ORIGINAL ARTICLE

Humanized liver mouse model with transplanted human hepatocytes from patients with ornithine transcarbamylase deficiency

Go Sugahara[1] | Chihiro Yamasaki[1] | Ami Yanagi[1] | Suzue Furukawa[1] |
Yuko Ogawa[1] | Akinari Fukuda[2] | Shin Enosawa[3] | Akihiro Umezawa[4] |
Yuji Ishida[1,5] | Chise Tateno[1,5] (ORCID)

[1]Research and Development Department, PhoenixBio Co., Ltd, Higashi-Hiroshima, Japan

[2]National Center for Child Health and Development, Tokyo, Japan

[3]Division for Advanced Medical Sciences, National Center for Child Health and Development, Tokyo, Japan

[4]Regenerative Medicine, National Center for Child Health and Development, Tokyo, Japan

[5]Research Center for Hepatology and Gastroenterology, Hiroshima University, Hiroshima, Japan

Correspondence
Chise Tateno, Research and Development Department, PhoenixBio Co., Ltd, 3-4-1 Kagamiyama, Higashi-Hiroshima, Japan.
Email: chise.mukaidani@phoenixbio.co.jp

Communicating Editor: Johannes Häberle

Abstract

Ornithine transcarbamylase deficiency (OTCD) is a metabolic and genetic disease caused by dysfunction of the hepatocytic urea cycle. To develop new drugs or therapies for OTCD, it is ideal to use models that are more closely related to human metabolism and pathology. Primary human hepatocytes (HHs) isolated from two patients (a 6-month-old boy and a 5-year-old girl) and a healthy donor were transplanted into host mice (hemi-, hetero-OTCD mice, and control mice, respectively). HHs were isolated from these mice and used for serial transplantation into the next host mouse or for in vitro experiments. Histological, biochemical, and enzyme activity analyses were performed. Cultured HHs were treated with ammonium chloride or therapeutic drugs. Replacement rates exceeded 80% after serial transplantation in both OTCD mice. These highly humanized OTCD mice showed characteristics similar to OTCD patients that included increased blood ammonia levels and urine orotic acid levels enhanced by allopurinol. Hemi-OTCD mice showed defects in OTC expression and significantly low enzymatic activities, while hetero-OTCD mice showed residual OTC expression and activities. A reduction in ammonium metabolism was observed in cultured HHs from OTCD mice, and treatment with the therapeutic drug reduced the ammonia levels in the culture medium. In conclusion, we established in vivo OTC mouse models with hemi- and hetero-patient HHs. HHs isolated from the mice were useful as an in vitro model of OTCD. These OTC models could be a source of valuable patient-derived

hepatocytes that would enable large scale and reproducible experiments using the same donor.

1 | INTRODUCTION

Urine cycle disorders (UCDs) are representative hereditary and metabolic diseases in newborns. UCDs feature hyperammonemia caused by a genetic defect in the metabolic pathway producing urea from ammonia, which is carried out primarily in the liver.[1,2] Ornithine transcarbamylase deficiency (OTCD) is one of the most common genetic causes of UCDs.[1-3] Unlike other types of UCDs, the gene coding for OTC is located on the X chromosome. Affected males typically present with severe hyperammonemia soon after birth. Affected females show a spectrum of severities ranging from profound hyperammonemia in newborns to an asymptomatic carrier state in adults due to mosaic inactivation of the X chromosome. Patients with mild OTCD or asymptomatic carriers may reveal mild cognitive impairments and executive function deficits on neuropsychological testing and may be at a risk of life-threatening hyperammonemia at any age due to several stressors.[4,5] Patients require lifelong treatment including protein restriction and nitrogen scavengers to control blood ammonia levels and may require liver transplantation.

In the last decade, several chimeric mice with humanized livers have been developed. We recently established a new chimeric mouse (PXB-Mouse) with high and stable replacement rates (>70%) of human hepatocytes (HHs) using urokinase-type plasminogen activator-cDNA/ severe combined immunodeficient (cDNA-uPA/SCID) mouse as a host.[6,7] A number of studies have demonstrated the utility of this chimeric model and the isolated HHs (PXB-cells) for the prediction of human drug metabolism and pharmacokinetics[8-12] and as a susceptible model of infection by hepatitis viruses.[13,14] To mimic normal human liver functions, normal HHs are generally transplanted to produce chimeric mice with humanized livers. However, only two reports have used HHs from OTCD patients,[15,16] and the OTCD pathological condition in humanized mice harboring HHs from OTCD patients has not been demonstrated. We hypothesized that highly repopulated chimeric mice are essential to represent the disease phenotype. However, not all cryopreserved hepatocytes obtained from patients can be used to produce highly repopulated chimeric mice due to the varying conditions of the cryopreserved HHs. To solve this problem, we isolated hepatocytes from primary transplanted and poorly repopulated chimeric mice, purified the HHs and serially transplanted into subsequent host mice. This protocol was successful in generating humanized chimeric mice with a high replacement ratio of HHs.

In vitro studies of patient hepatocytes have been hampered by the limited availability of HHs and the lack of an efficient cell propagation system. In our passaging system using chimeric mice, normal HHs can proliferate 500 to 1000 times in a mouse liver. A previous study described that the isolated HHs (PXB-cells) from the PXB-Mouse can maintain highly differentiated phenotypes for at least 21 days.[17] Here, we applied this propagation system and in vitro culture system to patient HHs to investigate the efficacy of drugs prescribed for OTCD patients.

In this study, we established a new chimeric mouse model displaying the pathological condition of OTCD by transplanting HHs from patients with OTCD into mice. The isolated patient HHs propagated in chimeric mice could be useful for screening of potential therapeutic compounds for liver disease.

2 | MATERIAL AND METHODS

2.1 | Preparation of HHs from patients

Liver tissues from two OTCD patients were used. The patients included a 5-year-old girl with a heterozygous *OTC* mutation at exon 8 (NM_000531.5: c.770G > A, p. Gly257Asp) and a 6-month-old boy with a hemizygous *OTC* mutation (deletion of exon 1 region). The hepatocytes were isolated from the resected liver tissue through the collagenase perfusion and cryopreserved in liquid nitrogen until use, as described previously[18] with the exception that collagenase of experimental grade (1 mg/mL in Hanks solution, Collagenase Type I for cell dispersion, 035-17604, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used. Hepatic parenchymal cells were isolated by low-speed centrifugation (50g).

Cell numbers and viability were assessed using the Trypan blue exclusion test. The cells were cryopreserved in a programmed freezer and preserved in liquid nitrogen until required. Control hepatocytes from a 2-year-old Hispanic female were purchased from BD Biosciences (BD195; Woburn, MA).

2.2 | Generation of chimeric mice with humanized livers, isolation of HHs, and serial transplantation

Cryopreserved HHs isolated from the two OTCD patients were thawed and 0.5 or 1.0×10^6 cells were transplanted into the spleens of 2 to 4-week-old, female, homozygous cDNA-uPA/SCID mice as previously described.[6] Briefly, a small incision (approximately 5 mm) was made on the skin and muscle layer on the left side of a mouse anesthetized using 2.5% isoflurane. The edge of the pancreas was held using tweezers, and the spleen was gently pulled out from the incision. The tip of the spleen was inserted into a looped suture. The needle filled with the HHs suspension was inserted into the tip of the spleen and injected for 1 second (approximately 0.5 or 1×10^6 cells/20 µL/ mouse). Hemostasis of the spleen was performed by tying the tip of the spleen with the looped suture, after which both the pancreas and spleen were returned to the abdominal cavity. Finally, the skin and the rectus abdomen were sutured together using a surgical needle and a suture. These chimeric mice were designated as OTCD mice and were further classified as hetero-OTCD mice or hemi-OTCD mice depending on the sex of the patient, female or male, respectively.

To perform serial transplantation, HHs were isolated from OTCD mice and purified as previously described with some modifications.[17] Briefly, HHs were isolated by a two-step collagenase perfusion method using type IV collagenase (Sigma-Aldrich Japan, Tokyo, Japan). To eliminate contaminating mouse cells, the isolated HHs were incubated for 30 minutes with Dynabead-conjugated with the 66Z antibody specific for mouse cells. HHs with >99% purities were obtained using this procedure. The ratio of beads lacking HHs and beads conjugated with HHs was determined using microscopy. The HHs were purified using the Dynal MPC-1 magnetic particle concentrator (Dynal Biotech, Oslo, Norway).[19] The purified HHs were immediately used as the donor cells for serial transplantation and for in vitro study. Serial transplantations were performed in up to the third generation of hetero-OTCD mice and fourth generation of hemi-OTCD mice using the method mentioned above. Chimeric mice with second generation BD195 HHs were used as the control.

2.3 | Blood examination

The concentration of human albumin (h-Alb) in blood samples obtained from chimeric mice was measured 3 and 6 weeks after transplantation and once every 2 weeks thereafter by immunonephelometry in a model BM6050 autoanalyzer (JEOL, Tokyo, Japan) using LX Reagent Seiken Alb II (Eisen Chemical, Tokyo, Japan). Plasma ammonia was determined using DRI-CHEM 7000 (Fujifilm, Tokyo, Japan).

2.4 | Allopurinol challenge test and measurement of urinary orotic acid

Allopurinol (Wako, Tokyo, Japan) dissolved in 0.5% methyl cellulose (Wako) was orally administered at a concentration of 5 mg/kg body weight. To collect urine samples, mice were kept in metabolic cages for 24 hours after administration of the vehicle or allopurinol. Urinary orotic acid was measured using an established colorimetric method.[20] The absorbance was determined at 490 nm using a SpectraMax i3 Multimode microplate platform (Molecular Devices, Sunnyvale, CA). Urinary orotic acid concentration was corrected according to urinary creatinine levels determined with a model 7170 autoanalyzer (Hitachi Ltd., Tokyo, Japan).

2.5 | OTC enzyme activity

OTC enzyme activity was determined by a colorimetric method based on the production of L-citrulline as described previously.[20,21] Citrulline production was determined by measuring the absorbance at 490 nm using a SpectraMax i3 Multimode microplate platform (Molecular Devices). The enzyme activity was expressed as citrulline µmol/mg protein/h.

2.6 | Histological analysis

Liver tissues obtained from mice were immediately fixed in 10% neutral buffered formaldehyde, dehydrated using graded alcohol, and embedded in paraffin for light microscopy examination. Paraffin sections with a thickness of 5 µm were prepared from the liver tissues for hematoxylin and eosin staining. Liver tissues were also embedded in O.C.T compound (Sakura Finetek, Tokyo, Japan) and snap frozen. Frozen sections (5 µm) were prepared and fixed in 10% neutral buffered formaldehyde for 10 minutes at room temperature and washed with PBS. Immunofluorescence (IF) was performed using a 1:100

dilution of anti-human cytokeratin 8/18 (hCK8/18) mouse monoclonal antibody (Cappel Laboratory, Cochranville, PA, USA), specific for HHs and a 1:250 dilution of anti-OTC rabbit polyclonal antibody (GeneTex, Irvine, CA) cross-reactive to mouse and human epitopes. After washing in PBS containing Tween 20, the sections were incubated with Alexa 488 labeled donkey anti-mouse and Alexa 594 labeled goat anti-rabbit IgG secondary antibodies (Life Technologies Japan, Tokyo, Japan), for 1 hour at room temperature. The replacement index (RI) of HHs and human OTC positive rate were calculated using double immunostaining sections from two liver lobes. The RI of HHs was the ratio of the hCK8/18 positive HHs to the entire area. The human OTC positive rate was the ratio of the OTC and hCK8/18 double-positive area to the hCK8/18 positive area.

2.7 | Selection of animals and time points for each experiment

The time points of each analysis are summarized in Figure S1. Mice with the highest blood h-Alb levels were used for perfusion experiments. In the final generations, the third generation of Hetero- and fourth generation of Hemi-OTCD mice, three mice showing the averages of h-Alb values in each group were chosen for perfusion. For histological analysis, three mice showing the average h-Alb values in each group were chosen for each generation. In the case of the third generation of Hemi-OTCD mice, all surviving animals except for the mice perfused for serial transplantation were used for histological observation. In the third generation of Hetero-OTCD mice, two different points were set: one at the same time point as that in the first generation and the other at the time point of perfusion. Orotic acid was measured before euthanasia of the animals used for histological analysis in third and fourth generations of Hetero- and Hemi-OTCD mice, respectively. Plasma ammonia levels of all animals in the group were measured before the final sampling.

2.8 | Culture of HHs, ammonia loading, and drug treatment

HHs isolated from chimeric mice were seeded in type I collagen-coated 24-well plates at 2.13×10^5 cells/cm^2 and cultured as reported in our previous study.[17] The medium was changed every 3 to 4 days. Six days after seeding, the medium was replaced by serum-free medium containing GlutaMax, which is a more stable form of L-glutamine that does not deaminate and release additional ammonia.

Twenty-four hours after pretreatment with the medium, HHs were treated with 10 mM NH$_4$Cl for 24 and 48 hours in the same medium. The cell culture supernatant was collected for determination of the levels of h-Alb secretion and ammonia concentrations by the same methods as used for blood examination. For drug treatment, on the day following seeding, HHs were cultured with 0.01, 0.1, and 1 mM sodium 4-phenylbutyrate (4-PB) or sodium benzoate (SB) for 2 weeks.

3 | RESULTS

3.1 | Generation of OTCD mice

Transplanted HHs from patients were successfully engrafted in hetero- and hemi-OTCD mice as shown by the low h-Alb levels in the first generation (Table 1, Figure S1). Except for the third generation hemi-OTCD mice, all OTCD mice survived longer than 18 weeks of age (15 weeks after transplantation). Rapid elevation of blood h-Alb levels, hyperammonemia, and lower body weight were seen in the third generation hemi-OTCD mice compared with the other mice. Most of the mice became moribund or died prior to 8 weeks of age (5 weeks after transplantation). Therefore, fourth generation hemi-OTCD mice were produced by transplanting half the number of HHs (0.5×10^6 cells) isolated from third generation mice. The blood h-Alb concentration was significantly improved on serial transplantation in both hetero- and hemi-OTCD mice.

3.2 | Histological analysis of OTCD mice, proliferation of HHs, and OTC expression in host mouse liver

Histologically, as shown previously in humanized liver mice transplanted with normal HHs, cryopreserved HHs from OTCD patients or fresh HHs isolated from OTCD mice proliferated and occupied host mice liver tissues after transplantation (Figure 1A). Consistent with blood h-Alb levels, hCK8/18 positive rate (RI) in OTCD mice was significantly improved by serial transplantation, especially in hemi-OTCD mice, and exceeded 80% in both third generation OTCD mice (Figure 1B,C). Immuno-staining of OTC revealed different expression patterns in liver tissue of hetero- and hemi-OTCD mice, representing its X-linked inheritance (Figure 1B). In liver tissues of hetero-OTCD mice, OTC displayed a mosaic pattern in the hCK8/18-positive area, suggesting X chromosome mosaic inactivation, which was demonstrated in hetero OTCD mice previously.[22] In contrast, OTC was

TABLE 1 Blood h-Alb levels, body weight and survival rates in OTCD-mouse

Type	Gen	Week-old[a] (weeks after TP)	Number of transplanted cells (×10^6)	N[b]	h-Alb (mg/ml)	BW (g)	RI (%)	hOTC positive rate (%)	Survival rate (%)[c]
Hetero-OTCD	1	23 (20)	1.0	5/5	3.7 ± 2.3 (4)[d]	15.7 ± 2.4 (4)[d]	68.3 ± 13.3 (3)	56.6 ± 10.7 (3)	100
	2	23 (20)		4/5	8.8 ± 1.2 (4)	17.3 ± 1.7 (4)	n.a.	n.a.	100
	3	23 (20)		9/10	7.8 ± 1.1 (6)	14.3 ± 1.5 (6)	91.9 ± 7.7 (6)	59.0 ± 5.1 (6)	100
Hemi-OTCD	1	18 (15)	1.0	5/6	0.6 ± 0.4 (4)	16.1 ± 3.2 (6)	6.5 ± 1.9 (3)	n.d.	100
	2	18 (15)		3/3	7.3 ± 0.5 (3)	15.1 ± 1.1 (3)	n.a.	n.a.	100
	3	8 (5)		14/15	7.8 ± 1.5 (9)	8.0 ± 1.5 (10)	83.0 ± 3.3 (5)	n.d.	0
	4	18 (15)	0.5	6/7	5.3 ± 1.5 (6)	15.5 ± 1.5 (6)	82.4 ± 4.6 (3)	n.d.	100

Abbreviations: BW, body weight; Gen, generation; h-Alb, human albumin; hOTC, human ornithine transcarbamylase; n.a., not available; n.d., not detectable; OTCD, ornithine transcarbamylase deficiency; TP, transplantation; RI, replacement index.

[a]Week-old and weeks after TP of the animals at the time of measurement of h-Alb and body weight.

[b]Number of animals showing detectable h-Alb levels in total transplanted animals.

[c]Survival rate at 18 (Hemi) or 23 (Hetero)-week-old.

[d]Number of animals at the time of measurement of h-Alb, body weight, histological RI and hOTC positive rate.

completely absent in the hCK8/18-positive area in hemi-OTCD mouse liver tissue. The human OTC positive rate confirmed that serial transplantation did not influence the human OTC positive rate in subsequent generations of both hetero- and hemi-OTCD mice, suggesting the similar growth of OTC positive and negative hepatocytes (Figure 1D). The expression of several hepatocyte functional genes, including OTC, was evaluated and compared between generations to assess the influence of serial transplantation on the genes (Figure S2). Differences in gene expression were observed between each group but were not observed between each generation in all groups except for Cytochrome P450 3A4 (CYP3A4) in Hetero-OTCD which is less than a twofold difference.

3.3 | Biochemical analyses of OTCD mouse and OTC enzyme activities and protein expression of isolated hepatocytes

Compared with control humanized liver mice, blood ammonium levels were significantly increased in third and fourth generation hetero- and hemi-OTCD mice, especially in the third generation hemi-OTCD mice (which showed a low survival rate) (Figure 2A). Allopurinol challenge test revealed the significant elevation of orotic aciduria in hetero-OTCD mice treated by allopurinol, while severe orotic aciduria was observed in

hemi-OTCD mice even without allopurinol (Figure 2B). Isolated HHs showed significant reduction in OTC enzyme activity (Figure 2C) and protein expression (Figure S3A,B) in patient hepatocytes from hetero- and hemi-OTCD mice compared with control HHs.

3.4 | Cell culture of hepatocytes isolated from OTCD mice

Isolated HHs from OTCD mice cultured on collagen-coated plates displayed a similar morphology as control hepatocytes. A bi- or monocular polygonal cell shape, clear cell boundaries, and bile canaliculi-like structures were evident more than 2 weeks after plating (Figure 3A). HHs from control chimeric mice stably secreted albumin into the culture medium for over 2 weeks, whereas albumin secretion was significantly lower in OTCD mouse HHs (Figure 3B). To assess the urea cycle function of cultured HHs, the ammonia load test was performed. Measurements of ammonium and urea levels in culture media revealed the complete lack of ammonia metabolism and urea synthesis in HHs isolated from hemi-OTCD mice (Figure 3C,D). On the other hand, accumulation of ammonia in the culture media only 24 hours after ammonia loading suggested a partial dysfunction of ammonia metabolism in hetero-OTCD HHs. However, the synthesis of urea in hetero-OTCD HHs was similar to normal HHs,
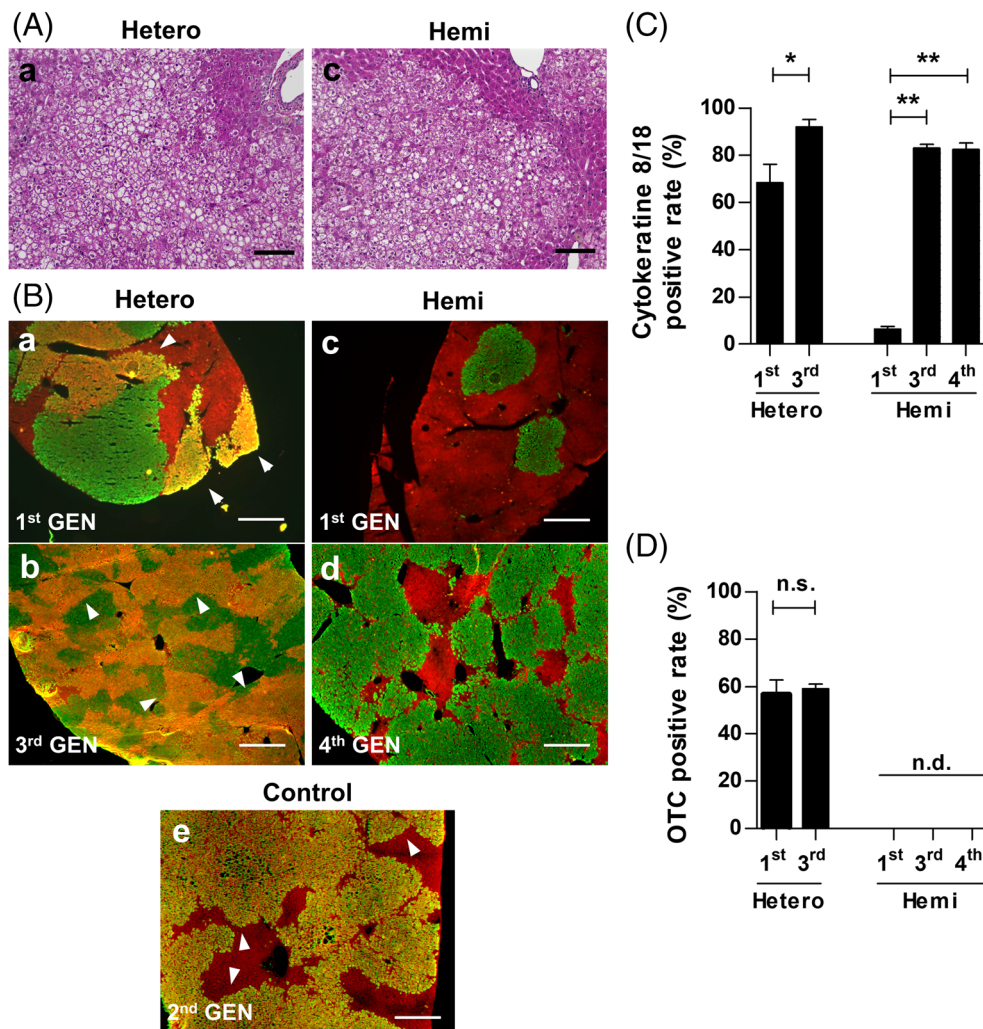
FIGURE 1 Histological analysis of ornithine transcarbamylase deficiency (OTCD) mouse liver. A, Hematoxylin and eosin staining for liver section of hetero-OTCD (third generation [GEN] at 20 weeks after transplantation [WAT]) and hemi-OTCD (fourth GEN at 15 WAT) mice. Scale bars denote 100 μm. B, Double immunofluorescence for hCK8/18 (green) and ornithine transcarbamylase (OTC) (red) of hetero-OTCD mouse liver tissue (a, first GEN; b, third GEN), hemi-OTCD mouse liver tissue (c, first GEN; d, fourth GEN), and control (e) humanized mouse liver tissue. Arrowheads indicate human OTC (hCK8/18 and OTC double) positive area. Scale bars denote 400 μm. C, hCK8/18 positive rate (%) per entire area in each generation of hetero- (n = 3-6) and hemi-OTCD mouse liver (n = 3-6). D, Human OTC positive rates calculated by OTC and hCK8/18 double-positive area to hCK8/18 positive area in hetero- and hemi-OTCD mouse liver (n = 3-6). Graph bars represent mean ± SD; * and ** indicate $P < .05$ and $P < .01$ by one-way ANOVA or Student's t-test; n.s., not significant; n.d., not detectable

which was elevated over time after ammonia loading (Figure 3C,D). Treatment with 4-PB and SB produced significant reductions in the levels of ammonia in the culture medium of OTCD HHs. The efficiency was higher in 4-PB treated samples than in SB treated samples, and hetero-OTCD HHs were more susceptible than hemi-OTCD HHs (Figure 3E).

4 | DISCUSSION

Several recent studies have demonstrated that HHs isolated from patients with genetic metabolic diseases, including OTCD and other UCDs, proliferate in humanized liver

mouse models.[15,16,23,24] Furthermore, some have a disease phenotype, such as carbamoyl phosphate synthetase-1 (CPS-1) deficiency[24] and familial hypercholesterolemia,[21] but not OTCD. In these studies, another representative humanized liver mouse model, FRG mice, with a genetic background of fumarylacetoacetate hydrolase (FAH) deficiency and immunodeficiency (Rag2$^{-/-}$ IL2rg$^{-/-}$) were used. There are several differences between the two models,[7] the major difference being the mechanism of hepatotoxicity in the host mouse, which is an essential factor for the proliferation of HHs. FAH deficiency in FRG mice causes lethal defects and requires frequent administration of nitisinone, an inhibitor of the tyrosine metabolic pathway, to maintain animal viability. In contrast, uPA/SCID
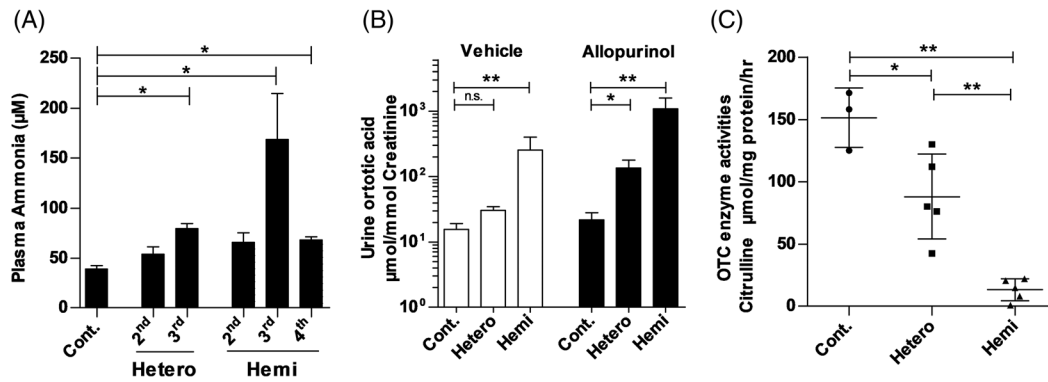
FIGURE 2 Biochemical parameters of ornithine transcarbamylase deficiency (OTCD) mouse, ornithine transcarbamylase (OTC) enzyme activities, and protein expression of isolated HHs. A, Plasma ammonia levels in hetero-OTCD mice (second GEN at 24 WAT [n = 4], and third GEN at 25-28 WAT [n = 6]), hemi-OTCD mice (second GEN at 18 WAT [n = 3], third GEN at 8 WAT [n = 6] and fourth GEN at 15 WAT [n = 7]), and control PXB-mice [n = 5]. B, Urine orotic acid in hetero-OTCD mice (third GEN at 25 WAT [n = 3]), hemi-OTCD mice (fourth GEN at 15 WAT [n = 3]), and PXB-mice (n = 4) with or without allopurinol challenge test. C, OTC enzyme activity in isolated hepatocytes from hetero-OTCD mice (n = 5), hemi-OTCD mice (n = 5), and control PXB-mice (n = 3). Graph bars represent mean ± SD; * and ** indicate $P < .05$ and $P < .01$ by Kruskal-Wallis test or one-way ANOVA; n.s., not significant
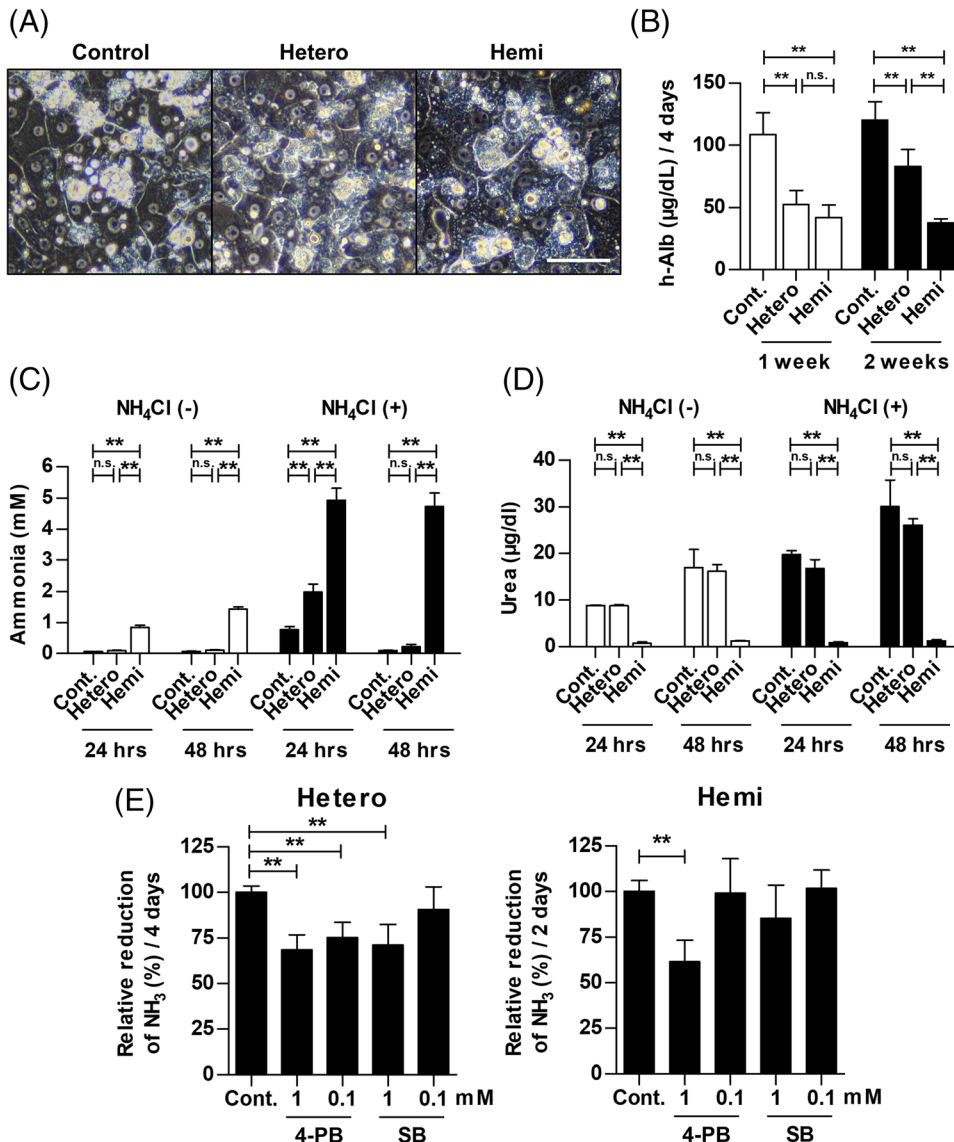
FIGURE 3 Cell culture of hepatocytes isolated from ornithine transcarbamylase deficiency (OTCD) mice. A, Phase-contrast photographs of human hepatocytes (HHs) isolated from OTCD and control mice at 14 days after plating. Scale bar denote 50 μm. B, Human albumin levels in culture media of HHs at 1 and 2 weeks after plating. C, Ammonium and, D, urea levels in cell culture media after medium change with/ without NH₃Cl loading and, E, the effect of 4-PB or SB treatment. Graph bars represent mean ± SD; ** indicate $P < .01$ by Kruskal-Wallis test or one-way ANOVA; n.s., not significant

mice do not require maintenance, and HHs are not unnecessarily exposed to the drug. In addition, even adult FRG mice are acceptable for HHs transplantation, whereas uPA/SCID mice have a narrow window for cell transplantation (2-4 weeks old). uPA/SCID mice can achieve a high RI in a relatively short period (8-12 weeks) compared with FRG mice (approximately 28 weeks). Theoretically, as long as HHs transplanted into mice have normal proliferation ability, humanization would be achieved regardless of the host mouse strain. In this study, OTCD HHs proliferated in our uPA base host mice, and humanized mice highly repopulated with OTCD HHs presented pathological states.

The study findings also emphasize the enormous benefits of serial transplantation in achieving high repopulation of mouse livers with patient HHs. We experientially know that have shown that cryopreserved and commercially supplied HHs vary in their efficacy of engraftment and replacement during the first transplantation. The success of engraftment and replacement in host mice can depend on the vial lots used. In the present study, the poor engraftment and repopulation abilities of low-quality HHs could be significantly improved by serial transplantation. Considering the functional reserve capacity of the liver, when the RI of mouse liver with genetic or metabolic disease HHs was low, the remaining host mouse hepatocytes might compensate for the defective hepatic functions. Therefore, it is assumed that a high RI is required to reproduce the particular pathological state of the genetic and metabolic disease. In this study, only serially transplanted OTCD mice with highly repopulated HHs displayed symptoms that included hyperammonemia and orotic aciduria. Especially in third generation hemi-OTCD mice, the rapid and extensive repopulation of HHs derived from patients caused severe hyperammonemia and high mortality, which reflected the severity of OTCD in male patients. In immature animals, rapid repopulation at an early stage could cause underdevelopment and poor growth conditions but not mortality. Thus, rapid repopulation might also contribute to high mortality together with hyperammonemia. Transplantation of reduced numbers of cells resulted in the coexistence of long-term survival and symptoms in fourth generation hemi-OTCD mice, indicating that modifying the number of transplanted cells might result in a severity of disease that is appropriate to the experimental objective. In addition to hyperammonemia, OTCD mice displayed another clinical feature, orotic acid excretion that results from the transfer of carbamoyl phosphate into the pyrimidine synthesis pathway and production of orotate and orotidine. In general, while potent orotic aciduria is observed in male patients, female patients show several degrees of orotic acid excretion.[1,25-27] A remarkable increase in orotic acid excretion after

administering allopurinol, which inhibits orotidine decarboxylase, is considered a diagnostic feature in patients suspected of OTCD or in carrier patients without orotic aciduria.[26-29] Therefore, the enhanced excretion of orotic acid by allopurinol that was observed in OTCD mice suggests an activated pyrimidine synthesis pathway that is similar to that observed in OTCD patients.

Concerning serial transplantation, the repeated expansion and isolation of patient HHs might promote selective proliferation of the cell with healthier state, which produces a milder disease phenotype than the previous generation mouse. However, histological measurements showed that the human OTC positive rate did not change between generations, indicating that OTCD in HHs did not affect the engraftment and proliferation in livers of the host mice. However, further analyses are necessary to validate this, as no conclusive data, such as those obtained after bromodeoxyuridine or immunostaining, have been generated in this study to assess the proliferation ability in each cell type. Additionally, consistent gene expression through the generations (Figure S2) suggests that serial transplantation does not affect the phenotype of transplanted HHs at least after the third or fourth generations.

We also demonstrated the utility of isolated patient HHs as an in vitro model of OTCD. While hemi-OTCD HHs completely lacked ammonia metabolism and urea synthesis, hetero-OTCD HHs showed decreased ammonia metabolic activity only at 24 hours after ammonia loading, with no difference in urea synthesis. Although CPS-1 is the first step and is considered the rate-limiting enzyme in the urea cycle, differences in the activities of other enzymes have also been reported. Arginosuccinate synthetase and arginosuccinate lyase, the downstream enzymes that follow OTC in urea cycle, show much lower activity than OTC.[30,31] This may explain the similar urea production ability of hetero-OTC with control HHs, despite the significant difference in OTC enzyme activity and ammonia metabolism. Consistent with this, the clinically measured BUN levels of girl donors (6.1 mg/dL) were within the reference range (5-18 mg/dL),[32] while the boys showed extreme reduction (2.4 mg/dL).

Furthermore, we demonstrated the utility of patient HHs for pharmacological examination by using 4- PB and SB as representative ammonia-scavenging drugs. The difference in susceptibility between hetero- and hemi-OTCD HHs to these drugs may be a result of the extraordinarily high basal level of ammonia in hemi-OTCD HHs (Figure 3C). Phenylbutyrate and benzoyl-CoA, which are products of 4- PB and SB metabolism, respectively, are conjugated with glutamine and glycine, respectively, in hepatocytes to excrete these ammonia-genic amino acids in urine.[33-35] Theoretically, the scavenging efficacy per

molecule of 4-PB should be double that of benzoate because glutamine has two nitrogen atoms compared to one in glycine. Further, a previous study involving healthy volunteers demonstrated the higher nitrogen scavenging efficiency of 4-PB compared to SB.[35] This might explain the difference of the scavenging efficacy between 4-PB and SB in vitro in this study. Of note, suitable animal models to assess the drug efficacy of 4-PB are lacking because nonprimate animals conjugate glycine, not glutamine, with phenylbutyrate.[36,37] This is a good example of the interspecies difference in drug metabolism between humans and experimental animals that must be considered in drug development. Therefore, the OTCD mouse and HHs isolated from the mouse established in this study may become suitable in vivo and in vitro models, respectively having both genetic defect and human type drug metabolism. These features differ from the several existing OTCD mouse models, which feature the native (mouse type) drug metabolism.

In addition to the demonstrated utility in drug screening studies, mice with humanized livers containing HHs isolated from human patients may be useful for studies with genetic approaches such as gene therapy or gene editing. Ginn et al demonstrated that point mutations in the *OTC* gene could be repaired at certain levels in proliferating OTCD patient-derived HHs transplanted into Fah-knockout mice using an AAV vector-based approach involving CRISPR-Cas9-mediated cleavage and homology-directed repair (HDR).[15] As they discussed, this successful repair could be overestimated in humanized liver chimeric mice with a robust proliferation of HHs in the host mouse liver, since classical HDR occurs only in proliferating cells. The current issues in clinical applications of this model include the development of cell cycle-independent approaches and a highly efficient liver-targeted gene transfer technology.

One week after plating, the levels of h-Alb were lower in the culture medium with hetero- and hemi-OTCD HHs (approximately 48% and 38%, respectively) than in the control, and ammonia loading reduced the h-Alb levels in control and hetero-OTCD HHs (Figure S4). These findings are partially consistent with a previous report that demonstrated the reduced Alb levels in primary HHs cell culture by ammonia loading and a relationship between the plasma ammonia levels and liver dysfunction in patients with OTCD showing acute liver failure.[38] However, blood h-Alb levels of OTCD mouse were lower than control (approximately 61% and 75% of control, respectively), although the histological RI were similar in both hetero- and hemi-OTCD mice without any relation to the ammonia levels. Therefore, other possibilities concerning hepatic dysfunction in OTCD hepatocytes should be considered. For example, the defect of urea cycle itself should cause the same intracellular alteration of amino acid status as systemic changes, which may affect protein production. Of course, it may be due to individual differences of each donor cell. Further study using many OTCD cells from the same patients may help us to address the pathology of hepatic dysfunction in OTCD hepatocytes.

In summary, we established a novel in vivo OTC mouse model harboring patient HHs by exploiting the benefits of serial transplantation, such as improvement of RI and robust expansion of HHs. Furthermore, we demonstrated the usefulness of isolated patient HHs as an in vitro model of OTCD. This novel OTC mouse model will be a promising source of valuable patient-derived hepatocytes in vitro, which will enable large scale and reproducible experiments using the same OTCD donor.

CONFLICT OF INTEREST
Go Sugahara, Chihiro Yamasaki, Ami Yanagi, Suzue Furukawa, Yuko Ogawa, Yuji Ishida, and Chise Tateno are employees of PhoenixBio Co., Ltd. Akinari Fukuda, Shin Enosawa and Akihiro Umezawa declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
Go Sugahara conducted experiment and drafted the manuscript. Chihiro Yamasaki, Ami Yanagi, and Suzue Furukawa conducted experiment. Yuko Ogawa carried out the statistical analyses. Akinari Fukuda, Shin Enosawa and Akihiro Umezawa provided hepatocytes from patients. Chise Tateno and Yuji Ishida participated in research design, led and supervised the study and contributed to revision of the manuscript. All authors approved the final, submitted version of the manuscript.

ETHICS STATEMENT
The procedures involving human liver tissues were approved by the Research Ethics Committee of the National Center for Child Health and Development and PhoenixBio Research Ethics Committee of Human Tissue. All experimental procedures were conducted in accordance with the guidelines provided by the Proper Conduct of Animal Experiments (June 1, 2006; Science Council of Japan). The procedures were approved by the Animal Care and use Committee of PhoenixBio Co., Ltd.

A PATIENT CONSENT STATEMENT

Patient liver tissues were collected in National Research Institute for Child Health and Development in a standardized manner with the permission of the patients' families. Written informed consent was obtained in each case.

ORCID

Chise Tateno https://orcid.org/0000-0002-2640-3268

REFERENCES

1. Ah Mew N, Simpson KL, Gropman AL, Adam MP, Ardinger HH. Urea cycle disorders overview (updated 2017), *Gene Reviews*. Seattle, WA: University of Washington; 1993. https://www.ncbi.nlm.nih.gov/books/NBK1217/.

2. Stone WL, Basit H, Jaishankar GB. *Urea Cycle Disorders*. Treasure Island, FL: StatPearls; 2020. https://www.ncbi.nlm.nih.gov/books/NBK482363/.

3. Lichter-Konecki U, Caldovic L, Morizono H, Simpson K. Ornithine transcarbamylase deficiency (updated 2016). *Gene Reviews*. Seattle, WA: University of Washington; 2013. https://www.ncbi.nlm.nih.gov/books/NBK154378/.

4. Matsuda I, Nagata N, Matsuura T, et al. Retrospective survey of urea cycle disorders: part 1. Clinical and laboratory observations of thirty-two Japanese male patients with ornithine transcarbamylase deficiency. *Am J Med Genet*. 1991;38(1):85-89.

5. Gyato K, Wray J, Huang ZJ, Yudkoff M, Batshaw ML. Metabolic and neuropsychological phenotype in women heterozygous for ornithine transcarbamylase deficiency. *Ann Neurol*. 2004;55(1):80-86.

6. Tateno C, Kawase Y, Tobita Y, et al. Generation of novel chimeric mice with humanized livers by using hemizygous cDNA-uPA/SCID mice. *PLoS One*. 2015;10(11):e0142145.

7. Sugahara G, Ishida Y, Sun J, Tateno C, Saito T. Art of making artificial liver: depicting human liver biology and diseases in mice. *Semin Liver Dis*. 2020;40(2):189-212.

8. Kanamori T, Iwata YT, Segawa H, et al. Metabolism of Butyrylfentanyl in fresh human hepatocytes: chemical synthesis of authentic metabolite standards for definitive identification. *Biol Pharm Bull*. 2019;42(4):623-630.

9. Kanamori T, Togawa-Iwata Y, Segawa H, et al. Use of hepatocytes isolated from a liver-humanized mouse for studies on the metabolism of drugs: application to the metabolism of fentanyl and acetylfentanyl. *Forensic Toxicol*. 2018;36(2):467-475.

10. Sanoh S, Horiguchi A, Sugihara K, et al. Prediction of in vivo hepatic clearance and half-life of drug candidates in human using chimeric mice with humanized liver. *Drug Metab Dispos*. 2012;40(2):322-328.

11. Watari R, Kakiki M, Oshikata A, et al. A long-term culture system based on a collagen vitrigel membrane chamber that supports liver-specific functions of hepatocytes isolated from mice with humanized livers. *J Toxicol Sci*. 2018;43(8):521-529.

12. Watari R, Kakiki M, Yamasaki C, et al. Prediction of human hepatic clearance for cytochrome P450 substrates via a new culture method using the collagen Vitrigel membrane chamber and fresh hepatocytes isolated from liver humanized mice. *Biol Pharm Bull*. 2019;42(3):348-353.

13. Ishida Y, Yamasaki C, Yanagi A, et al. Novel robust in vitro hepatitis B virus infection model using fresh human hepatocytes isolated from humanized mice. *Am J Pathol*. 2015; 185(5):1275-1285.

14. Uchida T, Imamura M, Kan H, et al. Usefulness of humanized cDNA-uPA/SCID mice for the study of hepatitis B virus and hepatitis C virus virology. *J Gen Virol*. 2017;98(5):1040-1047.

15. Ginn SL, Amaya AK, Liao SHY, et al. Efficient in vivo editing of OTC-deficient patient-derived primary human hepatocytes. *JHEP Rep*. 2020;2(1):100065.

16. Gramignoli R, Tahan V, Dorko K, et al. New potential cell source for hepatocyte transplantation: discarded livers from metabolic disease liver transplants. *Stem Cell Res*. 2013;11(1):563-573.

17. Yamasaki C, Ishida Y, Yanagi A, et al. Culture density contributes to hepatic functions of fresh human hepatocytes isolated from chimeric mice with humanized livers: novel, long-term, functional two-dimensional in vitro tool for developing new drugs. *PLoS One*. 2020;15(9):e0237809.

18. Enosawa S. Isolation of GMP grade human hepatocytes from remnant liver tissue of living donor liver transplantation. *Methods Mol Biol*. 2017;1506:231-245.

19. Yamasaki C, Kataoka M, Kato Y, et al. In vitro evaluation of cytochrome P450 and glucuronidation activities in hepatocytes isolated from liver-humanized mice. *Drug Metab Pharmacokinet*. 2010;25(6):539-550.

20. Oishi Y, Kakimoto T, Yuan W, Kuno S, Yamashita H, Chiba T. Fetal gene therapy for ornithine transcarbamylase deficiency by intrahepatic plasmid DNA-micro-bubble injection combined with hepatic ultrasound Insonation. *Ultrasound Med Biol*. 2016;42(6):1357-1361.

21. Tarasenko TN, Rosas OR, Singh LN, Kristaponis K, Vernon H, McGuire PJ. A new mouse model of mild ornithine transcarbamylase deficiency (spf-j) displays cerebral amino acid perturbations at baseline and upon systemic immune activation. *PLoS One*. 2015;10(2):e0116594.

22. Shiojiri N, Imai H, Goto S, Ohta T, Ogawa K, Mori M. Mosaic pattern of ornithine transcarbamylase expression in spfash mouse liver. *Am J Pathol*. 1997;151(2):413-421.

23. Bissig-Choisat B, Wang L, Legras X, et al. Development and rescue of human familial hypercholesterolaemia in a xenograft mouse model. *Nat Commun*. 2015;6:7339.

24. Srinivasan RC, Zabulica M, Hammarstedt C, et al. A liver-humanized mouse model of carbamoyl phosphate synthetase 1-deficiency. *J Inherit Metab Dis*. 2019;42(6):1054-1063.

25. Bachmann C, Colombo JP. Diagnostic value of orotic acid excretion in heritable disorders of the urea cycle and in hyperammonemia due to organic acidurias. *Eur J Pediatr*. 1980;134(2):109-113.

26. Hauser ER, Finkelstein JE, Valle D, Brusilow SW. Allopurinol-induced orotidinuria. A test for mutations at the ornithine carbamoyltransferase locus in women. *N Engl J Med*. 1990;322 (23):1641-1645.

27. Oexle K. Biochemical data in ornithine transcarbamylase deficiency (OTCD) carrier risk estimation: logistic discrimination and combination with genetic information. *J Hum Genet*. 2006; 51(3):204-208.

28. Burlina AB, Ferrari V, Dionisi-Vici C, Bordugo A, Zacchello F, Tuchman M. Allopurinol challenge test in children. *J Inherit Metab Dis*. 1992;15(5):707-712.

29. Grünewald S, Fairbanks L, Genet S, et al. How reliable is the allopurinol load in detecting carriers for ornithine

transcarbamylase deficiency? *J Inherit Metab Dis*. 2004;27(2): 179-186.

30. Mukarram Ali Baig M, Habibullah CM, Swamy M, et al. Studies on urea cycle enzyme levels in the human fetal liver at different gestational ages. *Pediatr Res*. 1992;31(2):143-145.

31. Snodgrass PJ, DeLong GR. Urea-cycle enzyme deficiencies and an increased nitrogen load producing hyperammonemia in Reye's syndrome. *N Engl J Med*. 1976;294(16):855-860.

32. Pagana KDPT. *Mosby's Manual of Diagnostic and Laboratory Tests*. 6th ed. Louis, MO: Elsevier Mosby; 2017.

33. De Las Heras J, Aldámiz-Echevarría L, Martínez-Chantar ML, Delgado TC. An update on the use of benzoate, phenylacetate and phenylbutyrate ammonia scavengers for interrogating and modifying liver nitrogen metabolism and its implications in urea cycle disorders and liver disease. *Expert Opin Drug Metab Toxicol*. 2017;13(4):439-448.

34. Marini JC, Lanpher BC, Scaglia F, et al. Phenylbutyrate improves nitrogen disposal via an alternative pathway without eliciting an increase in protein breakdown and catabolism in control and ornithine transcarbamylase-deficient patients. *Am J Clin Nutr*. 2011;93(6):1248-1254.

35. Nagamani SCS, Agarwal U, Tam A, et al. A randomized trial to study the comparative efficacy of phenylbutyrate and benzoate on nitrogen excretion and ureagenesis in healthy volunteers. *Genet Med*. 2018;20(7):708-716.

36. James MO, Smith RL, Williams RT, Reidenberg M. The conjugation of phenylacetic acid in man, sub-human primates and some non-primate species. *Proc R Soc Lond B Biol Sci*. 1972;182 (1066):25-35.

37. Jones AR. Some observations on the urinary excretion of glycine conjugates by laboratory animals. *Xenobiotica*. 1982;12(6):387-395.

38. Laemmle A, Gallagher RC, Keogh A, et al. Frequency and pathophysiology of acute liver failure in ornithine Transcarbamylase deficiency (OTCD). *PLoS One*. 2016;11(4):e0153358.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.