Ex Vivo Hepatocyte Reprograming Promotes Homology-Directed DNA Repair to Correct Metabolic Disease in Mice After Transplantation

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Ex vivo **CRISPR/Cas9-mediated gene editing in hepatocytes using homology-directed repair (HDR) is a potential alternative curative therapy to organ transplantation for metabolic liver disease. However, a major limitation of this approach in quiescent adult primary hepatocytes is that nonhomologous end-joining is the predominant DNA repair pathway for double-strand breaks (DSBs). This study explored the hypothesis that *ex vivo* hepatocyte culture could reprogram hepatocytes to favor HDR after CRISPR/Cas9-mediated DNA DSBs. Quantitative PCR (qPCR), RNA sequencing, and flow cytometry demonstrated that within 24 hours, primary mouse hepatocytes in *ex vivo* monolayer culture decreased metabolic functions and increased expression of genes related to mitosis progression and HDR. Despite the down-regulation of hepatocyte function genes, hepatocytes cultured for up to 72 hours could robustly engraft *in vivo*. To assess functionality long-term, primary hepatocytes from a mouse model of hereditary tyrosinemia type 1 bearing a single-point mutation were transduced *ex vivo* with two adeno-associated viral vectors to deliver the Cas9 nuclease, target guide RNAs, and a 1.2-kb homology template. Adeno-associated viral Cas9 induced robust cutting at the target locus, and, after delivery of the repair template, precise correction of the point mutation occurred by HDR. Edited hepatocytes were transplanted into recipient fumarylacetoacetate hydrolase knockout mice, resulting in engraftment, robust proliferation, and prevention of liver failure. Weight gain and biochemical assessment revealed normalization of metabolic function. *Conclusion:* The results of this study demonstrate the potential therapeutic effect of *ex vivo* hepatocyte-directed gene editing after reprogramming to cure metabolic disease in a preclinical model of hereditary tyrosinemia type 1. (*Hepatology Communications* 2019;3:558-573).**

Liver transplantation remains the only curative therapy for metabolic liver disease. However, the procedure is severely limited by a shortage of donor organs, potential for graft loss, and requirement for life-long immunosuppression. Allogeneic hepatocyte transplantation, in which hepatocytes are isolated from cadaveric organs deemed unsuitable for transplantation, has shown some efficacy in the clinic.[1] However, this procedure has the same limitations as liver transplantation with even more pronounced loss of transplanted hepatocytes over time, presumably due to immune rejection.[2] Autologous

Abbreviations: AAV, adeno-associated viral; AAV-HT, AAV vector carrying a second Cas9 guide RNA and 1.2-kb homology region of the Fah *gene with corrected hereditary tyrosinemia mutation and modified protospacer adjacent motif sequence; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; bp, base pair; DSB, double-strand break;* Fah*, fumarylacetoacetate hydrolase, AAV-Cas9, adeno-associated viral vector carrying Staphylococcus aureus Cas9 and a guide RNA targeting the* Fah *locus; gRNA, guide RNA; HDR, homology-directed repair; HT1, hereditary tyrosinemia type I; mo, month old; MOI, multiplicity of infection; NGS, next-generation sequencing; NHEJ, nonhomologous end-joining; ns, not significant; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexadione; PCR, polymerase chain reaction; qPCR, quantitative PCR; RNA-Seq, ribonucleic acid sequencing; sgRNA, single-guide RNA; TBIL, total bilirubin; Tx, transplant.*

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hepatocyte transplantation, in which the patient's own hepatocytes are isolated after partial hepatectomy and corrected using gene therapy, is a potential curative therapy and has been used in the clinic for the treatment of familial hypocholesteremia using integrating gammaretroviral vectors.[3] More recently, the application of lentiviral vectors for curative gene therapy in hepatocytes has been demonstrated in a number of animal model systems,[5] including a pig model of hereditary tyrosinemia type I (HT1).[6]

CRISPR/Cas9-mediated gene editing is one such potential gene therapy platform by which mutations in genes can be corrected using a homology repair template.[7] However, for precise editing to occur, the cell of interest must be primarily repairing double-strand breaks (DSBs) using homology-directed repair (HDR). For most cells, including hepatocytes, DNA repair using HDR occurs strictly during an active cell cycle.[8] As most adult hepatocytes are in the G_0 phase of the cell cycle,[9] DSBs are repaired by nonhomologous end-joining (NHEJ), making adult hepatocytes minimally predisposed to precisely correcting breaks. The suggestion that cells in a more active phase of the cell cycle with up-regulated DSB repair genes are more inclined to HDR has been made by a number of studies,[10,11] including recent *in vivo* hepatocyte-directed CRISPR/Cas9-mediated gene editing, in which optimal gene repair occurred in 2-day-old neonatal mice, whose hepatocytes are actively dividing.[7] The ability to optimize HDR in hepatocytes will bring gene-editing therapies for adult patients with liver disease one step closer to clinical application.

Previous studies have noted differential gene regulation in hepatocytes in culture,[12] but have not identified the effects that this has on gene editing. In this study, we explored the potential for hepatocytes cultured *ex vivo* to activate necessary DNA repair machinery for CRISPR/Cas9-mediated gene correction to occur by HDR. The results herein demonstrate that hepatocytes have the inherent ability to rapidly change expression of genes related to DNA repair by HDR. *Ex vivo*–cultured hepatocytes are able to undergo CRISPR/Cas9-mediated gene repair, and corrected hepatocytes can then engraft and proliferate *in vivo* to correct metabolic disease in a mouse model of HT1.

Materials and Methods

PLASMID AND VECTOR CONSTRUCTION

Two guides targeting the point mutation in exon 8 of the *Fah* (fumarylacetoacetate hydrolase) gene

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were designed using Benchling.com. For the first single-guide RNA (sgRNA), oligonucleotides containing *BsaI*-clonable overhangs were ligated downstream of a U6 promoter in an adeno-associated viral (AAV) vector expressing *S. aureus* Cas9 (pX601-AAV-CMV:NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA was a gift from Feng Zhang [Addgene plasmid #61591]) (AAV-Cas9). A second *Fah*-targeting sgRNA was constructed using a synthesized gBlock (IDT, Inc., Coralville, IA) comprising a U6 promoter and sgRNA that was cloned into a second AAV vector containing a 1,192–base pair (bp) fragment of homology to the *Fah* exon 8 locus (AAV-HT). The repair template contained two modified bases that could be used for PCR screening of corrected alleles and modified protospacer adjacent motif sites at each guide to prevent recutting by Cas9. Both the AAV-Cas9 and the AAV-HT were created by standard triple-plasmid transfection of HEK-293T cells using Fugene6 (Promega, Madison, WI) with serotype DJ. Cells were incubated for 72 hours, collected, and the AAV particles were purified and stored at −80°C. Vector titers were determined by qPCR using the Luna Universal qPCR Mix (NEB, Ipswich, MA) with the following primers: 5′-TTGCATATACGATACAAGGCTGTT, 5′-AAAACTGCAAACTACCCAAGAAA.

ANIMALS AND ANIMAL CARE

All animals received humane care in compliance with the regulations of the institutional animal care and use committee at the Mayo Clinic. Mice were housed in static, nondisposable cages with corncob bedding, paper nesting material, and a microisolator filter top. Irradiated Rodent Laboratory Chow (Purina®, St. Louis, MO) was fed *ad libidum* through a wire rack. All interventions were performed during the light cycle. Fah^{5981SB} mice ($Fah^{-/-}$ mice), an established model of HT1 that bears a single-point mutation at the exon 8 locus, were a gift from Markus Grompe (Oregon Health & Science University, Portland, OR).[13,14] The protective medication, NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione), prevents weight loss and liver failure due to HT1 and was administered to the $Fah^{-/-}$ mice in their drinking water at 8 mg/L, unless withdrawal was indicated.[15] Hepatocytes for the comparative qPCR experiment were isolated from a 3.5-month-old (mo) female $Fah^{-/-}$ mouse. Hepatocytes for ribonucleic acid sequencing (RNA-Seq) experiments were isolated from a 7-mo female $Fah^{-/-}$ mouse. Hepatocytes for the flow cytometry experiment were isolated from a 3.5-mo female $Fah^{-/-}$ mouse. Hepatocytes for the Ki-67 flow cytometry positive control were isolated from a 6-mo male $Fah^{-/-}$ mouse. Hepatocytes for bioluminescent imaging experiments were isolated from a 3-mo female C57BL/6 mouse and were transplanted into nine 6-mo male C57BL/6 mice. Hepatocytes for *in vitro* testing of the dual AAV system were isolated from a 2-mo female $Fah^{-/-}$ mouse. Hepatocytes transduced *ex vivo* with AAV-Cas9 and AAV-HT and transplanted into six $Fah^{-/-}$ mice (3 male and 3 female) were isolated from a 3.5-mo female $Fah^{-/-}$ mouse. The four control $Fah^{-/-}$ mice used to monitor weight change were same-sex littermates to their respective experimental animals. To encourage positive selection for FAH+ hepatocyte proliferation, NTBC was withheld from the six $Fah^{-/-}$ transplant recipient mice and their respective weight controls until they showed signs of severe weight decline, at which point it was administered in their drinking water at 8 mg/L for 5-7 days before it was withdrawn again. The control animals were sacrificed when they—but not the treated experimental animals—could not tolerate NTBC withdrawal. Control animals for the blood data were either 9.5-mo male $Fah^{-/-}$ mice who were withdrawn from NTBC for 20 days before sacrifice (−NTBC; n = 5) or 4.5-mo male $Fah^{-/-}$ mice who were on NTBC until sacrifice (+NTBC; n = 5). For serial transplantations, the original donor mouse was a 2.5-mo $Fah^{-/-}$ male. Hepatocytes were isolated and transplanted into two 2-mo $Fah^{-/-}$ male mice, which were cycled on and off NTBC until NTBC-independent weight gain was achieved. The livers of these two animals were perfused and those hepatocytes were transplanted into seven 2-mo $Fah^{-/-}$ female mice that were again cycled on and off NTBC until NTBC-independent weight gain was achieved.

HEPATOCYTE AND FIBROBLAST ISOLATION AND CELL CULTURE

Fibroblast cell lines were derived from neonatal $Fah^{-/-}$ mouse tissue and were immortalized using a lentiviral vector expressing the small and large SV40 T antigens. Fibroblasts were kept in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific,

Waltham, MA) containing 10% heat-inactivated fetal bovine serum (Corning, Herndon, VA) and 1% penicillin/streptomycin (Corning, Inc., Herndon, VA). Primary hepatocytes were harvested from an anesthetized donor mouse by a standard *in situ* perfusion of the liver using Collagenase NB (SERVA, Heidelberg, Germany) and Thermolysin (MilliporeSigma, St. Louis, MO). Hepatocytes were plated into 6-well Primaria plates (BD Biosciences, San Jose, CA) containing the following media: DMEM, 10% fetal bovine serum, 10 mM HEPES, 10 μM Dexamethasone (MilliporeSigma), 10 ng/mL murine epidermal growth factor (PeproTech, Rocky Hill, NJ), and 1% penicillin/streptomycin. Both murine epidermal growth factor and corticosteroid dexamethasone are known to increase viral vector transduction in primary hepatocytes.[6,16] The culture medium for *Fah*$^{-/-}$ hepatocytes contained an additional 7 mg/L NTBC. One hundred minutes after plating, the culture medium was replaced. At this time, AAV-Cas9 and AAV-HT were added, if indicated. Cells were cultured for 72 hours, unless otherwise specified. All cells were kept at 37°C and 5% CO_2.

QPCR, RNA-Seq, AND BIOINFORMATICS

RNA from *Fah*$^{-/-}$ primary mouse hepatocytes cultured for 0 hours, 24 hours, or 48 hours was isolated with a Direct-zol RNA isolation kit (Zymo Research, Irvine, CA). A comparative computed tomography qPCR was run on generated complementary DNA with Luna Universal qPCR Master Mix (NEB, Ipswich, MA) on standard speed using the following parameters: 95°C, 3 minutes; 40 cycles of 95°C, 15 seconds, 60°C, 30 seconds, read; melt curve. Primers can be found in Supporting Table S2. Results were normalized to *B2m* expression. RNA-Seq was performed using a high-throughput sequencer (BGISEQ-500 service provided by Beijing Genomics Institute, Hong Kong, China). After sequencing, Fastq files were processed by the Mayo Clinic's internal MAP-RSeq pipeline (Version 2.1.1).[17] The aligning and mapping of reads were performed using the TopHat2 spliced aligner against mm10 reference genome.[18] The gene and exon counts were generated by FeatureCounts using gene definition files from the Ensembl v79 database.[19] Quality control was carried out using the RSeqQC quality control package[20] and some additional metrics[21] to ensure the results from each sample were reliable and could be used for differential expression analysis. Differential expression analysis was carried out using the Bioconductor package, edgeR (Version 3.20.1).[22] Differential expressed genes (DEGs) were identified with average expression of more than 2 counts per million, more than 1 absolute $\log_2$ fold change, and adjusted *P* value of less than 0.05. All bioinformatics statistical analysis was evaluated using the computing software R (Version 3.4.2). A total of 480 "DNA repair" genes and 112 "cellular and amino acid catabolism process" genes as defined by the Gene Ontology collection within the Molecular Signatures Database (Version 6.1)[23] were analyzed in detail (gene list in Supporting Table S1). Statistical significance and concordant differences were calculated using an associated gene set enrichment analysis.

Ki-67 FLOW CYTOMETRY

Hepatocytes were isolated as described previously. Cells were plated and cultured for 0 hours, 24 hours, or 48 hours, at which point they were harvested, fixed, and permeabilized using 5 mL of cold ethanol and incubated at −20°C. A Ki-67 positive control was created by performing a two-thirds partial hepatectomy in a mouse, perfusing the liver at 48 hours, and fixing the cells in cold ethanol. Samples were then washed twice with 30 mL staining buffer (phosphate-buffered saline, 1% fetal bovine serum, 0.09% sodium azide) and centrifuged for 10 minutes at 200*g*. A total of 1×10^6 hepatocytes were stained with 0.125 μg phycoerythrin/Cy7 antimouse Ki-67 antibody (BioLegend, San Diego, CA) and incubated for 30 minutes in the dark. The cells were washed using staining buffer. Stained hepatocytes were run by the Mayo Clinic Flow Cytometry Core and analyzed with FlowJo12.0 software (FlowJo, LLC, Ashland, OR). Forward and side scatter were used to define single cells, and an unstained control sample was used to define the boundary of the Ki-67 positive population.

LUCIFERASE TRANSFECTION AND BIOLUMINESCENT IMAGING

Primary hepatocytes were cultured for 24 hours, 48 hours, or 72 hours, and 3 hours before harvest, each well of 300,000 cells was transfected with

3 μg messenger RNA (mRNA) for Firefly Luciferase (TriLink Biotechnologies, San Diego, CA). At each time point, hepatocytes were harvested and transplanted into three mice through splenic injection. Recipient mice were given an intraperitoneal injection of 150 mg/kg D-luciferin (Regis Technologies, Inc., Morton Grove, IL) 10 minutes before *in vivo* imaging in a Xenogen IVIS Imager (PerkinElmer, Inc., Waltham, MA). Recipient mice were imaged at 2 hours and 26 hours following transplantation. At the 26-hour time point, livers were excised and imaged *ex vivo*.

PCR AND T7 ENDONUCLEASE ANALYSIS

Total cells from *in vitro* assays were collected and DNA was purified using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). For PCR amplification of Cas9-disrupted sequence, the following primers were used: 5′-GGGCTCATGTGAGTAGACTTCC, 5′-TCAGCTCCATCCTTCCACTT. PCR amplification was conducted using Phusion polymerase (NEB, Ipswich, MA) as follows: 98°C, 3 minutes; 35 cycles of 98°C, 10 seconds, 63°C, 20 seconds, 72°C, 30 seconds; 72°C, 5 minutes. Products were ~452 bp in length. The sequences were processed using a T7 endonuclease Alt-R Genome Editing Detection Kit (IDT, Inc., Coralville, IA). For selective amplification of corrected sequence, the following primers were used: 5′-TGGAGCGGTAATGCCTCC for binding selectively to the corrected sequence and 5′-AAAATGCAGGATCCACCAAG for binding outside of the homology region. Only DNA that had successfully incorporated the AAV-HT would amplify by this method. PCR conditions were as follows: 98°C, 3 minutes; 35 cycles of 98°C, 10 seconds, 61°C, 20 seconds, 72°C, 45 seconds; 72°C, 5 minutes. Products were ~859 bp in length. PCR and T7 products were electrophoresed on a 2% Tris-acetate-ethylenediamine tetraacetic acid buffer agarose gel and visualized with ethidium bromide staining.

OFF TARGET ANALYSIS AND DNA SEQUENCING

For examination of indels created at the *Fah* locus in fibroblasts by AAV-Cas9 alone, a 452-bp PCR amplicon (primers above) surrounding the *Fah* single-nucleotide polymorphism region was generated and sent to GENEWIZ (South Plainfield, NJ) to undergo their Amplicon-EZ service on an Illumina 2 × 250 bp platform. To detect disruption and editing efficiency in cultured hepatocytes treated with both AAV-Cas9 and AAV-HT, as well as to find the top 10 sequences in edited mouse livers, snap-frozen pieces of liver tissue from six experimental mice were digested using a DNeasy Blood and Tissue Kit (QIAGEN), and the same 452-bp amplicon was generated. Samples were sent to GENEWIZ and underwent their Amplicon-EZ. To identify potential off-target sites, the online software program Cas-OFFinder (Center for Genome Engineering, Seoul, South Korea) was used.[24] Based on previous studies,[25,26] parameters for each of the two guide RNAs (gRNAs) were set at equal or less than two mismatches, equal or less than one DNA bulge size, and equal or less than one RNA bulge size. Primers were designed to amplify 200 bp to 250 bp regions around these sites (Supporting Table S3). DNA was isolated from the livers of six treated animals and three untreated controls as previously. PCR products were generated for each sample at each of the seven sites, and the purified samples were sent to GENEWIZ to undergo their Amplicon-EZ service.

HEPATOCYTE TRANSPLANTATION

Primary hepatocytes transduced with AAV-Cas9 and AAV-HT at multiplicity of infection (MOI) 500,000 genome copies and 200,000 genome copies, respectively, were cultured for 48 hours and were then harvested with 0.05% trypsin/ethylene diamine tetraacetic acid, centrifuged, and suspended for transplantation in 200 μL media containing 2 ug/mL DNaseI (Sigma-Aldrich, St. Louis, MO). The cells were then injected into recipient *Fah*$^{-/-}$ mice through an intrasplenic injection, as described previously.[27] For serial transplantations, the first iteration of edited *Fah*$^{-/-}$ hepatocytes underwent this treatment and was transplanted into recipient *Fah*$^{-/-}$ mice through intrasplenic injection. These hepatocytes were again isolated after proliferation and were transplanted into another set of *Fah*$^{-/-}$ recipients without further *ex vivo* treatment or culture.

HISTOLOGY AND BIOCHEMICAL ANALYSIS

For histological analysis, tissue samples were fixed in 10% neutral buffered formalin (Protocol; Fisher-Scientific, Pittsburgh, PA) and processed for paraffin embedding and sectioning. For hematoxylin and eosin staining, slides were prepared with standard protocols. FAH immunohistochemistry using a polyclonal rabbit anti-FAH primary antibody[28] was performed with a Bond III automatic stainer (Leica Biosystems, Buffalo Grove, IL) with a 20-minute antigen retrieval step using Bond Epitope Retrieval Solution 2 (Leica Biosystems) and stained with diaminobenzidine (Leica Biosystems). Quantification of FAH+ cells was obtained using a cytoplasmic stain algorithm in Aperio ImageScope (Leica Biosystems). Fifteen rectangular areas totaling 954,829.4 μm^2 were randomly selected and analyzed. Reported results were calculated as the total percentage of cytoplasmic FAH positivity among the cells selected. For biochemical analysis of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total bilirubin (TBIL), plasma was analyzed with the VetScan VS2 benchtop analyzer (Mammalian Liver Profile; Abaxis, Union City, CA). Tyrosine values were determined using tandem mass spectrometry and chromatography through Mayo Clinic's internal biochemical phenylketonuria test.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software (Version 7; GraphPad Software, La Jolla, CA). Experimental groups were compared using an unpaired, two-tailed Student *t* test. Differences between multiple groups were compared using a one-way analysis of variance followed by Tukey's multiple comparisons test. A *P* value of less than 0.05 was considered statistically significant.

Results

EX VIVO CULTURE RAPIDLY REPROGRAMS PRIMARY HEPATOCYTES

Cellular quiescence has been associated with less active DNA repair pathways, which are a key component of successful site-specific gene editing.[29] To characterize the changes in hepatocyte gene expression in *ex vivo* cultured mouse hepatocytes, we performed a comparative qPCR on five genes required for homologous recombination: *Brca1*, *Brca2*, *Rad51*, *Rad52*, and *Pcna*.[30-32] When grouped, these genes are up-regulated over time (Fig. 1A), and each gene individually showed a similar progressive up-regulation (Fig. 1B). To determine whether these changes were influenced by entry into the cell cycle, we conducted a flow cytometry assay for Ki-67+ mouse hepatocytes after 0 hours, 24 hours, or 48 hours of culture. This nuclear protein, whose absence reliably differentiates cells in G_0 from those in an actively proliferating phase,[33] was found in a greater percentage of cultured hepatocytes over the time course, suggesting that *ex vivo* culture advanced normally quiescent hepatocytes from G_0 phase into more active phases of cycling (Fig. 1C,D).

To further explore these gene expression trends, we performed RNA-Seq analysis on mouse hepatocytes cultured for 24 hours or 48 hours. We compared this to freshly isolated hepatocytes that were not cultured (0 hours). Overall, there was a high concordance among samples in the same group, and most of the differences were observed between the 0-hour group and the 24-hour or 48-hour groups (Fig. 1E). A principal component analysis confirmed these relationships (Supporting Fig. S1B). In total, 5,254 differentially expressed genes were identified between 0 hours and 48 hours (Fig. 1F and Supporting Fig. S1C). Gene set enrichment analysis between 0 hours and 48 hours found a striking enrichment of up-regulation in function annotations related to cell cycle and DNA repair regulations, and down-regulation in function annotations related to metabolic processes (Fig. 1G). Subsequently, two selected function annotations—DNA repair and the cellular amino acid catabolic process—were further examined (Fig. 1H), showing a clear enrichment of genes up-regulated at 48 hours for DNA repair function and genes down-regulated at 48 hours for the cellular amino acid catabolic process. Detailed summary statistics regarding these two selected function annotations are shown in Supporting Fig. S1D,E.

HEPATOCYTES CAN ENGRAFT *IN VIVO* AFTER *EX VIVO* CULTURE FOR AT LEAST 72 HOURS

As gene expression was significantly altered in hepatocytes after *ex vivo* culture, we assessed whether

cultured hepatocytes could engraft *in vivo* after intrasplenic injection. Qualitatively, no major differences among the three transplant groups were noted using noninvasive bioluminescence imaging at 26 hours following transplant (Fig. 2A). To quantify luciferase expression (given the inconsistencies of *in vivo* bioluminescence imaging), animals were euthanized at 26 hours following injection, and excised livers were subjected to bioluminescence imaging, also showing little qualitative difference among the groups (Fig. 2B). No significant differences in quantified total excised liver bioluminescence flux were noted among the groups, indicating that hepatocytes cultured for up to 72 hours did not lose their engraftability compared with 24 hours in culture—a common time point for multiple previous hepatocyte transplant experiments in mice.[4,6,16]

A DUAL-AAV VECTOR SYSTEM CAN EDIT A TARGET LOCUS IN THE *Fah* GENE

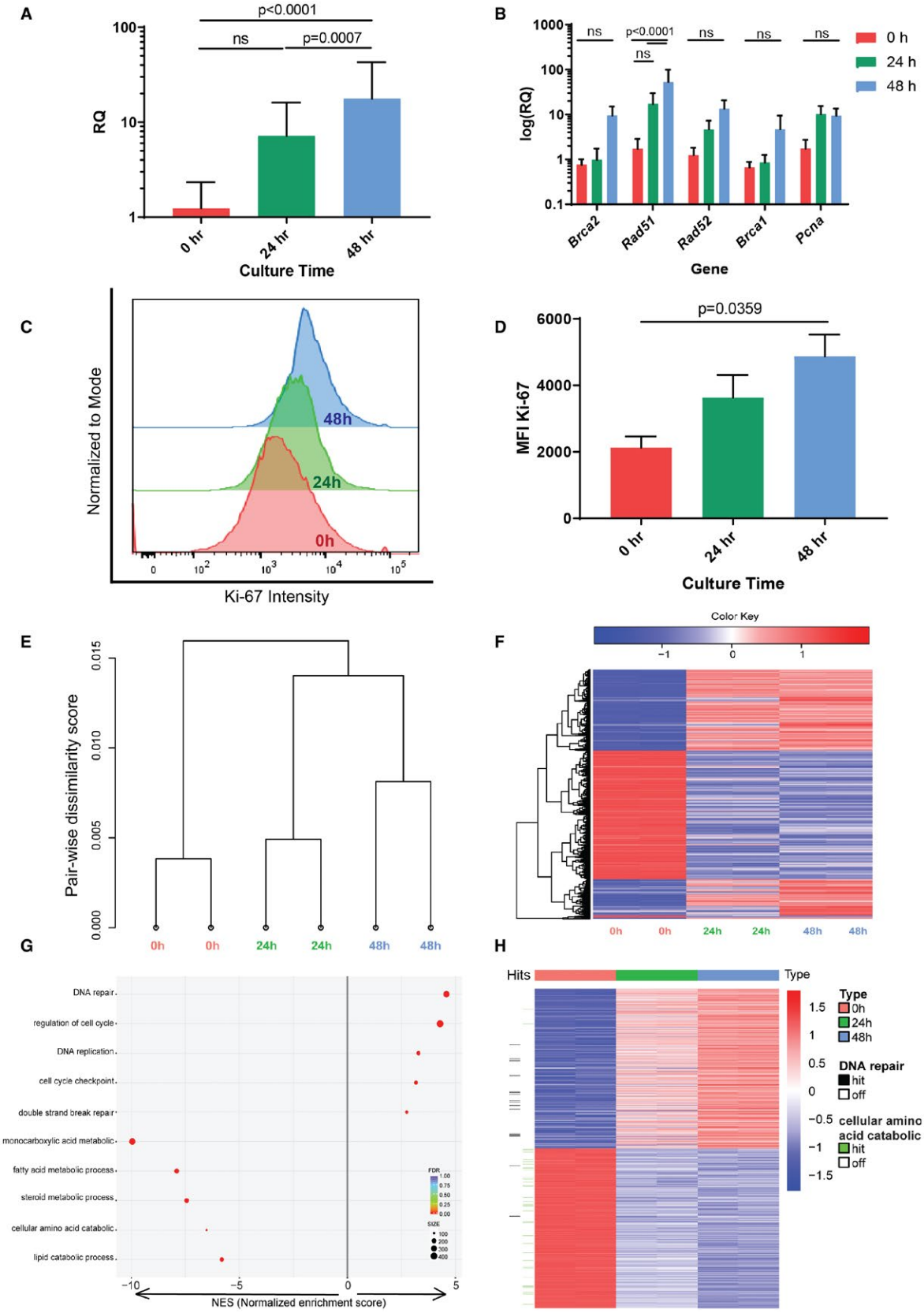
Because bioluminescence data suggested hepatocytes cultured *ex vivo* retained the ability to engraft *in vivo*, we set out to test the long-term effect of culturing and modifying primary hepatocytes. The *Fah*$^{-/-}$ locus and the two AAV vectors used are shown in Fig. 3A. To initially test this system *in vitro*, fibroblasts from *Fah*$^{-/-}$ mice were transduced with AAV-Cas9 to determine activity of *Staphylococcus aureus* Cas9 at the *Fah* locus (Supporting Fig. S2A-D). We then applied these vectors to cultured primary hepatocytes from *Fah*$^{-/-}$ mice. PCR amplicon next-generation sequencing (NGS) around the *Fah* locus indicated a variety of possible alleles at both guide sites, ranging from an unedited sequence to both HDR and NHEJ, caused by targeted disruption of the locus using CRISPR/Cas9 (Fig. 3B, and top 50 sequences are found in Supporting Table S4). In congruence with the *in vitro* data from fibroblasts, correction of the point mutation was dependent on expression of Cas9, and no gene correction could be detected in cells receiving only receiving AAV-HT (Fig. 3C). We also analyzed the timing of correction in hepatocytes after transduction with both AAV-HT and AAV-Cas9, harvesting the cells at 24 hours, 48 hours, and 72 hours for analysis. PCR analysis revealed a time-dependent correction of the mutation, with correction occurring after 48 hours and maximally at 72 hours (Fig. 3D).

TRANSPLANTED HEPATOCYTES CAN CURE LIVER DISEASE IN *Fah*$^{-/-}$ MICE

Based on the positive data showing gene correction in primary hepatocytes between 24 hours and 48 hours, we proceeded to test the efficacy of transplanting these gene-edited hepatocytes *in vivo* (Fig. 4A). Hepatocytes from *Fah*$^{-/-}$ mice were plated in media containing murine epidermal growth factor and dexamethasone and cultured for 48 hours after being transduced with both AAV-HT and AAV-Cas9. Hepatocytes were trypsinized and transplanted into adult male and female syngeneic *Fah*$^{-/-}$ mice by intrasplenic injection. Mice were cycled on/off the protective drug NTBC to provide a selective advantage for corrected FAH+ cells to proliferate. Female mice became NTBC-independent after 68 days, whereas male mice became NTBC-independent after 90 days (Fig. 4B). Control *Fah*$^{-/-}$ mice that were not transplant recipients never became weight stable off NTBC and were euthanized at the indicated times. All transplanted mice were euthanized 165 days after transplantation, and a thorough biochemical analysis was performed (Fig. 4C). In congruence with the weight data, transplanted mice had significantly reduced markers of liver injury, including ALP, TBIL, AST, and ALT. Considering the HT1 phenotype, tyrosine levels were significantly reduced to the levels seen in wild-type mice, indicating correction of metabolic disease. Albumin levels were also significantly improved compared with *Fah*$^{-/-}$ mice off NTBC.

GENE-CORRECTED HEPATOCYTES CAN PROLIFERATE EXTENSIVELY *IN VIVO*

To assess the *in vivo* potential for CRISPR/Cas9-edited hepatocytes to engraft and proliferate, we performed histopathological analyses on transplanted livers using FAH immunohistochemistry (Fig. 5A). A liver from an untreated *Fah*$^{-/-}$ control mouse showed no staining for FAH (Fig. 5B). Although female mice took one fewer NTBC cycle to rescue, they did not have a significantly increased percentage of FAH+ cells in the liver, nor did they have an increased number of FAH+ nodules. Next, a histopathological assessment of hematoxylin and eosin–stained


A
p<0.0001
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−1
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DNA repair
regulation of cell cycle
DNA replication
cell cycle checkpoint
double strand break repair
monocarboxylic acid metabolic
fatty acid metabolic process
steroid metabolic process
cellular amino acid catabolic
lipid catabolic process
FDR
SIZE
−10
−5
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5
NES (Normalized enrichment score)
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Hits
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48h
DNA repair
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−1.5

FIG. 1. *Ex vivo* reprogramming of cultured hepatocytes up-regulates DNA repair pathways. (A) Averaged relative quantification values showing expression levels of five genes involved in homologous recombination (*Brca1*, *Brca2*, *Rad51*, *Rad52*, and *Pcna*) relative to the length of culture as determined by comparative qPCR on RNA isolated from primary hepatocytes. Samples are standardized to *B2m* expression. (B) Deconvolution of these five genes, showing consistent up-regulation over time. (C) Representative histogram of Ki-67 intensity in primary hepatocytes after increasing time in culture, with peak heights normalized to mode of sample. (D) Quantification of mean fluorescence intensity in biological replicates (n = 2). (E) Hierarchical clustering of six RNA-Seq samples at three different time points (0 hours, 24 hours, and 48 hours). The 24-hour and 48-hour time points are more similar to one another than either is to 0 hours. (F) Heat map of gene expression in all samples for differentially expressed genes between times 0 hours and 48 hours (n = 5,254). (G) Enrichment analysis of highly affected gene sets clustered by biologic functions between cells in culture for 48 hours relative to 0 hours. (H) Detailed enrichment summary illustrating differences in the regulation of two selected Gene Ontology function annotations of interest—DNA repair (horizontal black bars on the left) and cellular amino acid catabolic process (horizontal green bars on the left)—among the three time points collected (presented by column). The heat map includes the top and bottom 500 differentially expressed genes ranked by fold change between times 0 hours and 48 hours. Individual DNA repair genes are up-regulated, whereas individual cellular and amino acid catabolic process genes are down-regulated in the 24-hour and 48-hour groups. Abbreviations: FDR, false discovery rate; and ns, not significant at $\alpha = 0.05$.

transplanted livers was performed by a pathologist. Healthy hepatocyte and parenchymal architecture correlated with FAH+ regions, with evidence of hyperplasia seen primarily in the FAH–negative regions (data not shown). No evidence of hepatocellular carcinoma was noted. Finally, high-throughput NGS at the *Fah* locus indicated that the corrected point mutation without the modified protospacer adjacent motif site (TCC present in AAV-HT) was the most common edited allele in 9.8% of sequences, whereas the corrected point mutation with the modified protospacer adjacent motif site was present in only 2.6% of alleles

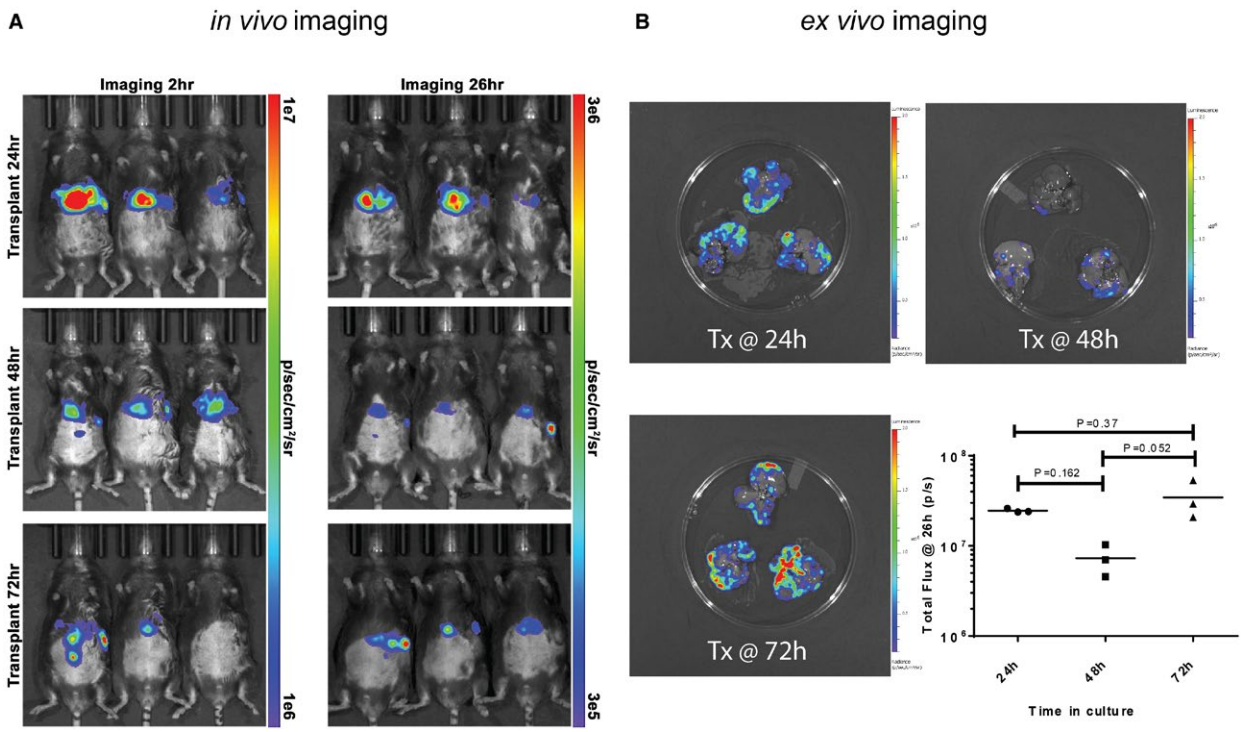


FIG. 2. Hepatocytes cultured *ex vivo* can engraft in the liver after at least 72 hours in culture. (A) *In vivo* imaging of hepatocyte transplant (Tx) recipients 2 hours and 26 hours following Tx. Hepatocytes were cultured for t = 24 hours, 48 hours, or 72 hours and transplanted through splenic injection (n = 3 for each Tx time point). Hepatocyte engraftment in the liver can be seen 26 hours after Tx, even in the group that received cells after 72 hours in culture. (B) All livers imaged *ex vivo* at the 26-hour time point showed luciferase activity from engrafted hepatocytes. Flux was standardized and quantified, showing no significant difference among the three groups.

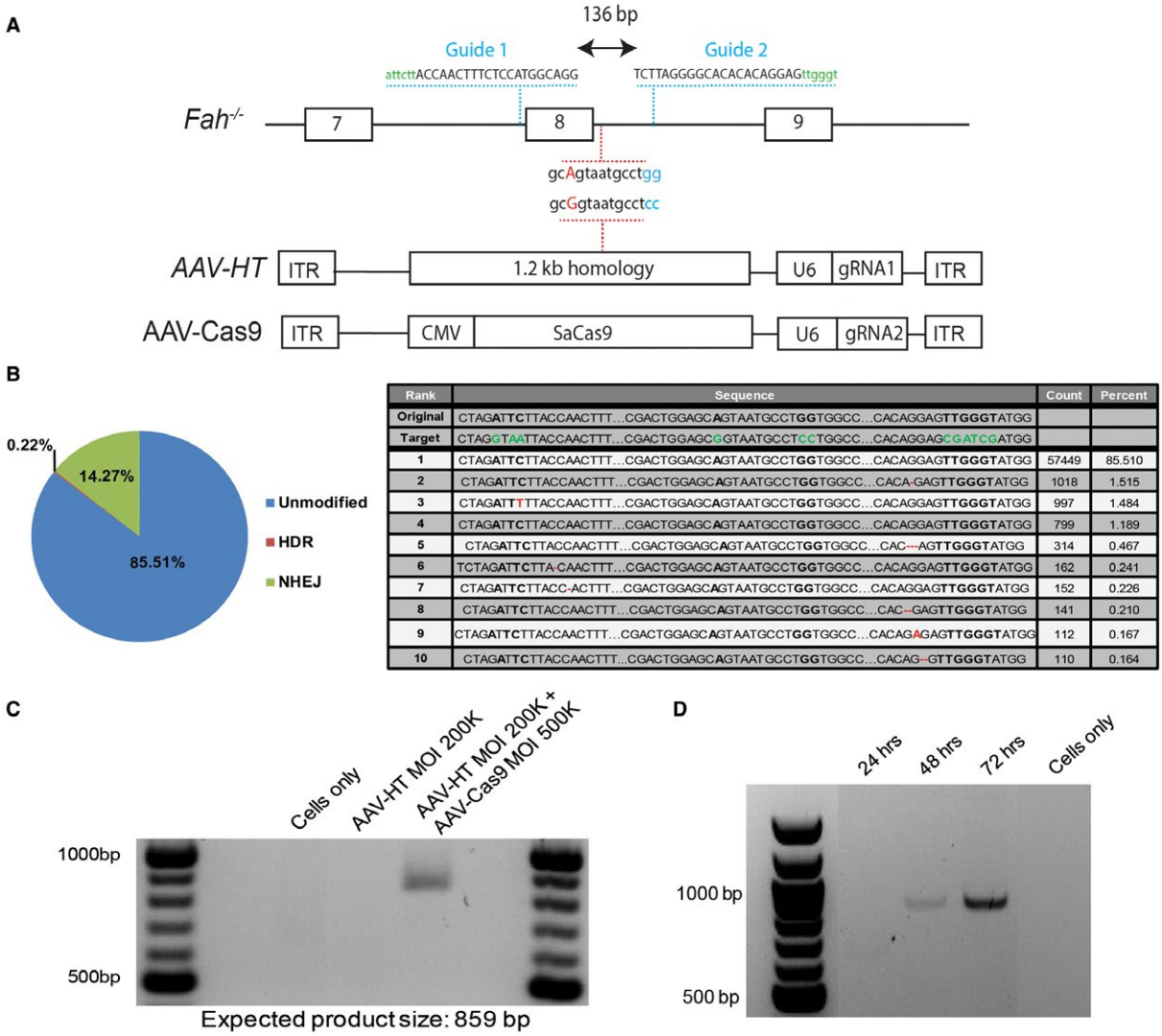


FIG. 3. AAV-Cas9 and AAV-HT can disrupt and correct the *Fah* locus in primary *Fah*$^{-/-}$ hepatocytes transduced *ex vivo*. (A) Schematic illustration of the position of the two Cas9 gRNAs and protospacer adjacent motif sequences (in green) that were used relative to the *Fah* locus and the HT1 mutation (in red). Also shown are the two AAV vectors used in the subsequent experiments. AAV-HT contains the corrected mutation, additional silent sequence modifications introduced for selective PCR (in blue), and Cas9 Guide 1. AAV-Cas9 carries the *Staphylococcus aureus* Cas9 gene under the cytomegalovirus promotor, as well as Cas9 Guide 2. (B) PCR amplicon deep sequencing of the *Fah* locus from primary *Fah*$^{-/-}$ hepatocytes transduced with AAV-Cas9 and AAV-HT showed that low gene-editing rates in hepatocytes are detectable after 72 hours in culture. Sequence distribution and top 10 hits are shown. Both complete and partial homologous recombination were classified as HDR. (C) Selective PCR amplification of the corrected sequence showed that correction was occurring in primary hepatocytes transduced with both AAV-Cas9 and AAV-HT, but not in hepatocytes transduced with AAV-HT alone. (D) Primary hepatocytes were transduced with AAV-Cas9 and AAV-HT, and were harvested at 24 hours, 48 hours, and 72 hours, respectively. The cells only control is primary hepatocytes that were not transduced and were collected at 72 hours. Abbreviations: CMV, cytomegalovirus; ITR, inverted terminal repeat.

(Fig. 5D and top 50 sequences in Supporting Table S5). However, given the natural polyploidy of hepatocytes and the need to have only one corrected allele to stain FAH+, the total number of FAH+ cells likely would be significantly higher than this percentage, as corroborated by immunohistochemistry.[34] A total of seven potential off-target loci were identified by algorithm, three of which were outside of gene-coding regions

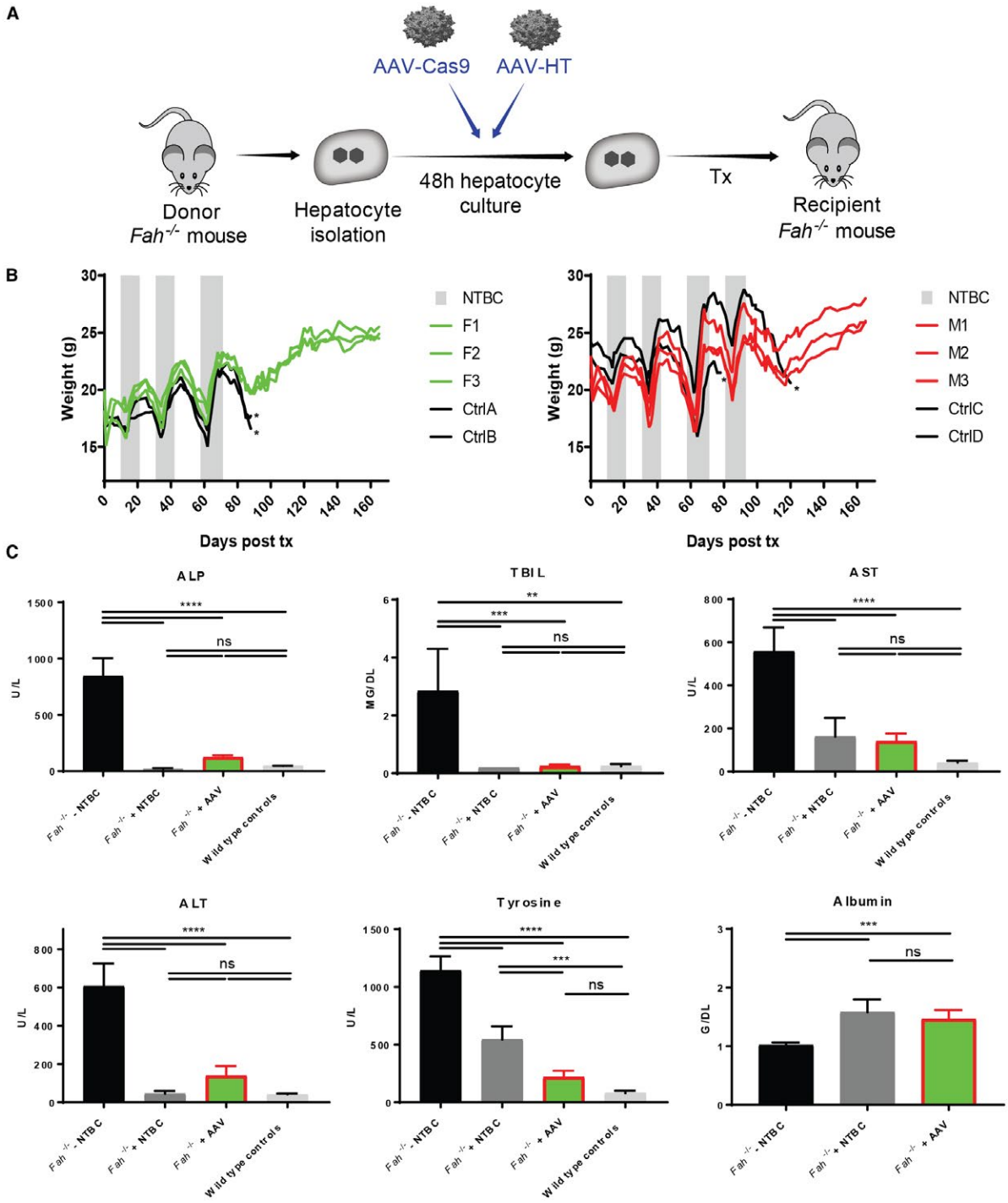
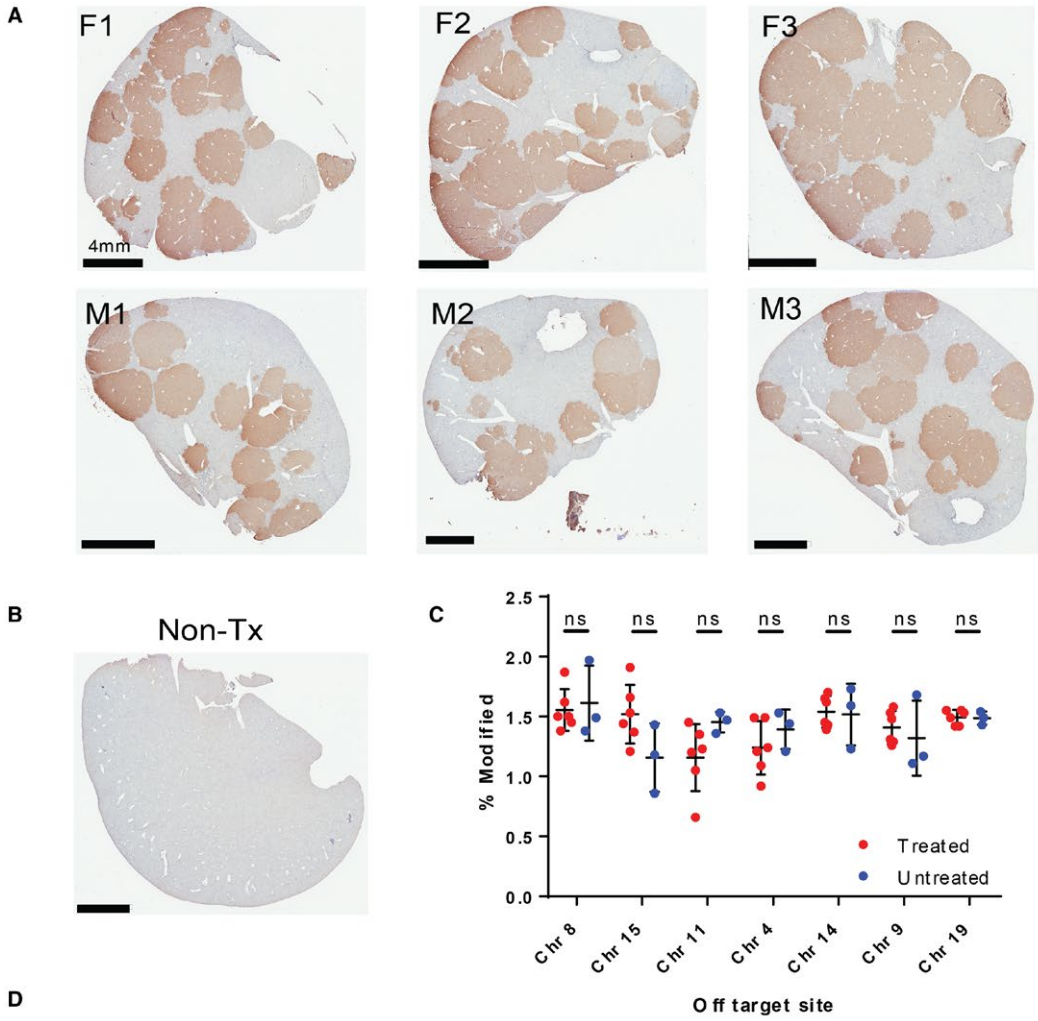


FIG. 4. Primary hepatocytes transduced with AAV-Cas9 and AAV-HT and cultured *ex vivo* can phenotypically correct a mouse model of hereditary tyrosinemia type I. (A) Workflow of *ex vivo* transplant experiments. Hepatocytes are isolated from a *Fah*$^{-/-}$ donor mouse, transduced with AAV-Cas9 and AAV-HT, cultured for 48 hours, and transplanted into a *Fah*$^{-/-}$ recipient mouse through splenic injection. (B) Weight charts for female recipients (green; n = 3), male recipients (red; n = 3), and control mice (black; n = 4). Gray bars indicate time on the protective drug, NTBC. *Euthanization of control mice. Females phenotypically rescued from liver failure with one fewer NTBC cycle than did the males. (C) Blood data for *Fah*$^{-/-}$ mice sacrificed after 20 days of NTBC withdrawal (black; n = 5), *Fah*$^{-/-}$ mice that were never withdrawn from NTBC (dark gray; n = 5), experimental mice treated with AAV-Cas9 and AAV-HT (green/red; n = 6), and wild-type control mice (light gray; n = 4). $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.



D

Rank	Sequence	Count	Percent
Original	TCATGAACGACTGGAGC**A**GTAATGCCT**GG**TGGCCCAGCTTCCTCTGATG		
Target	TCATGAACGACTGGAGC**G**GTAATGCCT**CC**TGGCCCAGCTTCCTCTGATG		
1	TCATGAACGACTGGAGC**A**GTAATGCCT**GG**TGGCCCAGCTTCCTCTGATG	346669	86.0
2	TCATGAACGACTGGAGC**G**GTAATGCCT**GG**TGGCCCAGCTTCCTCTGATG	39567	9.8
3	TCATGAACGACTGGAGC**G**GTAATGCCT**CC**TGGCCCAGCTTCCTCTGATG	10460	2.6
4	TC**G**TGAACGACTGGAGC**G**GTAATGCCT**CC**TGGCCCAGCTTCCTCTGATG	652	0.16
5	TCATGAACGACTGGAGC**A**GTAA----------GGCCCAGCTTCCTCTGATG	641	0.16
6	TCATGAACGACTGGAGC**G**GTAATGCCT**CC**TGGCCCAGCTTCCTCTG**C**TG	178	0.044
7	TCATGAACGACTGGAGC**A**GTAATGCCT**CC**TGGCCCAGCTTCCTCTGATG	147	0.036
8	TC**G**TGAACGACTGGAGC**A**GTAATGCCT**GG**TGGCCCAGCTTCCTCTGATG	88	0.022
9	TCATGAAC**A**ACTGGAGC**A**GTAATGCCT**GG**TGGCCCAGCTTCCTCTGATG	81	0.020
10	TCATGAACGACTGGAGC**A**GTAA**T**TGCCT**GG**TGGCCCAGCTTCCTCTGATG	43	0.011

FIG. 5. An *ex vivo* protocol using AAV-Cas9 and AAV-HT can molecularly correct a mouse model of hereditary tyrosinemia type I. (A) Immunohistochemistry for fumarylacetoacetate hydrolase in liver sections of all six experimental mice. Black scale bar is 4 mm. (B) Untreated *Fah*$^{-/-}$ mouse liver stained for FAH. (C) PCR amplicon deep sequencing of seven predicted off-target sites, showing percentage of non-wild-type alleles from the livers of both treated and untreated control mice. (D) NGS data of the Fah locus from liver samples from all six experimental mice showed that 86% of alleles were unmodified. Green letters indicate HDR-corrected bases; red letters indicate other changed bases; and bolded letters indicate bases targeted for change in the homology template. Perfect correction at this locus was seen in 2.6% of alleles, although the point mutation change was seen in over 12.6% of alleles.

(Supporting Table S3). Prevalence of sequence variation was highly similar between treated and untreated DNA, indicating that there were no off-target effects from Cas9 activity at these high-probability genomic loci (Fig. 5E).

GENE CORRECTION IS DURABLE

Gene-edited hepatocytes were isolated from rescued *Fah*$^{-/-}$ mice and transplanted into another generation of recipient *Fah*$^{-/-}$ mice. These serial transplant recipients became NTBC-independent after only one or—in the case of one mouse—two NTBC cycles (Fig. 6A). After sacrifice, their plasma levels of tyrosine, ALP, and ALT had been corrected to wild-type levels in comparison to control *Fah*$^{-/-}$ mice off NTBC, whereas their AST levels had corrected to the level of *Fah*$^{-/-}$ mice on NTBC (Fig. 6B). Immunohistochemistry for FAH showed nearly complete repopulation of the liver by FAH+ hepatocytes in all treated animals with no signs of hepatocellular carcinoma in FAH+ regions after 220 total days of hepatocyte expansion (Fig. 6C,D and Supporting Fig. S4).

Discussion

Liver transplantation remains the only curative therapy for multiple inborn errors of metabolism, including HT1. Over the past 3 decades, multiple iterations of cell therapy approaches have been applied to these genetic disorders with varying success. The first regenerative medicine approach attempted continues to be the most clinically used: allogeneic hepatocyte transplantation. However, despite many attempts to disrupt the immune response against the allogeneic cells, graft loss continues to limit the long-term efficacy of this approach, with most engraftments lost by 6 months.[1,2] Autologous hepatocyte transplantation after gene therapy is a promising option for treating metabolic diseases, as an immune response is less likely to be initiated. The efficacy of this approach with integrating retroviral vectors has been demonstrated in small and large animal models of metabolic disease.[6,35] However, concerns remain about the potential for insertional mutagenesis using retroviral vectors that randomly integrate throughout the genome.[36] Consequently, we and others believe targeted gene editing at the specific genomic locus is a preferred alternative for future clinical application. To this end, CRISPR/Cas9-mediated gene editing has emerged as the leading candidate for specific gene repair in a number of rodent models, including liver diseases in which the underlying genetic mutation occurs in hepatocytes.[7,37]

Ex vivo gene therapy in hepatocytes presents several potential barriers to effective gene correction using CRISPR/Cas9, including cell loss during culture of primary hepatocytes, delivery of Cas9 and repair templates, ability to perform HDR in nondividing hepatocytes, and engraftment of modified cells after transplantation. The issue regarding HDR is often underappreciated and underreported in studies relating to gene therapy. The problem exists because adult hepatocytes are normally quiescent and in G_0 phase of the cell cycle[38]—a phase of the cell cycle in which NHEJ remains the predominant DNA repair pathway.[10] For gene editing to occur using HDR, hepatocytes must be targeted in young (commonly neonatal) livers that are actively dividing or potentially in livers that have undergone a regenerative stimulus, such as a partial hepatectomy.[39,40] Therefore, although *in vivo* approaches to perform gene editing in neonatal livers offer great potential using CRISPR/Cas9, alternative approaches are needed for gene editing to occur in adult hepatocytes.

Consequently, we hypothesized that *ex vivo* culturing of hepatocytes from adult mice could reprogram these cells to re-enter the cell cycle, thereby enhancing CRISPR/Cas9-mediated gene repair using HDR followed by transplantation. The data presented here demonstrate the potential for large-scale genomic changes to occur in cultured hepatocytes, as determined by qPCR, RNA-Seq, and flow cytometry. Cultured hepatocytes rapidly down-regulated genes related to metabolic function, but concurrently increased expression of genes related to cell cycle entry, DNA DSB repair, and mitosis. Importantly, these changes did not affect the ability of cultured hepatocytes to engraft *in vivo*, as demonstrated using bioluminescence imaging of luciferase-labeled hepatocytes that were cultured for up to 72 hours and subsequent cure of HT1 animals undergoing this technique.

In addition to tracking early stages of engraftment using bioluminescence, we used the HT1 mouse model to demonstrate that *ex vivo* cultured and corrected hepatocytes could still engraft and repopulate

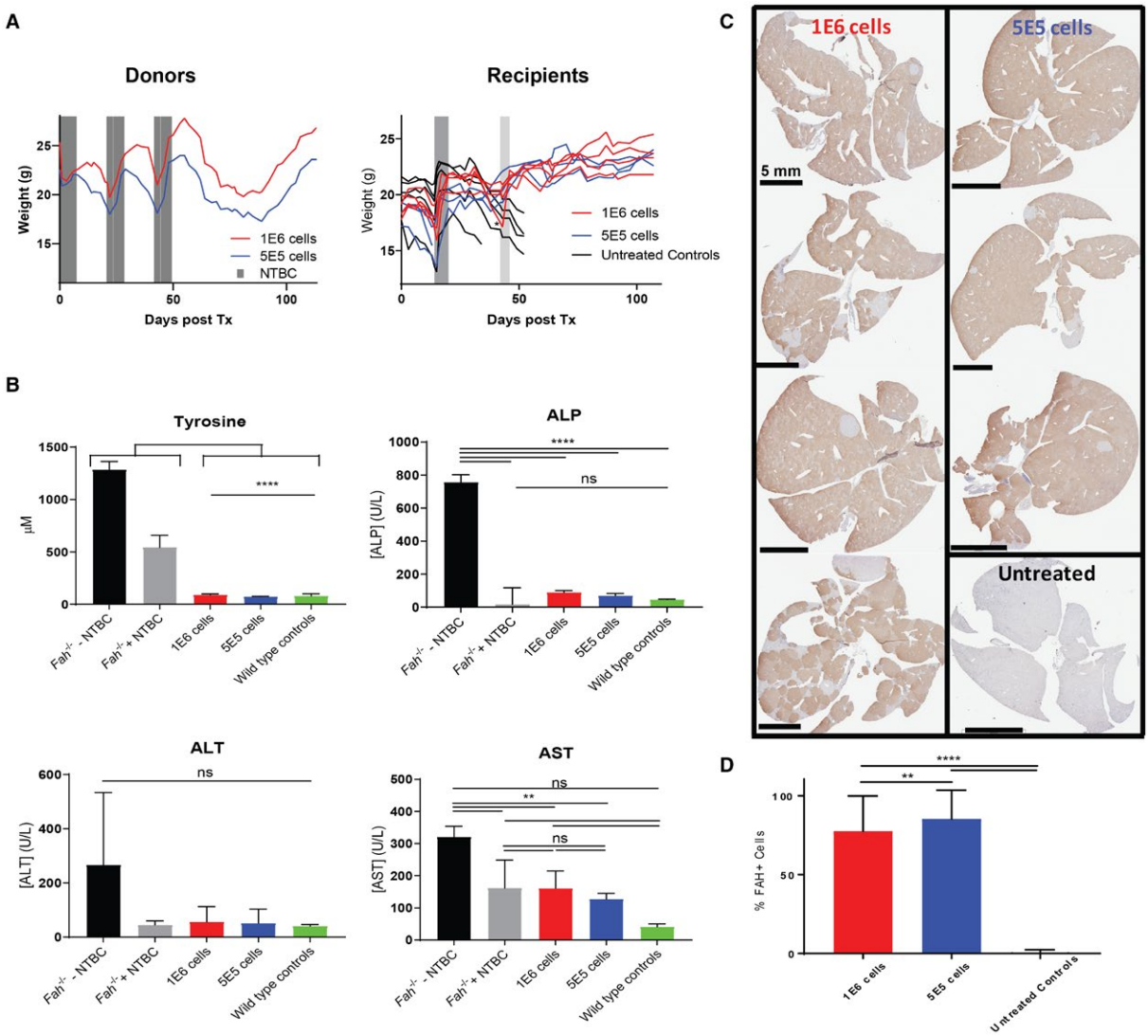


FIG. 6. HDR-mediated correction of the *Fah* single-nucleotide polymorphism is repeatable and durable. (A) Weight curves for two hepatocyte transplant donors that were cured following the dual AAV treatment, as well as weight curves for seven serial recipient mice transplanted with hepatocytes from the first two mice after cycling and rescue. The dark gray bar indicates time on NTBC for every animal, and the light gray bar indicates time on NTBC for only the starred red animal. (B) Serum data for $Fah^{-/-}$ mice sacrificed after 20 days of NTBC withdrawal (black; n = 5), $Fah^{-/-}$ mice who were never withdrawn from NTBC (gray; n = 5), experimental serial transplant recipient mice (red, 1×10^6 hepatocytes, n = 4; blue, 5×10^5 hepatocytes, n = 3), and wild-type control mice (green; n = 4). (C) Immunohistochemistry staining for FAH in treated mice and an untreated control. (D) Quantification of the levels of FAH expression in the livers of both treated groups and their untreated controls at time of sacrifice. $^*P < 0.05$; $^{**}P < 0.01$; and $^{****}P < 0.0001$.

the $Fah^{-/-}$ liver. Although the HT1 model may not be representative of other metabolic diseases because of the strong selective advantage for FAH+ cells, we used this model to show that *ex vivo* modified hepatocytes do not lose proliferative potential after culture and gene editing. It is well known that hepatocytes rapidly undergo apoptosis in primary culture[41]; however, the strong selective advantage in HT1 mitigates the loss of cell viability over the course of culture period. Future studies will be needed in other metabolic disease models, to optimize engraftment conditions and to demonstrate that this *ex vivo* gene editing approach will be successful. Additional studies are needed to overcome three key limitations of any cell therapy

approach, including *ex vivo* gene therapy. First, this approach used AAV to deliver Cas9 to hepatocytes. Although this method showed robust induction of cutting, future studies should focus on selection of nonviral vectors—possibly mRNA and ribonucleoprotein complexes—for delivery of Cas9, to reduce the potential for off-target cutting caused by continued activity of AAV-mediated expression. Second, engraftment of transplanted cells *in vivo* is low (discussed previously), and normally represents 10%-15% of total cells transplanted.[42] Ongoing work related to improving engraftment includes partial liver irradiation[43] and partial hepatectomy.[44] Third, to overcome the absence of selective pressure for corrected cells in other diseases, a clinically relevant protocol will need to be optimized for enhancing proliferation of transplanted cells to phenotypically relevant levels. Currently, a number of genetic and drug-induced injury methods have demonstrated efficacy in preclinical models.[45,46] However, to date, no clinically relevant methodology has been demonstrated.

In conclusion, the data presented here provide a proof-of-concept that *ex vivo* gene therapy in primary hepatocytes is feasible. Although much work needs to be done to make this approach clinically relevant, we believe that this work should lay the foundation for future studies related to gene editing in hepatocytes for HT1 and other metabolic liver diseases.

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REFERENCES

1) Dhawan A, Puppi J, Hughes RD, Mitry RR. Human hepatocyte transplantation: current experience and future challenges. Nat Rev Gastroenterol Hepatol 2010;7:288-298.
2) Jorns C, Nowak G, Nemeth A, Zemack H, Mork LM, Johansson H, et al. De Novo Donor-specific HLA antibody formation in two patients with Crigler-Najjar syndrome type I following human hepatocyte transplantation with partial hepatectomy preconditioning. Am J Transpl 2016;16:1021-1030.
3) Grossman M, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ 3rd, et al. A pilot study of *ex vivo* gene therapy for homozygous familial hypercholesterolaemia. Nat Med 1995;1:1148-1154.
4) Rittelmeyer I, Rothe M, Brugman MH, Iken M, Schambach A, Manns MP, et al. Hepatic lentiviral gene transfer is associated with clonal selection, but not with tumor formation in serially transplanted rodents. Hepatology 2013;58:397-408.
5) Menzel O, Birraux J, Wildhaber BE, Jond C, Lasne F, Habre W, et al. Biosafety in *ex vivo* gene therapy and conditional ablation of lentivirally transduced hepatocytes in nonhuman primates. Mol Ther 2009;17:1754-1760.
6) Hickey RD, Mao SA, Glorioso J, Elgilani F, Amiot B, Chen H, et al. Curative *ex vivo* liver-directed gene therapy in a pig model of hereditary tyrosinemia type 1. Sci Transl Med 2016;8:349ra399.
7) **Yang Y**, **Wang L**, Bell P, McMenamin D, He Z, White J, et al. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. Nat Biotechnol 2016;34:334-338.
8) Iyama T, Wilson DM 3rd. DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst) 2013;12:620-636.
9) Michalopoulos GK. Liver regeneration. J Cell Physiol 2007;213:286-300.
10) Lin S, Staahl B, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife 2014;3:e04766.
11) Gutschner T, Haemmerle M, Genovese G, Draetta GF, Chin L. Post-translational regulation of Cas9 during G1 enhances homology-directed repair. Cell Rep 2016;14:1555-1566.
12) **Richert L**, **Liguori MJ**, Abadie C, Heyd B, Mantion G, Halkic N, et al. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. Drug Metab Dispos 2006;34:870-879.
13) Aponte JL, Sega GA, Hauser LJ, Dhar MS, Withrow CM, Carpenter DA, et al. Point mutations in the murine fumarylacetoacetate hydrolase gene: animal models for the human genetic disorder hereditary tyrosinemia type 1. Proc Natl Acad Sci U S A 2001;98:641-645.
14) 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.
15) 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.
16) Hickey RD, Mao SA, Amiot B, Suksanpaisan L, Miller A, Nace R, et al. Noninvasive 3-dimensional imaging of liver regeneration in a mouse model of hereditary tyrosinemia type 1 using the sodium iodide symporter gene. Liver Transpl 2015;21:442-453.
17) **Kalari KR**, **Nair AA**, Bhavsar JD, O'Brien DR, Davila JI, Bockol MA, et al. MAP-RSeq: Mayo Analysis Pipeline for RNA sequencing. BMC Bioinformatics 2014;15:224.
18) Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 2013;14:R36.
19) Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2014;30:923-930.
20) Wang L, Wang S, Li W. RSeQC: quality control of RNA-seq experiments. Bioinformatics 2012;28:2184-2185.
21) **Chen HI**, **Liu Y**, Zou Y, Lai Z, Sarkar D, Huang Y, et al. Differential expression analysis of RNA sequencing data by incorporating non-exonic mapped reads. BMC Genom. 2015;16(Suppl 7):S14.
22) McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res 2012;40:4288-4297.

23) Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-15550.
24) Bae S, Park J, Kim JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 2014;30:1473-1475.
25) Anderson EM, Haupt A, Schiel JA, Chou E, Machado HB, Strezoska Z, et al. Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. J Biotechnol 2015;211:56-65.
26) Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N, et al. CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. Nucleic Acids Res 2014;42:7473-7485.
27) Ponder KP, Gupta S, Leland F, Darlington G, Finegold M, DeMayo J, et al. Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. Proc Natl Acad Sci U S A 1991;88:1217-1221.
28) 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.
29) St. Clair WH, Dwarakanath BS, Zhang H, Wheeler KT. Influence of proliferation on DNA repair rates in liver. Exp Cell Res 1991;197:323-325.
30) Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol 2008;9:297-308.
31) Kowalczykowski SC. An overview of the molecular mechanisms of recombinational DNA repair. Cold Spring Harb Perspect Biol 2015;7:a016410.
32) Li J, Holzschu DL, Sugiyama T. PCNA is efficiently loaded on the DNA recombination intermediate to modulate polymerase delta, eta, and zeta activities. Proc Natl Acad Sci U S A 2013;110:7672-7677.
33) Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-322.
34) Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components *in vivo*. Nat Biotechnol 2016;34:328-333.
35) Kay MA, Baley P, Rothenberg S, Leland F, Fleming L, Ponder KP, et al. Expression of human alpha 1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc Natl Acad Sci U S A 1992;89:89-93.
36) Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest 2008;118:3132-3142.
37) Shao Y, Wang L, Guo N, Wang S, Yang L, Li Y, et al. Cas9-nickase-mediated genome editing corrects hereditary tyrosinemia in rats. J Biol Chem 2018;293:6883-6892.
38) Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276:60-66.
39) Jirtle RL, Michalopoulos G. Effects of partial hepatectomy on transplanted hepatocytes. Cancer Res 1982;42:3000-3004.
40) Wang C, Zhang L, He Q, Feng X, Zhu J, Xu Z, et al. Differences in Yes-associated protein and mRNA levels in regenerating liver and hepatocellular carcinoma. Mol Med Rep 2012;5:410-414.
41) Bailly-Maitre B, de Sousa G, Zucchini N, Gugenheim J, Boulukos KE, Rahmani R. Spontaneous apoptosis in primary cultures of human and rat hepatocytes: molecular mechanisms and regulation by dexamethasone. Cell Death Differ 2002;9:945-955.
42) Gupta S, Lee CD, Vemuru RP, Bhargava KK. 111Indium labeling of hepatocytes for analysis of short-term biodistribution of transplanted cells. Hepatology 1994;19:750-757.
43) Yamanouchi K, Zhou H, Roy-Chowdhury N, Macaluso F, Liu L, Yamamoto T, et al. Hepatic irradiation augments engraftment of donor cells following hepatocyte transplantation. Hepatology 2009;49:258-267.
44) Jirtle RL, Michalopoulos G. Effects of partial-hepatectomy on transplanted hepatocytes. Cancer Res 1982;42:3000-3004.
45) Nygaard S, Barzel A, Haft A, Major A, Finegold M, Kay MA, et al. A universal system to select gene-modified hepatocytes *in vivo*. Sci Transl Med 2016;8:342ra379.
46) **Borel F**, **Tang Q**, Gernoux G, Greer C, Wang Z, Barzel A, et al. Survival advantage of both human hepatocyte xenografts and genome-edited hepatocytes for treatment of alpha-1 antitrypsin deficiency. Mol Ther 2017;25:2477-2489.

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