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ORIGINAL ARTICLE

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A liver-humanized mouse model of carbamoyl phosphate synthetase 1-deficiency

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Abstract

A liver-humanized mouse model for CPS1-deficiency was generated by the high-level repopulation of the mouse liver with CPS1-deficient human hepatocytes. When compared with mice that are highly repopulated with CPS1-proficient human hepatocytes, mice that are repopulated with CPS1-deficient human hepatocytes exhibited characteristic symptoms of human CPS1 deficiency including an 80% reduction in CPS1 metabolic activity, delayed clearance of an ammonium chloride infusion, elevated glutamine and glutamate levels, and impaired metabolism of [^{15}N]ammonium chloride into urea, with no other obvious phenotypic differences. Because most metabolic liver diseases result from mutations that alter critical pathways in hepatocytes, a model that incorporates actual disease-affected, mutant human hepatocytes is useful for the investigation of the molecular, biochemical, and phenotypic differences induced by that mutation. The model is also expected to be useful for investigations of modified RNA, gene, and cellular and small molecule therapies for CPS1-deficiency. Liver-humanized models for this and other monogenic liver diseases afford the ability to assess the therapy on actual disease-affected human hepatocytes, in vivo, for long periods of time and will provide data that are highly relevant for investigations of the safety and efficacy of gene-editing technologies directed to human hepatocytes and the translation of gene-editing technology to the clinic.

KEYWORDS

CPS1-deficiency, liver-humanized mice, urea cycle defects

1 | INTRODUCTION

Excess nitrogen in the form of ammonia is accumulated during the breakdown of proteins and amino acids. The urea cycle is a pathway that predominantly occurs in liver to eliminate excess ammonia in the blood by converting it into urea, which is excreted in urine, but unlike ammonia is non-toxic. Six separate enzymes comprise the functional urea cycle, N-acetylglutamate synthase, carbamoyl phosphate synthetase I, ornithine transcarbamylase (OTC),

argininosuccinate synthetase, argininosuccinate lyase, and arginase and the two transporters ORNT1 (or SLC25A15) and citrin, or aspartate-glutamate carrier (SLC25A13). Mutations in any of the urea cycle genes or of the two-inner mitochondrial membrane transport proteins can result in a urea cycle defect (UCD).[16,20,28,32]

A rare autosomal recessively inherited metabolic disorder, CPS1-deficiency (CPS1-D) results from a partial or complete loss of CPS1 activity, the first committed enzyme in the urea cycle. Patients deficient in CPS1 activity are frequently healthy at birth but within 24 to 72 hours, will exhibit more serious symptoms, including elevated blood ammonia, lethargy, and vomiting.[16,23] However, less severe conditions may present later in life. The incidence of CPS1-D is estimated to be 1 to 9 in 1 000 000 (https://www.orpha.net/consor/www/cgi-bin/OC_Exp.php?lng=EN&Expert=147) and 1 in 1 300 000 live births in the United States.[33] More than 262 CPS1-D–associated genetic variants have been identified.[9,15] If undiagnosed or untreated, the resultant hyperammonemia can lead to life-threatening encephalopathy and coma.[3,5,21,23,35] Severe hyperammonemic episodes can result in irreversible neurological damage. The mechanisms of neurological injury includes neurotransmitter abnormalities and cerebral edema due to accumulation of glutamine which is osmotically active.

Currently, there is no cure for CPS1-D other than orthotopic liver transplantation. However, due to a limited availability of donor organs, all patients who might require a liver transplant may not receive one.[22] The scarcity of organ donors has initiated a search for alternative treatments for UCD patients[4,6,35,38] including human hepatocyte transplantation.[26,30]

Animal models are of undeniable importance to study pathophysiological aspects of human diseases and to validate treatments. Current murine models available for each UCD have shown both advantages and disadvantages.[7,8,27–29] A *CPS1* knockout mouse was generated, and while it recapitulates aspects of the human disease phenotype, including severe hyperammonemia, all affected mice died within 36 hours of birth, making it difficult to study or treat the disease or even to maintain the animal model.[28] The original *CPS1* gene knockout mouse model may no longer exist.[7] A more recent study reported the conditional disruption of the *CPS1* gene in mice that survived only for 4 weeks and provided a phenotype with hyperammonemia but without orotic aciduria .[19] In addition, rodent models have disadvantages, particularly for investigating potential therapies, because there are differences in the immune system, drug metabolism, DNA synthesis and repair pathways, and other biochemical processes between mice and humans that can make extrapolation of the data from animal studies to human patients challenging. Because most metabolic liver diseases result from mutations that alter a specific function or functions of parenchymal hepatocytes, a hypothesis was generated that a useful model for a human metabolic liver disease would be one that incorporated actual, disease-affected human hepatocytes. An improvement to the model could be realized if studies of the disease-affected human hepatocytes could be conducted, in vivo.

We and others have generated chimeric mice where mouse hepatocytes can be almost completely replaced with human hepatocytes.[2,10,13,14,17,25,31,34,36] Successful repopulation of the liver with hepatocytes requires a mouse model that allows engraftment of donor cells, avoids rejection of the cellular xenograft, and also provides a selective growth advantage to the donor cells. The model reported here, utilized triple knockout mice, the so-called FRGN mice, where the fumarylacetoacetate hydrolase (*Fah*), recombination activating 2 gene (*Rag2*), and the common gamma chain of interleukin 2 receptor (*IL2rg*) genes were knocked out,[2] and mice were subsequently cross-bred with non-obese diabetic (NOD) mice.[36] Mice were rendered severely immunodeficient, so that they would not reject the human xenograft, by knocking out the *Rag2* and *IL2rg* genes. *Fah*-deficient mice are the murine equivalent of the human liver disease hereditary tyrosinemia type 1 (HT-1). The *Fah* deficiency in the mouse hepatocytes creates a mechanism to provide a growth advantage to the transplanted human hepatocytes. In HT-1 patients, the severe liver disease can be prevented by providing a protective drug, 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC). In the mouse model, when NTBC is withdrawn, toxic tyrosine metabolites accumulate and severely injure the *Fah*-deficient mouse hepatocytes creating a strong liver regeneration stimulus. During this time, if *FAH*-proficient human hepatocytes are transplanted, they can survive in the absence of NTBC and respond to the signals for liver regeneration and highly repopulate the mouse liver, frequently replacing more than 90% of the mouse hepatocytes. In the present study, chimeric mice were generated by the transplantation of human hepatocytes from normal donors as well as from a patient with CPS1-D, and the resulting phenotypes were investigated.

2 | MATERIALS AND METHODS

2.1 | FRGN mice

All institutional and national guidelines for the care and use of laboratory animals were followed. All animal studies are conducted according to Karolinska Institutet guidelines and under approved ethical protocol ID400 42-17. FRGN mice were generated by knocking out *Fah*, *Rag2*, and *IL2rg*, the

so-called FRG mouse, and cross-breeding with NOD mice.[2,36] The FRGN mouse does not develop diabetes because they cannot mount a sufficient immune response. Mice used for this study were between 5 and 6 weeks old, originally obtained from Yecuris Corporation (Tualatin, Oregon) and maintained in breeding colonies at Karolinska Institutet Animal Facility. Mice were maintained throughout the study on PicoLab High Energy Mouse Diet with 18.9% protein (Animal Specialties and Provisions, Quakertown, Pennsylvania) and supplemented with NTBC to avoid lethal liver damage. After cell transplantation, animals were cycled on-and-off of NTBC to support engraftment and the proliferation of donor-human hepatocytes. All animals that achieved 4 mg/mL of circulating human albumin were used in the study.

2.2 | Cell preparation

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 to 2005. Informed consent was obtained from all patients before being included in the study. Human hepatocytes were procured from donor liver tissue according to ethical protocol (human studies: 2014/1561-32; animals ID 40042-17). Organ donors were tested, and were negative, for hepatitis viruses B and C, and human immunodeficiency virus. All human hepatocytes were isolated as previously described.[12] Normal (CPS1-proficient) liver tissues were collected from a 50-year-old female, an 8-year-old male, a 6-year-old male, and a 4-day-old female organ donor. The CPS1-D hepatocytes were isolated from the explanted liver of a 1.5-year-old female who received an orthotopic liver transplant. All cells were cryopreserved before transplantation in University of Wisconsin solution (BEL GEN 1000, Lissieu, France) supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri). Hepatocytes were thawed on the day of transplantation in a 37°C water bath until the ice is barely visible and cells are diluted with 10 volumes of cold Williams medium E with 10% calf serum and centrifuged at 90*g* at 4°C for 5 minutes. The cell pellet was suspended in cold plasmalyte (Baxter, Norfolk, UK) for transplantation. The viability for control and CPS1-D hepatocytes was $72 \pm 9\%$ and $72 \pm 5\%$, respectively, after thawing for transplantation.

The two disease-causing variants identified in the CPS1-D patient were c.2339G>A (p.Arg780His) on one (maternal) allele and c.3559-745A>G on the other (paternal) allele. The latter generates a new donor splice site causing the insertion of an intronic region in the *CPS1* mRNA between exons 29 and 30. A truncated protein is produced (p.Arg1186_Val1187insKPRLSK*), most likely non-functional, due to a premature stop codon.

2.3 | Hepatocyte transplantation

The FRGN mice were pretreated approximately 24 hours before transplantation with a adenovirus vector expressing urokinase plasminogen activator (Yecuris Corp., Tualatin, Oregon).[2,36] Hepatocytes were injected directly into the parenchyma of the spleen by making a small incision on the left abdominal region and the organ was exposed. Each mouse received one million viable hepatocytes suspended in 200 μL plasmalyte delivered over about a 1-minute period with a syringe and a 29G needle. The spleen was returned to the abdominal cavity, and the incision was sutured using absorbable 5.0 vicryl suture (product no: V391H, Ethicon, Diegen, Belgium). The mice were anesthetized during the entire procedure with isoflurane (Baxter, Norfolk, UK). Mice received appropriate analgesia at the time of surgery and 24 hours after the surgical procedure.

2.4 | Blood sampling and ELISA

Starting from the fifth week after hepatocyte transplantation, human albumin levels were monitored. Blood samples were collected from mice twice a month. The mice were placed in a restrainer, and blood samples were collected from the tail vein using 27G needle. From each mouse, 2 μL blood was collected, diluted in 198 μL diluent, and assayed using the Quantitative Human Albumin ELISA Quantitation Kit (Bethyl Laboratory, Montgomery, Texas) according to the manufacturer's protocol. Multiple measurements were performed on each sample, at different dilutions (1:100-100 000), to quantify the level of "humanization". It is estimated that 1 mg/mL of circulating human albumin represents a 20% level of repopulation with human hepatocytes.[2,36] A fresh standard curve (ranging from 15 to 200 ng/mL) made with human reference serum RS10-110-4 (Bethyl laboratory Inc.) was included in each analysis.

2.5 | Ammonia measurement and ureagenesis

Ammonia levels were measured by taking 5 μL of blood diluted in 20 μL of water. Samples were analyzed immediately using an Arkray pocket chem (Arkray, AT, Netherlands) according the manufacturer's instructions.

Ureagenesis assay was performed on animals repopulated with human hepatocytes to an estimated level of 80% or higher, or > 4 mg/mL of circulating human albumin. Enrichment of urea from [^{15}N]-labeled ammonia was measured following an intraperitoneal injection of [^{15}N]ammonium chloride (product number 299251,

>99% pure, Sigma-Aldrich) 4 mmol/kg of body weight, dissolved in 1 × phosphate-buffered saline, as originally described by Yudkoff et al[39] and modified by Allegri et al.[1] Mice were off of NTBC a minimum of 4 days before the assay. Samples for the ureagenesis assay were collected pre, and 30 minutes post, injection of [^{15}N] ammonium chloride where 5 μL of blood was diluted with 100 μL of water and frozen immediately. Alternatively, 5 μL of blood was added to a filter paper (IDBS-226; Perkin Elmer, Shelton, CT) and stored at −20°C in a sealed bag containing desiccant and analyzed as described by Allegri et al.[1]

2.6 | Amino acid detection and CPS1 and OTC enzyme assays

Amino acid analysis was performed in the clinical laboratory from dried blood spots using tandem MS. CPS1 and OTC assays were performed according to Gautschi et al.[11] In summary, the frozen and pulverized liver samples were homogenized on ice with a glass Teflon homogenizer. Enzyme activities were determined in duplicates at 37°C, with liver homogenates diluted with water, 1:100 for the OTC and 1:300 for CPS1 measurements. For OTC activity, 300 μL of ornithine (2.5 mM) and carbamoylphosphate (5 mM), were added to the 25 μL diluted homogenate. The reaction was stopped after 10 minutes by addition of 300 μL 5% trichloroacetic acid and 0.5% phosphotungstic acid. The reaction product, citrulline, was measured spectrophotometrically at 464 nm after reaction with diacetylmonoxime, antipyrine, Fe and quantified using a citrulline standard curve. CPS1 activities were measured as a coupled reaction with addition of excess OTC. In summary, 150 μL of reaction solution containing ammonium hydrogen carbonate (109 mM), N-acetylglutamate (10 mM), ornithine (7 mM), magnesium sulfate (20 mM), and ATP (24 mM) were incubated for 15 minutes with 25 μL of liver homogenate and stopped as above. The final reaction product, citrulline, was quantified as described previously. Citrulline measurements were corrected for blank reagents and endogenous citrulline (addition of stop solution before addition of homogenate). Protein concentrations were determined with a Lowry test.

2.7 | Tissue collection and reverse transcriptase polymerase chain reaction

Liver tissue was collected from mice repopulated with control or CPS1-D hepatocytes. Portions of each liver lobe were fixed in formalin. Remaining liver tissue was crushed to a powder in liquid nitrogen and mixed homogeneously for molecular and enzymatic analysis. Tissue samples were lysed in Trizol^{tm} solution (Thermo Fisher, Waltham, Massachusetts) and RNA isolated according to the manufacturer's instructions. Total RNA was converted to complimentary DNA using a high-capacity cDNA kit (Thermo Fisher, Illinois). Gene expression was assessed using TaqMan assays (Thermo Fisher) for *CYP3A4* (Hs00430021_m1), *CYP1A2* (Hs01070374_m1), *CYP2B6* (Hs03044634_m1), *UGT1A6* (Hs01592477_m1), *CPS1* (Hs00157048_m1), *FAH* (Hs00908445_m1), *OTC* (Hs00166892_m1), *ALB* (Hs00609411_m1), and *A1AT* (Hs01097800_m1). The Ct values were normalized to the house keeping gene cyclophilin A (*PPIA*) (Hs99999904_m1). All assays were conducted with TaqMan primers that are specific for the human genes and do not cross react with the mouse homologs. Nontransplanted FRGN mouse liver tissue was used as a negative control, and the expression of all genes reported here was undetectable in untransplanted FRGN mouse liver tissue.

2.8 | Statistical analysis

Results are expressed as individual values and as Box and Whisker plots showing median, 25- and 75-percentiles. Data sets were compared by Mann-Whitney nonparametric tests, because the data were not normally distributed. *P*-value <0.05 was considered as significant ($*P < 0.05$; $**P < 0.001$; $***P < 0.0001$). Data were analyzed with GraphPad Prism software, version 6 (GraphPad Software Inc., San Diego, California).

3 | RESULTS

3.1 | Human album levels in liver-humanized mice

Human albumin was detected in the blood of transplanted mice, indicating that the human hepatocytes engrafted and proliferated post-transplant. All animals that achieved a minimum of 4 mg/mL of human albumin,estimated to be ~80% repopulation were used for the subsequent study. There was no statistical difference between the level of human albumin in mice that were transplanted with control or CPS1-D human hepatocytes (Figure 1).

3.2 | Blood ammonia and ureagenesis

Mice repopulated with CPS1-D hepatocytes showed a numerically higher basal blood ammonia of 182 μM as compared with 148 μM in control animals that received CPS1 proficient hepatocytes (Figure 2A); however, this difference did not reach statistical significance. Mice were examined for their ability to metabolize ammonia and to synthesize

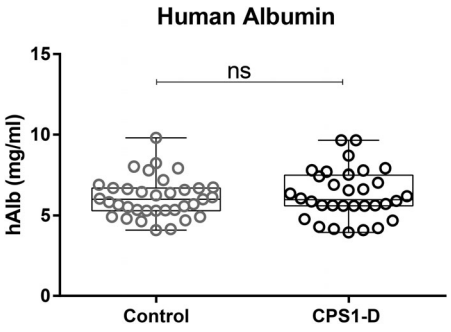

FIGURE 1 Circulating human albumin levels in mice transplanted with control and CPS1-deficiency (CPS1-D) human hepatocytes. The extent of repopulation of the mouse liver is estimated in live mice by the levels of circulating human albumin. There was no significant difference in level of human albumin between control and CPS1-D mice. The number of highly repopulated mice analyzed with CPS1-D (n = 33) or control human hepatocytes (n = 35) are shown as individual dots

urea from exogenous [^{15}N]ammonium chloride. As shown in Figure 2B, the control mice maintained significantly lower ammonia levels post injection with a median of 494 μM while CPS1-D mice displayed impaired clearance of ammonia from the blood with a median blood ammonia of 818 μM (Figure 2).

The percentage of enrichment of [^{15}N] into urea following the infusion of a [^{15}N]H_4Cl bolus is a measure of ureagenesis after a 30 minutes period. As shown in Figure 3, there was a significant difference between mice liver-humanized with healthy control (11% enrichment) as compared with those repopulated with the CPS1-D hepatocytes (7% enrichment; Figure 3).

As compared with control animals, mice repopulated with CPS1-D human hepatocytes showed a different response to the ammonium infusion. At post injection, CPS1-D mice most became lethargic. Their reaction time to a startle or an attempt to pick them up was obviously impaired. Mice repopulated with control human hepatocytes displayed normal behavior through the 30-minute observation period.

3.3 | Blood amino acid levels

The blood samples were collected from mice repopulated with control or CPS1-D human hepatocytes for amino acid quantification. In the standard analysis performed on blood, 18 different amino acids were analyzed including citrulline, arginine, alanine, glutamine, and glutamate. Only the blood levels of glutamine and glutamate were statistically different between control and CPS1-D mice. Both glutamine and glutamate levels were significantly increased in CPS1-D mice when compared with control mice (Figure 4A,B). Median glutamine levels in control and CPS1-D mice were 390 and 547 μM, respectively. Median glutamate levels in control and CPS1-D were 261 and 318 μM, respectively.

3.4 | CPS1 and OTC enzyme activity

Enzyme analyses to quantify OTC and CPS1 metabolic activities were performed on liver tissue from mice selected at random from animals highly repopulated with control or CPS1-D hepatocytes (Figure 5). There was a significant difference in the levels of CPS1 activity in CPS1-D mice as compared with those that were highly repopulated with control human hepatocytes. The liver of the CPS1-D mice expressed approximately 20% of the CPS1 activity measured in control mice (Figure 5A). A second urea cycle enzyme, OTC activity was also measured in samples from the same liver, but there was no significant difference between OTC activities in the liver of CPS1-D and control mice.

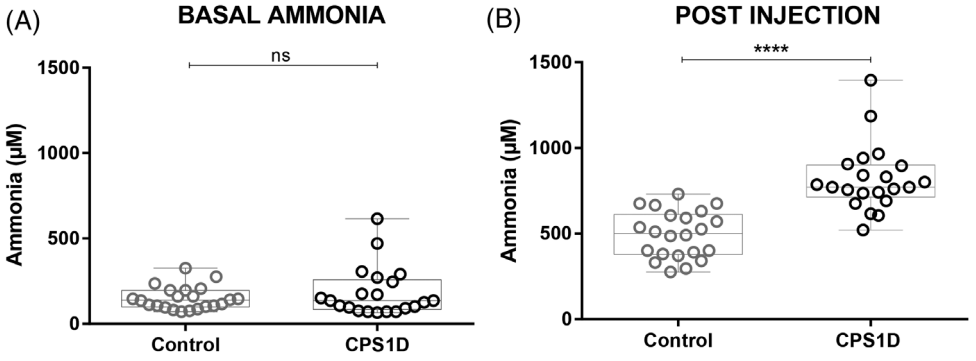

FIGURE 2 Blood ammonia levels in liver-humanized mice. Ammonia levels in the blood of control and CPS1-D liver-humanized mice were measured before (basal, 2A) and 30 min post, (2B) an [^{15}N]ammonium chloride infusion as described in the Methods. The median basal ammonia levels were not significantly higher in the CPS1-D group as compared to control, 148 μM and 182 μM for control and CPS1-D animals, respectively. Blood ammonia levels were significantly higher 30 min post-injection of ammonium chloride in the CPS1-D group as compared to controls, 494 μM and 818 μM for control and CPS1-D animals, respectively. ns, not significantly different; ****, significantly different, $p<0.001$

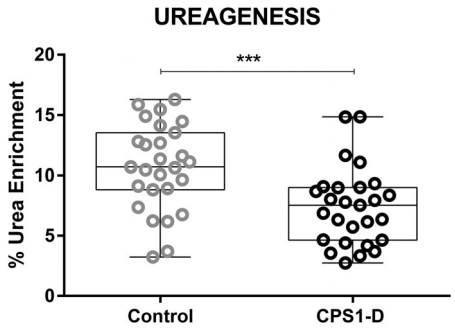

FIGURE 3 Isotopic enrichment of [^{15}N] ammonia into urea in control and CPS1-deficiency (CPS1-D) mice. The incorporation of [^{15}N]ammonium into urea in the blood after 30 minutes post injection of [^{15}N] ammonium chloride (4 mmol/kg). The enrichment [^{15}N] into urea was significantly different between control (11%) and CPS1-deficient animals (7%) ($P < 0.001$)

3.5 | Mature liver gene expression in liver-humanized mice

Given the large differences in CPS1 activity observed in the CPS1-proficient and deficient livers, the expression of several mature liver genes was analyzed in the liver samples to determine if other metabolic pathways were altered in the CPS1-D mice. Gene expression was determined using quantitative, reverse-transcription polymerase chain reaction and normalized to human cyclophilin A. The analysis included the genes for the enzymes that metabolize a major proportion of the Food and Drug Administration–approved drugs, the Cytochromes P450 in the 1A, the 2B, and the 3A family (CYP1A2, CYP2B6, and CYP3A4), and uridine diphosphate glucuronosyltransferase (UGT)1A6, an enzyme highly expressed in human liver and required for the excretion and elimination of many drugs and endogenous compounds from the body. In addition, analysis of genes was included that when mutated can create other liver-based diseases, that is, *CPS1*, *FAH*, *OTC*, and alpha 1 antitrypsin (*A1AT;* Figure 6). These genes were chosen because they are regulators of critical physiological processes in the normal human liver and the expression of each gene can vary greatly between individuals. Because of this variability, differences between the expression of these genes in mice humanized with cells from normal donors and the CPS1 donor would be more obvious. No other genes were analyzed, and as shown in Figure 6, there were no significant differences in the expression of these various liver genes between the two groups.

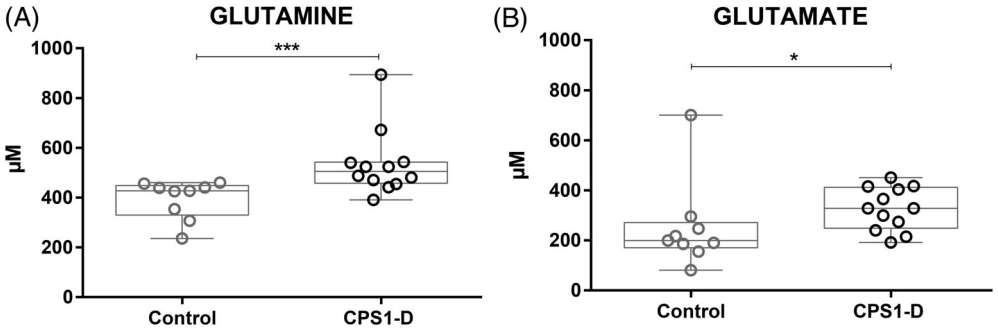

FIGURE 4 Glutamine and glutamate concentrations in control and CPS1-D mice. Blood glutamine (4A) and glutamate (4B) concentrations were analyzed as described in Methods. The glutamine levels were 390 μM and 547 μM for control and CPS1-deficient animals and the glutamate levels were 261 μM and 318 μM, respectively. Both glutamine (***p<0.001) and glutamate (*p<0.05) levels were significantly higher in the CPS1-D animals as compared to control animals

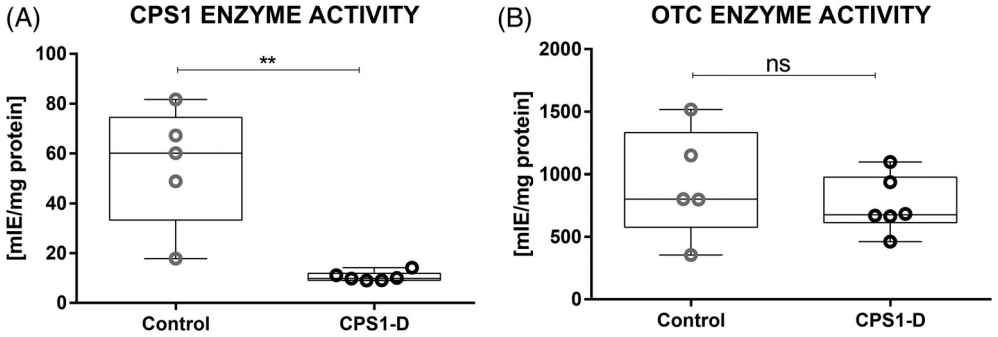

FIGURE 5 CPS1 and OTC metabolic activity in control and CPS1-D mice. The enzymatic activity of CPS1 (5A) and OTC (5B) in liver-humanized mice was measured as described in Methods. Mean CPS1 activities were significantly lower (**p<0.01) in CPS1-D mice as compared to controls, 55±24 mU/mg protein and 11±2 mU/mg protein for control and CPS1-D animals, respectively. There was no significant difference in the OTC activities between the two groups, with 924±434 mU/mg and 752±227 mU/mg protein for control and CPS1-D animals, respectively

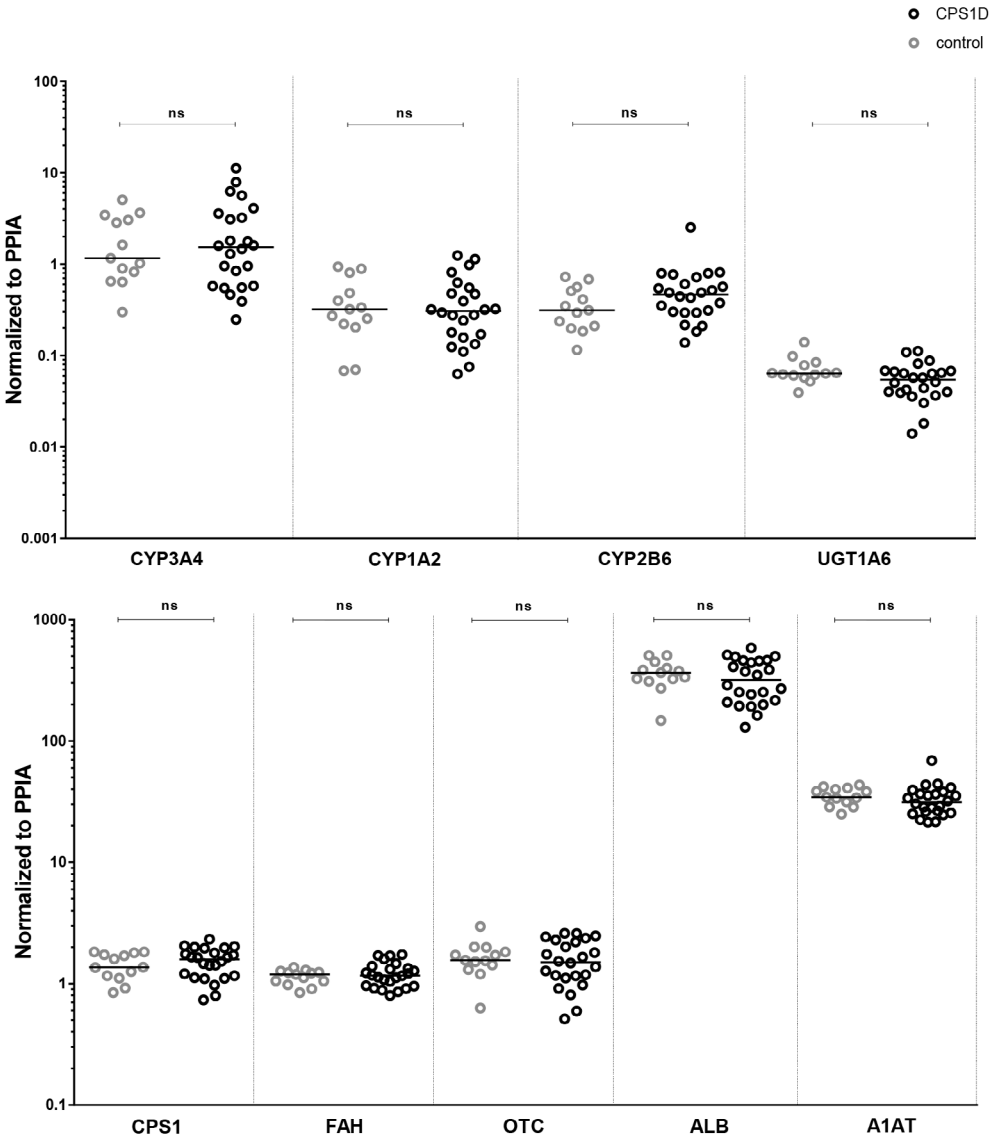

FIGURE 6 Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of cDNA made from mRNA isolated from liver tissue from control and CPS1-deficiency (CPS1-D) mice. No statistically significant difference in gene expression was detected between the two groups of animals

4 | DISCUSSION

Investigations of xenobiotic metabolism have revealed considerable species differences between rodents and human patients with respect to the uptake, metabolism and excretion of drugs. Perhaps one of the most noteworthy examples was Fialuridine, which showed little to no toxicity and great potential for the treatment of HBV infection in preclinical animal studies, but when the drug was provided to human patients, it caused severe liver failure.[24]

Studies with liver-humanized mice were initiated in efforts to make the extrapolation of preclinical studies more likely to translate to the clinic.[18,34,37] However, nearly all publications describing liver-humanized mice restrict their investigations to issues related to toxicology and drug metabolism since these endpoints are critical to drug development and are largely dependent on hepatocyte function. Because most liver-based metabolic diseases result from mutations in genes expressed in hepatocytes, a liver-humanized mouse, made with human hepatocytes isolated from a patient with a metabolic liver disease could be a useful model to investigate the pathology, as well as the therapy, of the disease. The FRG and the FRGN mouse models were initially developed by Grompe et al.[2] There was no evidence of cell fusion of donor hepatocytes with mouse hepatocytes in liver-humanized mice[2] and mice became highly repopulated with donor, human hepatocytes. This report expands our previous studies where liver-humanization was reported (but not characterized) with hepatocytes from several monogenic liver diseases.[13] This current report characterizes the phenotype of mice that are highly repopulated with human hepatocytes from a patient with severe CPS1-D, in comparison with animals transplanted with normal human hepatocytes. This model requires the transplantation and proliferation of the normal or disease-affected hepatocytes in each recipient mouse.

When compared with mice transplanted with normal human hepatocytes, the phenotype of mice transplanted with CPS1-D hepatocytes was consistent with what is observed in human patients. Mice repopulated with CPS1-D hepatocytes displayed elevated glutamate and glutamine levels on a normal protein diet (18.9% protein) and higher blood ammonia levels and delayed clearance of ammonia following an [^{15}N]ammonium chloride challenge. Ureagenesis, measured as the enrichment of ^{15}N into urea following the infusion of [^{15}N]H_4Cl was significantly lower in the CPS1-D recipient mice (Figure 3). The ureagenesis results are consistent with the greatly reduced CPS1 enzyme activity measured in CPS1-D mice, which was only 20% of the level measured in mice transplanted with normal human hepatocytes (Figure 5). As expected, the activity of another urea cycle enzyme, OTC, was not significantly different between CPS1-D and control mice.

Data in Figure 5 clearly show that while OTC activity is normal, the CPS1 activity is greatly reduced. Given the mutations identified in this CPS1-D patient, one could only speculate on the likely presence of both *CPS1*-mRNA and protein in the hepatocytes or the phenotypic changes that might result. Only so much can be inferred from DNA sequence alterations. This model allows the investigator to fully interrogate the molecular, biological, and pathological consequences that the mutation produces, in an in vivo model. In addition to being a useful model to investigate possible therapies for the disease, this liver-humanized model allows detailed investigations of the molecular changes as well as the pathology associated with that specific mutation in actual disease-affected human hepatocytes.

While CPS1 enzyme activity and function was reduced in the CPS1-D mice, there is evidence that other liver functions are maintained at normal levels, as was observed with OTC activity. Circulating human albumin levels were not significantly different between mice receiving normal human hepatocytes and those that received CPS1-D hepatocytes (Figure 1). In addition, 18 different amino acids were analyzed including citrulline, arginine, alanine, glutamine and glutamate, and several additional amino acids. A statistical analysis of the two groups only identified differences in the levels of glutamine and glutamate. The data with glutamine and glutamate are consistent with the CPS1-D phenotype but the lack of differences in the other amino acids supports a conclusion that other critical liver biochemical pathways were not altered in these liver-humanized mice. In addition, mRNA levels of nine hepatic genes expressed in mature human liver failed to identify any significant differences between the control and the CPS1-D mice. While not an exhaustive investigation of liver function and gene expression, there were no obvious phenotypic or genetic differences between the normal and the CPS1-D mice except those attributable to the CPS1-D itself.

Human albumin in the recipient mice was used to estimate the level of repopulation. Being conservative, and trying to avoid the most severe symptoms of CPS1-D in recipients, the study was conducted with mice that achieved a minimum of 4 mg/mL human albumin, which is estimated to correlate with ~80% level of repopulation with human hepatocytes (1 mg/mL $\simeq$ 20% humanization). In some mice, higher levels of repopulation with mutant human hepatocytes were likely achieved and mice became lethargic, failed to eat, lost weight, and had to be treated with ammonia scavengers to restore health. All mice investigated here displayed a high level of humanization as estimated from the circulating human albumin levels. The phenotype displayed in the CPS1-D mice, particularly with respect to the basal level of ammonia in the mice maintained on a regular protein diet could be expected in mice that maintained residual 20% of normal CPS1 activity and an 80% level of repopulation with mutant human cells. A more severe phenotype can be achieved in some mice at higher levels of repopulation with mutant cells, however, at the expense of animal health and welfare. At the level of humanization investigated here, the CPS1-D phenotype is readily quantified with standard tests in live animals. If a successful drug, genetic or cellular therapy were provided, a normalization of the CPS1-D phenotype would likely be verifiable with this mouse model.

In summary, a liver-humanized mouse model for CPS1-D was generated through the high-level repopulation of the liver of mice with CPS1-D human hepatocytes. The resultant mice exhibited characteristic symptoms of CPS1-D with no other obvious phenotypic differences when compared to mice transplanted with normal human hepatocytes. It is likely that preclinical studies conducted with liver-humanized mice will provide data more easily extrapolated and relevant to human CPS1-D. Liver-humanized mice in general are likely to be an important model to study small molecule, cellular and gene therapies, and especially gene correction technologies such as those mediated by CRISPR or other gene-editing procedures. These state-of-the-art technologies require interaction with the human genome at precise sequences and the interplay of the human DNA replication and repair pathways in the editing process, all of which can differ greatly between mice and humans. It would be advantageous to perform preclinical studies of gene-editing and other newer technologies with human hepatocytes, and when possible, with the actual disease-affected human hepatocytes. The value of the data obtained from such studies will be greatly enhanced if those studies can be conducted, in vivo. Studies with live animals will enable a comprehensive assessment of the long-term outcomes of these interventions. Following treated animals long-term will

allow, not just an investigation of the phenotypic correction of the disease, but other endpoints just as important such as the long-term safety of the procedures. Perhaps these liver-humanized models will be most informative for the investigation of the safety and efficacy of gene-editing technologies on human cells in vivo and the translation of the gene-editing technologies to the clinics.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

AUTHOR CONTRIBUTIONS

R.C.S., M.Z., C.H., T.W., R.G., K.K., E.E., A.K., R.F., G.A., V.R., B.T., J.H., J.-M.N., and S.C.S. contributed to conception and design, analysis, interpretation of data, drafting the manuscript, critical review, and revision.

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