by submandibular vein puncture. ALT levels were measured using an ALT color endpoint reagent kit (Genway). For mice used in CEHPOBA selection, serum was obtained by terminal cardiac puncture and submitted to IDEXX laboratories for a comprehensive blood chemistry panel.

Statistical Analysis

Statistical analyses were conducted with GraphPad Prism software v.4.0 (GraphPad). Experimental differences were evaluated by student two-tailed t-test assuming equal variance. P values <0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet.* 2011; 12:341–355. [PubMed: 21499295]
2. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, O'Beirne J, Smith K, Pasi J, Glader B, Rustagi P, Ng CY, Kay MA, Zhou J, Spence Y, Morton CL, Allay J, Coleman J, Sleep S, Cunningham JM, Srivastava D, Basner-Tschakarjan E, Mingozzi F, High KA, Gray JT, Reiss UM, Nienhuis AW, Davidoff AM. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med.* 2011; 365:2357–2365. [PubMed: 22149959]
3. Wang L, Bell P, Lin J, Calcedo R, Tarantal AF, Wilson JM. AAV8-mediated hepatic gene transfer in infant rhesus monkeys (*Macaca mulatta*). *Mol Ther.* 2011; 19:2012–2020. [PubMed: 21811248]
4. Pien GC, Basner-Tschakarjan E, Hui DJ, Mentlik AN, Finn JD, Hasbrouck NC, Zhou S, Murphy SL, Maus MV, Mingozzi F, Orange JS, High KA. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *J Clin Invest.* 2009; 119:1688–1695. [PubMed: 19436115]
5. Mingozzi F, High KA. Immune responses to AAV in clinical trials. *Curr Gene Ther.* 2007; 7:316–324. [PubMed: 17979678]
6. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, Dake M, Kaye R, Razavi M, Zajko A, Zehnder J, Rustagi PK, Nakai H, Chew A, Leonard D, Wright JF, Lessard RR, Sommer JM, Tigges M, Sabatino D, Luk A, Jiang H, Mingozzi F, Couto L, Ertl HC, High KA, Kay MA. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med.* 2006; 12:342–347. [PubMed: 16474400]
7. Wang PR, Xu M, Toffanin S, Li Y, Llovet JM, Russell DW. Induction of hepatocellular carcinoma by in vivo gene targeting. *Proc Natl Acad Sci U S A.* 2012; 109:11264–11269. [PubMed: 22733778]
8. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS. AAV vector integration sites in mouse hepatocellular carcinoma. *Science.* 2007; 317:477. [PubMed: 17656716]

9. Nault JC, Datta S, Imbeaud S, Franconi A, Mallet M, Couchy G, Letouze E, Pilati C, Verret B, Blanc JF, Balabaud C, Calderaro J, Laurent A, Letexier M, Bioulac-Sage P, Calvo F, Zucman-Rossi J. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet.* 2015; 47:1187–1193. [PubMed: 26301494]
10. Paulk NK, Loza LM, Finegold MJ, Grompe M. AAV-mediated gene targeting is significantly enhanced by transient inhibition of nonhomologous end joining or the proteasome in vivo. *Hum Gene Ther.* 2012; 23:658–665. [PubMed: 22486314]
11. Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA, Grompe M. Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology.* 2010; 51:1200–1208. [PubMed: 20162619]
12. Miller DG, Wang PR, Petek LM, Hirata RK, Sands MS, Russell DW. Gene targeting in vivo by adeno-associated virus vectors. *Nat Biotechnol.* 2006; 24:1022–1026. [PubMed: 16878127]
13. Li H, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature.* 2011; 475:217–221. [PubMed: 21706032]
14. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Kotliansky V, Sharp PA, Jacks T, Anderson DG. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol.* 2014; 32:551–553. [PubMed: 24681508]
15. Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Kotliansky V, Wolfe SA, Langer R, Xue W, Anderson DG. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol.* 2016
16. Kvittingen EA, Rootwelt H, Berger R, Brandtzaeg P. Self-induced correction of the genetic defect in tyrosinemia type I. *J Clin Invest.* 1994; 94:1657–1661. [PubMed: 7929843]
17. Yoshida Y, Tokusashi Y, Lee GH, Ogawa K. Intrahepatic transplantation of normal hepatocytes prevents Wilson's disease in Long-Evans cinnamon rats. *Gastroenterology.* 1996; 111:1654–1660. [PubMed: 8942746]
18. Ding J, Yannam GR, Roy-Chowdhury N, Hidvegi T, Basma H, Rennard SI, Wong RJ, Avsar Y, Guha C, Perlmutter DH, Fox IJ, Roy-Chowdhury J. Spontaneous hepatic repopulation in transgenic mice expressing mutant human alpha1-antitrypsin by wild-type donor hepatocytes. *J Clin Invest.* 2011; 121:1930–1934. [PubMed: 21505264]
19. De Vree JM, Ottenhoff R, Bosma PJ, Smith AJ, Aten J, Oude Elferink RP. Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. *Gastroenterology.* 2000; 119:1720–1730. [PubMed: 11113093]
20. Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou CN, Finegold M, Grompe M. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. *Nat Genet.* 1996; 12:266–273. [PubMed: 8589717]
21. Bateman RL, Bhanumoorthy P, Witte JF, McClard RW, Grompe M, Timm DE. Mechanistic inferences from the crystal structure of fumarylacetoacetate hydrolase with a bound phosphorus-based inhibitor. *J Biol Chem.* 2001; 276:15284–15291. [PubMed: 11154690]
22. Bateman RL, Ashworth J, Witte JF, Baker LJ, Bhanumoorthy P, Timm DE, Hurley TD, Grompe M, McClard RW. Slow-onset inhibition of fumarylacetoacetate hydrolase by phosphinate mimics of the tetrahedral intermediate: kinetics, crystal structure and pharmacokinetics. *Biochem J.* 2007; 402:251–260. [PubMed: 17064256]
23. Paulk NK, Wursthorn K, Haft A, Pelz C, Clarke G, Newell AH, Olson SB, Harding CO, Finegold MJ, Bateman RL, Witte JF, McClard R, Grompe M. In vivo selection of transplanted hepatocytes by pharmacological inhibition of fumarylacetoacetate hydrolase in wild-type mice. *Mol Ther.* 2012; 20:1981–1987. [PubMed: 22871666]
24. Endo F, Kubo S, Awata H, Kiwaki K, Katoh H, Kanegae Y, Saito I, Miyazaki J, Yamamoto T, Jakobs C, Hattori S, Matsuda I. Complete rescue of lethal albino c14CoS mice by null mutation of 4-hydroxyphenylpyruvate dioxygenase and induction of apoptosis of hepatocytes in these mice by in vivo retrieval of the tyrosine catabolic pathway. *J Biol Chem.* 1997; 272:24426–24432. [PubMed: 9305902]

25. Manning K, Al-Dhalimy M, Finegold M, Grompe M. In vivo suppressor mutations correct a murine model of hereditary tyrosinemia type I. *Proc Natl Acad Sci U S A*. 1999; 96:11928–11933. [PubMed: 10518553]
26. Lindstedt S, Holme E, Lock EA, Hjalmarson O, Strandvik B. Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. *Lancet*. 1992; 340:813–817. [PubMed: 1383656]
27. Larochelle J, Alvarez F, Bussieres JF, Chevalier I, Dallaire L, Dubois J, Faucher F, Fenyves D, Goodyer P, Grenier A, Holme E, Laframboise R, Lambert M, Lindstedt S, Maranda B, Melancon S, Merouani A, Mitchell J, Parizeault G, Pelletier L, Phan V, Rinaldo P, Scott CR, Scriver C, Mitchell GA. Effect of nitisinone (NTBC) treatment on the clinical course of hepatorenal tyrosinemia in Quebec. *Mol Genet Metab*. 2012; 107:49–54. [PubMed: 22885033]
28. Lisowski L, Lau A, Wang Z, Zhang Y, Zhang F, Grompe M, Kay MA. Ribosomal DNA integrating rAAV-rDNA vectors allow for stable transgene expression. *Mol Ther*. 2012; 20:1912–1923. [PubMed: 22990671]
29. Wang Z, Lisowski L, Finegold MJ, Nakai H, Kay MA, Grompe M. AAV vectors containing rDNA homology display increased chromosomal integration and transgene persistence. *Mol Ther*. 2012; 20:1902–1911. [PubMed: 22990673]
30. Barzel A, Paulk NK, Shi Y, Huang Y, Chu K, Zhang F, Valdmann PN, Spector LP, Porteus MH, Gaensler KM, Kay MA. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature*. 2015; 517:360–364. [PubMed: 25363772]
31. Chandler RJ, LaFave MC, Varshney GK, Trivedi NS, Carrillo-Carrasco N, Senac JS, Wu W, Hoffmann V, Elkahoul AG, Burgess SM, Venditti CP. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J Clin Invest*. 2015; 125:870–880. [PubMed: 25607839]
32. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006; 441:537–541. [PubMed: 16724069]
33. Hill-Kayser CE, Plastaras JP, Tochner Z, Glatstein E. TBI during BM and SCT: review of the past, discussion of the present and consideration of future directions. *Bone Marrow Transplant*. 2011; 46:475–484. [PubMed: 21113184]
34. Gerull S, Beard BC, Peterson LJ, Neff T, Kiem HP. In vivo selection and chemoprotection after drug resistance gene therapy in a nonmyeloablative allogeneic transplantation setting in dogs. *Hum Gene Ther*. 2007; 18:451–456. [PubMed: 17536975]
35. Adair JE, Johnston SK, Mrugala MM, Beard BC, Guyman LA, Baldock AL, Bridge CA, Hawkins-Daarud A, Gori JL, Born DE, Gonzalez-Cuyar LF, Silbergeld DL, Rockne RC, Storer BE, Rockhill JK, Swanson KR, Kiem HP. Gene therapy enhances chemotherapy tolerance and efficacy in glioblastoma patients. *J Clin Invest*. 2014; 124:4082–4092. [PubMed: 25105369]
36. Mitchell C, Mignon A, Guidotti JE, Besnard S, Fabre M, Duverger N, Parlier D, Tedgui A, Kahn A, Gilgenkrantz H. Therapeutic liver repopulation in a mouse model of hypercholesterolemia. *Hum Mol Genet*. 2000; 9:1597–1602. [PubMed: 10861286]
37. Mignon A, Guidotti JE, Mitchell C, Fabre M, Wernet A, De La Coste A, Soubrane O, Gilgenkrantz H, Kahn A. Selective repopulation of normal mouse liver by Fas/CD95-resistant hepatocytes. *Nat Med*. 1998; 4:1185–1188. [PubMed: 9771754]
38. Jorquera R, Tanguay RM. Cyclin B-dependent kinase and caspase-1 activation precedes mitochondrial dysfunction in fumarylacetoacetate-induced apoptosis. *FASEB J*. 1999; 13:2284–2298. [PubMed: 10593876]
39. Russell DW, Grompe M. Adeno-associated virus finds its disease. *Nat Genet*. 2015; 47:1104–1105. [PubMed: 26417859]
40. Holme E, Lindstedt S. Nontransplant treatment of tyrosinemia. *Clin Liver Dis*. 2000; 4:805–814. [PubMed: 11232358]
41. Leung L, Kalgutkar AS, Obach RS. Metabolic activation in drug-induced liver injury. *Drug Metab Rev*. 2012; 44:18–33. [PubMed: 21939431]
42. Urban TJ, Daly AK, Aithal GP. Genetic basis of drug-induced liver injury: present and future. *Semin Liver Dis*. 2014; 34:123–133. [PubMed: 24879978]

43. Grompe M, Al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes Dev.* 1993; 7:2298–2307. [PubMed: 8253378]
44. Grompe M, Lindstedt S, Al-Dhalimy M, Kennaway NG, Papaconstantinou J, Torres-Ramos CA, Ou CN, Finegold M. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. *Nat Genet.* 1995; 10:453–460. [PubMed: 7545495]
45. Fellmann C, Hoffmann T, Sridhar V, Hopfgartner B, Muhar M, Roth M, Lai DY, Barbosa IA, Kwon JS, Guan Y, Sinha N, Zuber J. An optimized microRNA backbone for effective single-copy RNAi. *Cell Rep.* 2013; 5:1704–1713. [PubMed: 24332856]
46. Grimm D, Kay MA, Kleinschmidt JA. Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. *Mol Ther.* 2003; 7:839–850. [PubMed: 12788658]
47. Wang X, Montini E, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. Kinetics of liver repopulation after bone marrow transplantation. *Am J Pathol.* 2002; 161:565–574. [PubMed: 12163381]

Fig. 1. Identification of an shRNA that rescues *Fah* deficiency

(A) The tyrosine catabolic pathway. Genetic deficiency of *Fah* causes hereditary tyrosinemia type 1 (HT1) due to accumulation of fumarylacetoacetate (FAA) in hepatocytes. The disease can be treated pharmacologically (NTBC) or by shRNA knock-down of the genes required for making FAA. CEHPOBA inhibits FAH and causes accumulation of FAA. TAT, tyrosine amino transferase; HPD, 4-OH-phenylpyruvate dioxygenase; HGD, homogentisic acid dioxygenase; MAI, maleylacetoacetate isomerase; FAH, fumarylacetoacetate hydrolase. (B) Lentiviral construct. shRNAs targeting *Hpd*, *Hgd*, or *Tat* were expressed from a U6 promoter. Vector expresses a GFP reporter. (C) Experimental time line. Neonatal *Fah*^{-/-} mice were injected with sh*Tat*, sh*Hpd*, sh*Hgd* lentiviruses or a vector devoid of any shRNA (control) and kept on NTBC until weaning. NTBC was then withdrawn to permit liver injury and selection of resistant hepatocytes. (D) Mouse weights during selection starting at age 5 weeks. Grey bars represent periods of intermittent NTBC therapy. Only the control, sh*Hgd* and sh*Tat* cohorts were given NTBC after week 6. Data are mean $\pm$ SD (downward tick) ($n=4-6$). (E) Mice were injected with a non-selectable vector (control) or sh*Hpd*. Liver tissues were stained for the reporter GFP and for α -fetoprotein (AFP), which is highly expressed in mutant *Fah*^{-/-} hepatocytes. Yellow arrow denotes the absence of AFP within a selected nodule, compared with AFP-positive surrounding tissue (black arrows). Scale bar = 100 μ m. (F) PCR amplification of genomic liver DNA with primers flanking the lentiviral-shRNA sequence (two primer sets).

Fig. 2. Selection of integrating rAAV vectors

(A) Ribosomal rAAV constructs capable of chromosomal integration. (Top) Standard vector control; (bottom) selectable *Hpd* shRNA vector. Both vectors contain ribosomal DNA (rDNA) homology arms to enhance chromosomal integration, and both expressed human F9. (B) Experimental time line. Selection: Periods of NTBC withdrawal were different between cohorts and are indicated in panel C. (C) Weights and NTBC administration in animals receiving control or *shHpd* vector. Black rectangles indicate periods of NTBC treatment in control mice, whereas grey bar is NTBC for mice receiving *shHpd*. Data are means $\pm$ SD ($n = 3-5$). (D) Plasma human F9 measured by ELISA. Data are means $\pm$ SD ($n=4-5$). * $p<0.05$, ** $p=0.01$ versus controls. Differences were evaluated by student two-tailed t-test assuming equal variance.

Fig. 3. Selection of targeted integrations in the albumin locus

(A) Generide rAAV constructs designed for chromosomal integration. (Top) The selectable *shHpd* construct is driven by the pol3 U6 promoter. (Below) The *shHpd* is embedded within a miRNA and controlled by the endogenous albumin promoter. Both albumin-targeted “generide” vectors encoded human *F9* cDNA flanked by mouse albumin homology arms. The structures of the wild-type and gene-targeted albumin locus are also shown. Homologous recombination led to generation of a fused mRNA transcript. RNA processing liberated the *shHpd* and ribosome skipping at the 2A-peptide coding sequence generated separate mouse albumin (Alb) and hF9 proteins. (B) Experimental time line. Selection cycles starting week 5: off NTBC for 3 weeks, then on for 5 days, until week 20. (C) Plasma human F9 measured by ELISA in mice treated with the selectable or control gene-ride rAAV. The 5% and 100% levels of normal human F9 blood levels are shown with a dashed line. Data are means $\pm$ SD ($n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$ versus controls. Differences were evaluated by student two-tailed t-test assuming equal variance. (D) Human F9 liver immunohistochemistry showing representative nodules from mice with plasma F9 levels of 38,000 and 27,000 ng/ml (high), 800 ng/ml (low), and a control (no selection). Scale bars, 100 μ m. (E) Plasma human F9 levels in mice treated with the selectable gene-ride rAAV and subjected to NTBC withdrawal from age 6-9 weeks, followed by reintroduction of NTBC

thereafter. The dashed line indicates the therapeutic level of hF9 (250 ng/ml, or 5%). Data are single measurements from individual mice ($n = 3$).

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Fig. 4. Histology of shHpd selected hF9-positive nodules

(A) Serial sections of representative fields from three separate *Fah*^{-/-} mice after multiple cycles of selection (NTBC withdrawal). Mice were given either the shHpd vector or saline control. The adjacent sections were stained for expression of hF9, glutamine synthase, a marker for hepatocytes adjacent to the central vein of the hepatic lobule (zone 3), H&E and the proliferation marker Ki67. The “x” and “o” symbols mark vessels as landmarks for lining up the serial sections. For shHpd mouse 1 (shHpd1), serial sections are the same area in low magnification (62×);. For shHpd mouse 2 (shHpd2) and the control mouse, serial sections are high magnification (150×). Black arrows indicate nodules expressing human F9. Scale bars, 100 μm. (B) In vivo selection after gene transfer in adults. Four adult male *Fah*^{-/-} mice treated from birth with NTBC were injected with 8×10^{11} vg of the generide vector (Fig. 3A) at day 52. NTBC therapy was stopped 10 days later. Human F9 levels were measured. Data are averages $\pm$ SD (n = 4). Differences between hF9 levels at different time points were evaluated by student two-tailed t-test assuming equal variance.

Fig. 5. Selection of gene-targeted hepatocytes in wild-type mice using pharmacologic FAH inhibition

(A) Experimental time line. Beginning at 4 weeks of age, the mice were given daily i.p. injections of CEHPOBA (1 μ mol/g) or saline until 8-weeks of age. (B) Plasma human F9 measured by ELISA in mice treated with CEHPOBA (red symbols) or saline (blue symbols). Data for individual mice are shown. Dashed line denotes 5% of normal F9 levels, which is considered therapeutic. The grey rectangle indicates CEHPOBA treatment periods. A second period of CEHPOBA treatment was administered to only one mouse (inverted triangle). (C) Human F9 immunohistochemistry showing representative liver nodules from two separate CEHPOBA-treated mice and two saline injected controls. Arrows denote hF9-positive hepatocytes. Scale bars, 100 μ m. (D) H&E staining of liver and kidney tissues from a representative CEHPOBA-treated animal and saline-treated control. Scale bars, 100 μ m.

Fig. 6. Histology of CEHPOBA-treated livers

(A) Liver histology of a representative CEHPOBA-treated and a saline control one month after stopping the drug. An untreated age-matched *Fah*^{-/-} off NTBC (a positive control from a separate experiment) is shown at right for comparison. Serial sections were stained with H&E, followed by antibody stains for Ki67, HNF4α, and CYP2E1. Black arrows show portal bile ducts. The “*” marks central veins and “o” delineates portal veins. Pleiocytosis is marked by yellow arrows. (B) Relative abundance of fused transcripts/mouse albumin transcripts from saline-treated C57Bl6 mice, CEHPOBA-treated C57Bl6 mice (8 weeks after completing selection), and NTBC-cycled *Fah*^{-/-} mice injected with either control vector or the generide vector harboring the shHpd selection cassette (age 20 weeks, see Fig.3C). Data are individual animals (n=3) with means +/- SD.

Table 1
Liver function tests after CEHPOBA selection

Liver function parameters one month after the end of CEHPOBA or saline selection. Data are means +/- SD. P-values were determined a student two-tailed t-test assuming equal variance.

Measurement	Saline (n = 3)	CEHPOBA (n = 3)	P-value
ALT (U/l)	17.3 +/- 2.1	15.3 +/- 1.5	0.13
AST (U/l)	68 +/- 32	39 +/- 8	0.10
Alkaline phosphatase (U/l)	111 +/- 30	114 +/- 47	0.46
Total bilirubin (mg/dl)	0.2 +/- 0.01	0.13 +/- 0.06	0.06
Albumin (g/dl)	2.9 +/- 0.2	2.9 +/- 0.2	0.5
Total protein (g/dl)	5.4 +/- 0.6	5.4 +/- 0.2	0.50