Hepatocyte transplantation in bile salt export pump-deficient mice: selective growth advantage of donor hepatocytes under bile acid stress

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Abstract

The bile salt export pump (Bsep) mediates the hepatic excretion of bile acids, and its deficiency causes progressive familial intrahepatic cholestasis. The current study aimed to induce bile acid stress in \( \text{Bsep}^{-/-} \) mice and to test the efficacy of hepatocyte transplantation in this disease model. We fed \( \text{Bsep}^{-/-} \) and wild-type mice cholic acid (CA) or ursodeoxycholic acid (UDCA). Both CA and UDCA caused cholestasis and apoptosis in the \( \text{Bsep}^{-/-} \) mouse liver. Wild-type mice had minimal liver injury and apoptosis when fed CA or UDCA, yet had increased proliferative activity. On the basis of the differential cytotoxicity of bile acids on the livers of wild-type and \( \text{Bsep}^{-/-} \) mice, we transplanted wild-type hepatocytes into the liver of \( \text{Bsep}^{-/-} \) mice fed CA or CA + UDCA. After 1–6 weeks, the donor cell repopulation and canalicular Bsep distribution were documented. An improved repopulation efficiency in the CA + UDCA-supplemented group was found at 2 weeks (4.76 ± 5.93% vs. 1.32 ± 1.48%, \( P = 0.0026 \)) and at 4–6 weeks (12.09 ± 14.67% vs. 1.55 ± 1.28%, \( P < 0.001 \)) compared with the CA-supplemented group. Normal-appearing hepatocytes with prominent nuclear staining for FXR were noted in the repopulated donor nodules. After hepatocyte transplantation, biliary total bile acids increased from 24% to 82% of the wild-type levels, among which trihydroxylated bile acids increased from 41% to 79% in the \( \text{Bsep}^{-/-} \) mice. We conclude that bile acid stress triggers differential injury responses in the \( \text{Bsep}^{-/-} \) and wild-type hepatocytes. This strategy changed the balance of the donor–recipient growth capacities and was critical for successful donor repopulation.

Keywords: cholestasis • cell therapy • spgp (sister of p-glycoprotein) • ATP-binding cassette transporters • bile acids • hepatocyte transplantation

Introduction

Progressive familial intrahepatic cholestasis (PFIC) is a clinical syndrome that has the features of chronic intrahepatic cholestasis and progresses to biliary cirrhosis and hepatic failure by the first or second decade of life [1–4]. Bile salt export pump (BSEP) deficiency, also known as PFIC-2, is caused by genetic defects in the \( \text{BSEP} (ABCB11) \) gene, which encodes a canalicular ATP-binding cassette transporter [4, 5]. This protein is crucial for the bile acid–dependent bile flow and for maintaining normal liver function. Current therapeutic options for PFIC-2 are limited. Most patients eventually require liver transplantation [6, 7]. Due to the limited number of donor livers and the high risk associated with transplantation at a young age, children with PFIC-2 usually have a poor prognosis. Liver-based cell therapy is a promising alternative therapy for inherited liver diseases [8, 9]. Two previously reported PFIC-2 patients receiving hepatocyte transplantation did not show successful donor cell repopulation, possibly due to the advanced stage of the disease [10].

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The Bsep (also known as sppg) knockout mice have the phenotype of non-progressive chronic intrahepatic cholestasis [5]. Transplantation of adult wild-type hepatocytes into Bsep−/− mice results in very low levels (<0.1%) of donor cell repopulation (unpublished data). We have reported bone marrow cell transplantation in this model with low levels of donor cell repopulation [11]. These sub-optimal results were likely due to an insufficient liver injury, or high liver proliferative activity in the recipient Bsep−/− mice. In the Bsep−/− mice, severe cholestasis can be induced by dietary cholic acid (CA) supplementation [12]. These mice display marked jaundice, elevated levels of bile acids and aminotransferase in plasma, and high mortality. Their phenotype is more similar to PFIC-2 than is the phenotype of Bsep−/− mice fed a normal diet. Thus, we believe that CA-challenged Bsep−/− mice may represent a more suitable model of human cholestasis for testing therapeutic hepatocyte transplantation.

Ursodeoxycholic acid (UDCA) is hepatoprotective and is widely used in treating liver diseases, including cholestasis and hepatitis. The UDCA has choleretic and cytoprotective effects by enhancing membrane stability and inhibiting apoptosis [6, 13]. However, because the Bsep−/− mice secrete very little UDCA [5, 14], the administration of UDCA could result in high bile acid levels in the hepatocytes and may potentially be harmful to these mice, as well as to PFIC-2 patients. We suggest that UDCA supplementation will add to the selective growth advantage of wild-type donor hepatocytes in the Bsep−/− mice treated with hepatocyte transplantation.

The aims of this study were to test whether bile acid stress would facilitate donor cell repopulation in the model of hepatocyte transplantation in Bsep−/− mice and to further test the effects of UDCA and CA in Bsep−/− mice in terms of cytotoxicity and liver regeneration. The results can help to clarify whether cell therapy could become a therapeutic option for BSEP-deficient patients, and they demonstrate the potential application of bile acids in liver-directed cell therapy.

Materials and methods

Animals

Mice with targeted inactivation of the Bsep (sppg) gene on a FVB/NJ background were generated as previously reported [5]. Animals were maintained in a 12-hr light and dark cycle at 25°C with free access to food and water in a specific pathogen-free environment in the animal facility of the National Taiwan University, College of Medicine. Experiments were performed according to the approved protocols from the Committee on Animal Care, National Taiwan University, College of Medicine. Wild-type FVB/NJ mice were used to provide donor hepatocytes.

Chemicals and antibodies

Ursodeoxycholic acid, CA, and bromodeoxyuridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies used were polyclonal Bsep (Sppg) antibody IW [4], monoclonal Ki-67 antibody (M7249; DakoCytomation, Glostrup, Denmark), monoclonal BrdU antibody (M0744) and polyclonal FXR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The fluorescein-conjugated secondary antibodies used were Alexa Fluor 594 goat anti-rabbit IgG (H + L) and Alexa Fluor 488 goat anti-rat IgG (H + L) (Molecular Probes, Grand Island, NY, USA). The secondary antibodies and reagents for immunohistochemical staining were biotinylated goat anti-rabbit IgG (H + L), biotinylated horse anti-mouse IgG (H + L) (BA-1000 and BA-2001; Vector Laboratories, Burlingame, CA, USA) and four Vectastain ABC kits (alkaline phosphatase, AK-5000; horseradish peroxidase, PK-6100; Vector Red; SK-5100; and DAB, SK-4100).

Liver injury induced by bile acids in the Bsep−/− mice

To test our hypothesis that UDCA would cause cell toxicity in Bsep−/− mice as opposed to cytoprotection in wild-type mice, we first examined the effects of UDCA and CA feeding on the Bsep−/− mice. Six- to 12-week-old Bsep−/− mice or wild-type mice were fed standard chow containing 0.2% (w/w) UDCA, 0.2% CA or 0.2% CA plus 0.2% UDCA. A 0.2% dose of UDCA or CA was chosen based on preliminary data of dose-response experiments; the dosage was appropriate to create a significant but non-fatal liver injury to the Bsep−/− mice that mimicking human PFIC phenotype. These were compared with mice fed a control diet. Five or more mice were tested in each group. Mice were killed after 5 days, and serum and liver specimens were collected.

Analysis of apoptosis and proliferation

Paraffin-embedded liver tissue sections were dewaxed and rehydrated. After antigen retrieval and proteolytic digestion by citrate buffer and proteinase K, each section was incubated with 50 units of TdT (M0315; New England Biolabs, Ipswich, MA, USA) and 20 nmol bromodeoxyuridine at 37°C for 90 min. After the TUNEL reaction, the labelled cells were probed with the mouse monoclonal antibody against bromodeoxyuridine followed by the biotinylated horse anti-mouse IgG and then detected using the Vectastain ABC alkaline phosphatase kit and its chromogen substrate Vector Red. The tissue sections were counter-stained using haematoxylin. Twenty portal areas in low-power fields were randomly selected and counted for positively labelled hepatocytes. The apoptotic index is shown as the number of TUNEL-positive hepatocytes/cm² of liver sections.

The proliferation assay was performed by immunohistochemical staining for Ki-67. The number of Ki-67-positive hepatocytes was calculated, and the area of each liver section was quantified by a similar approach as that used in the determination of the apoptotic index. The proliferation index is shown as positive Ki-67 hepatocytes/cm² of liver sections.

Hepatocyte transplantation

Donor hepatocytes were isolated from adult wild-type FVB/NJ mouse using a two-step collagenase method [15]. A 100-µl suspension containing 2 × 10⁶ freshly isolated hepatocytes was injected intrasplenically into Bsep−/− mice [16]. The Bsep−/− mice were treated with 2-acetylaminofluorene (AAF) at a dose of 16 mg/kg daily for 7 days before hepatocyte transplantation to inhibit recipient hepatocyte regeneration. CA (0.2%) was administered for 4 days until 24 hrs before hepatocyte transplantation. Monocrotaline (50–100 mg/kg) was injected intraperito-
neally 1 day before hepatocyte transplantation to enhance engraftment [17]. After cell transplantation, the mice were divided into two groups according to the bile acid regimen. Group 1 mice were fed the 0.2% CA–supplemented diet starting on the day of hepatocyte transplantation (day 0) until the end-point. Group 2 was fed the 0.2% CA–supplemented diet starting on day 0, plus 0.2% UDCA dietary supplementation starting on day 7 and continuing until the end-point (Fig. 1). Mice were killed at 1, 2 or 4–6 weeks after hepatocyte transplantation. Three or more mice were analysed in each set of experiments.

Analysis of donor hepatocytes repopulation

Genomic DNA was extracted from 25 to 30 mg of frozen liver samples from transplanted mice using the DNase Control Blood & Tissue Kit (QIAGEN Inc., Valencia, CA, USA). A total of 100 ng of DNA was used for each polymerase chain reaction (PCR) reaction. The PCR for the Bsep gene and its deleted region in the Bsep–/– mice before and after transplantation was performed using the following primers: forward 5′-GGAGGCATAGATACAGGACACCA-3′; reverse-mt 5′-GCCACCCACCACTGGTTTTCCC-3′; reverse-wt 5′-GSCAGCTATTGTCCATGACCAGTGC-3′ (annealing temperature 65°C, 40 cycles). The forward and reverse-wt primers were derived from the sequencing results of the original targeting vector when the knockout mouse was generated. The reverse-mt primer originated from the neo cassette used to construct the targeting vector. The primers were also used for genotyping in the knockout mouse colony.

Immunofluorescent staining of transplanted mouse livers was performed using cryostat liver sections (5–7 μm). Briefly, the slides were fixed with pre-cooled acetone for 5 min. After serum blocking, samples were incubated with primary antibodies for 2 hrs at room temperature and then with fluorescent secondary antibodies at room temperature for 1 hr. The slides were mounted and viewed using a fluorescent microscope (Leica DM1000, CoolSNAP, Leica Microsystems GmbH, Wetzlar, Germany).

The repopulation efficiency was calculated as the percentage area of Bsep-positive areas (donor cells) in the recipient liver sections using the image processing software Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA). At least 10 sections from four lobes of each mouse were analysed.

Liver histology and expression of FXR in the transplanted liver

The liver sections were paraffin-embedded and stained with haematoxylin and eosin to analyse liver pathology. The expression of Bsep and FXR were determined by immunohistochemical staining on serial liver sections. The primary and secondary antibodies and reagents used were as described above.

Bile acid analysis by electrospray ionization/ mass spectrometry (ESI/MS)

Bile samples from the gallbladder were obtained from the mice that had been fasted for 4 hrs. The ESI/MS analysis was performed as previously described [11]. Tandem-MS was performed using the Applied Biosystems API 3000 Tandem Mass Spectrometry coupled with the Perkin Elmer HPLC Series 200 System. A negative MRM scan mode was used.

**Fig. 1** The hepatocyte transplantation (HT) protocol: The Bsep–/– mice were pre-treated with AAF (day -7 to day 0) and monocrotaline (days -1). The Group 1 mice were fed with CA (0.2%) before and after transplantation (since day -4). For the Group 2 mice, in addition to the above treatment, UDCA (0.2%) was added into the mouse diet 7 days after transplantation (dotted arrow).

**The effect of bile acids on the liver regeneration in the Bsep–/– vs. wild-type mice**

To further analyse whether bile acids have differential effects on hepatocyte proliferation in the Bsep–/– and wild-type mice, we used the partial hepatectomy (PH) model in the untransplanted Bsep–/– and wild-type mice. We chose this model because it could show the changes in the hepatocyte growth rate in a short time and reflect the response of liver regeneration to bile acid stress. Mice were fed UDCA, CA, CA + UDCA or control diet for 5 days, followed by one-third PH. Mice were killed 24 or 72 hrs after PH. The liver weight/body weight (LW/BW) ratio and the liver re-growth rate (final LW/BW compared to baseline LW/BW) were analysed.

**Statistics**

Statistical comparisons between an experimental group and the control group were analysed using the Student’s two-sample t-test. Two-sided P-values < 0.05 were considered statistically significant, and P-values between 0.05 and 0.1 were considered borderline significant, indicating a difference trend.

**Results**

**Cholestatic injury induced by CA and UDCA in the Bsep–/– mice**

The Bsep–/– mice on the control diet suffered from mild chronic cholestatic liver injury. When fed CA for 5 days, aggravated cholestasis and marked cellular injury were observed in the Bsep–/– mice (Fig. 2A and B). In contrast, the bsep–/– mice fed UDCA showed a better-preserved lobular structure than mice fed CA. Infrequent focal hepatocyte necrosis, mild centrilobular disarray and portal inflammation were noted (Fig. 2C and D), which was not observed in the bsep–/– mice fed control diet. When Bsep–/– mice were fed a combination of CA and UDCA (0.2% each), a moderate extent of hepatocellular damage with focal cell ballooning was noted (Fig. 2E and F), but with a reduced area of confluent necrosis, compared with...
Fig. 2 After CA (0.2%) or UDCA (0.2%) feeding, the histopathology of the Bsep−/− livers showed confluent cell necrosis in mice fed CA (A and B), with less liver injury in mice fed UDCA (C and D). When Bsep−/− mice were fed the combination of CA + UDCA (0.2% each), a moderate extent of hepatocellular damage with focal cell ballooning was noted (E and F). (Scale bar: 100 μm in A, C, and E; 50 μm in B, D, and F) Serum bilirubin was elevated in the Bsep−/− mice fed CA or UDCA in comparison with mice fed the control diet (P = 0.015 or P = 0.010, respectively; G). ALT was extremely elevated in Bsep−/− mice fed CA (P = 0.008), but not in Bsep−/− mice fed UDCA (P = 0.480; H), in comparison with the control diet. CA + UDCA feeding resulted in a marked elevation of bilirubin (P = 0.013), but a lesser extent of ALT elevation (P = 0.114).
the CA-fed mice. Elevated serum bilirubin was noted in all groups fed a CA- UDCA- or CA + UDCA-supplemented diet. However, ALT was only elevated significantly in the CA-fed mice (Fig. 2G and H). When a higher dose (0.3%) of CA was administered for more than 5 days to the Bsep−/− mice, high mortality rates >70% were noted.

The Bsep−/− mice on a control diet had higher baseline levels of apoptosis, and when the mice were fed either CA or UDCA, the mean apoptotic index increased. The combination of CA + UDCA induced the highest apoptotic index (Fig. 3A-I). The above data suggest an acute and severe cholestatic injury and hepatocellular death in Bsep−/− mice fed a CA-supplemented diet, but a moderate extent of continuous cholestatic injury in mice fed the CA + UDCA diet. The highest apoptotic index indicated a synergistic effect of CA + UDCA on cell turnover. In contrast, the same concentration of UDCA, CA or UDCA + CA had no apparent effects on liver function or apoptosis in wild-type mice.

The proliferation index was increased in the wild-type mice when fed CA, UDCA or CA + UDCA. In the Bsep−/− mice, the proliferation index was not apparently elevated when fed only CA or UDCA compared to control diet. However, an elevated, highly variable increase of the proliferation index was observed in the CA + UDCA group (Fig. 3J).

**Donor hepatocyte repopulation after hepatocyte transplantation in the Bsep−/− mice**

At 7 days after hepatocyte transplantation, wild-type Bsep DNA from the donor cells was detected in the liver of Bsep−/− mice by PCR (Fig. 4A). The immunofluorescent staining confirmed the incorporation of donor hepatocytes into the liver lobules, with Bsep localized in the canalicular membrane (Fig. 4B). Small clusters of three–eight donor cells around the portal vascular space were noted 1 week after hepatocyte transplantation. At 2–6 weeks after hepatocyte transplantation, gradual donor cell repopulation and nodular expansion were observed in the mice with proliferative responses. The UDCA + CA mice exhibited a higher efficiency of donor hepatocyte repopulation, with larger regeneration nodules than the CA mice (Fig. 4B). An improvement in repopulation efficiency in the UDCA + CA fed mice as compared with CA fed mice was found at 2 weeks (4.76 ± 5.93% vs. 1.32 ± 1.48%, P = 0.0026) and at 4–6 weeks (12.09 ± 14.67% vs. 1.55 ± 1.28%, P < 0.001) (Fig. 4C). In the histopathology, the donor nodules consisted of small hepatocytes with a normal appearance, in contrast with the background recipient hepatocytes, which had variable levels of cell ballooning, necrosis and apoptosis (Fig. 5A and B). The transplanted donor nodules had higher levels of FXR expression and nuclear localization than the recipient hepatocytes (Fig. 5C–F).

**Correction of bile acid profiles in the transplanted mice**

Previously, the abnormalities in the bile composition of the Bsep−/− mice, which consists of low concentrations of total bile acids, low levels of tri-hydroxylated muricholate and abnormally high levels of tetra-hydroxylated bile acids have been reported [4]. We sampled the gallbladder bile samples from seven transplanted Bsep−/− mice 7–28 days after hepatocyte transplantation. The bile in the Bsep−/− mice tended to be stagnant or formed bile plugs, thus a significant proportion of mice had no sufficient amount and quality of bile to be analysed. The results show that the total bile acid concentration increased from 24.3 ± 5.7% of the wild-type liver in the untransplanted Bsep−/− mice to 82.3 ± 32.0% in the transplanted ones (P = 0.003, Fig. 6A). Tri-hydroxylated bile acid levels increased from 40.8 ± 7.3% to 78.7 ± 11.8% (P < 0.001), but levels of tetra-hydroxylated bile acids, which are abnormally secreted in the knockout mice, significantly decreased (P < 0.001, Fig. 6B and C).

**The effects of bile acids stress on liver regeneration**

It has been reported that CA promotes hepatocyte proliferation, and UDCA can do the same only when a proliferative stimulus is present [18]. We therefore tested the effect of bile acids on liver regeneration by using the PH model in the wild-type and in the Bsep−/− mice. After PH, the wild-type mice fed CA, UDCA or CA + UDCA showed higher liver regeneration rates than the mice fed the control diet, with a ranking of CA > UDCA + CA > UDCA (Table 1, Fig. 7). The Bsep−/− mice fed UDCA displayed a trend of higher liver regeneration rates as compared with the Bsep−/− mice fed the control diet 24 hrs after PH (97.01 ± 22.43 vs. 74.42 ± 3.24, P = 0.087). The high mortality rate above 60% in the Bsep−/− mice receiving CA or CA + UDCA after PH rendered the data analysis insufficient. Taken together, the data show that the CA and UDCA supplementations appeared to enhance liver regeneration and proliferation in the wild-type mice and, to a lesser extent, in the Bsep−/− mice.

**Discussion**

The current study is the first to show a significant donor repopulation in this inherited cholestatic mice model with defective bile salt excretion. The repopulated donor hepatocytes expressed the Bsep gene at the canalicular membrane. Furthermore, the transplanted mice showed partially corrected bile acid profiles. Bile acid stress enhanced the donor cell repopulation by providing selective growth advantages for the transplanted wild-type hepatocytes in the livers of the Bsep−/− mice.

Hepatocyte transplantation has been used to correct disease phenotypes for genetic liver diseases in other animal models, such as mdr2−/− and fah−/− mice [8, 19–21]. However, the efficacy of hepatocyte transplantation varies widely among different models, probably due to the type of recipient liver injury and the balance between the growth potentials of the donor and recipient cells. Different diseases and conditioning regimens should be individually tested for the therapeutic effect of cell transplantation. The success of donor cell repopulation depends on several key factors, including sufficient liver damage in the recipients, stimuli for donor cell proliferation and
Fig. 3 TUNEL staining (red) in the liver of wild-type (A, C, E, G) and Bsep−/− (B, D, F, H) mice fed the control (A, B), CA (C, D), UDCA (E, F), or CA + UDCA (G, H) diet. Haematoxylin was used to counterstain the nucleus. (scale bar: 100 μm) The control or bile acid–supplemented diet resulted in minimal apoptosis in the wild-type mice liver. The Bsep−/− mice liver showed greater apoptosis at baseline, and a marked increase of apoptosis was observed in mice fed CA, UDCA, or CA + UDCA (D, F, H), with the highest mean apoptotic index observed in mice fed CA + UDCA (I). The proliferation index (J) was greater in wild-type mice fed CA, UDCA or CA + UDCA in comparison with the control diet (P = 0.020, P = 0.014, or P = 0.001, respectively). The proliferation index was significantly increased in the Bsep−/− mice fed CA + UDCA (P = 0.001), but not in mice fed CA alone or UDCA alone (P = 0.093 or P = 0.039, respectively), in comparison with Bsep−/− mice fed the control diet.
growth competition between donor and recipient cells [19, 21, 22]. The model used in the current study showed that Bsep<sup>−/−</sup> mice displayed mild liver damage on a normal diet and had a high baseline proliferation rate, which was presumably caused by high intrahepatic bile acid levels that stimulated hepatocyte proliferation. Therefore, Bsep<sup>−/−</sup> mice are considered a difficult model for cell transplantation because donor cells have problems gaining a significant growth advantage over recipient hepatocytes.

CA induces pro-oxidant liver damage and facilitates donor-cell engraftment in the animal models for Wilson disease and PFIC-3 [8, 9].
The dietary supplementation of CA to Bsep−/− mice causes apparent cholestatic and apoptotic injury to the liver [12]. However, a higher dose of CA was found to cause a high mortality in the Bsep−/− mice before significant donor hepatocyte repopulation, which usually takes several weeks. This mortality appeared to be caused by the direct cytotoxicity of supplementary CA due to the lack of Bsep. The UDCA is also transported by Bsep, but it caused relatively mild liver injury in the Bsep−/− mice. Under these less toxic conditions, the Bsep−/− mice survived longer, which allowed enough time for the donor cells to repopulate and for other functional corrections to occur. Interestingly, we found that when a combination of CA + UDCA was administered, the mice manifested less acute liver damage, as shown by the modest elevation of ALT, but maintained a high apoptotic index. This may have been caused by the membrane stability effect of UDCA [13]. The highest apoptotic index indicated a synergistic effect of CA + UDCA on cell turnover, also creating a space or microenvironment for replacement of recipient cells by donor cells. This phenotype of chronic cholestatic injury is similar to the clinical condition of BSEP deficiency (PFIC-2) in humans. On the other hand, UDCA feeding did not damage the wild-type donor hepatocytes, and it has been shown in many studies to be cytoprotective to the liver by enhancing bile flow, maintaining membrane stability, inhibiting apoptosis and inhibiting inflammation [13, 24, 25]. We showed that either UDCA or CA alone or the combination of CA + UDCA did not damage wild-type hepatocytes but did increase their proliferation activity. Therefore, the donor hepatocytes could have a growth advantage when transplanted into the Bsep−/− liver of mice fed a combination of CA + UDCA.

Bile acids not only are essential molecules for the digestion of fat and fat-soluble vitamins but also influence hepatocyte growth and death [24, 26]. However, the detailed mechanisms by which bile acids affect hepatocyte proliferation are still under investigation. The CA potently enhances hepatocyte growth, whereas UDCA only promotes hepatocyte proliferation in the presence of proliferation stimuli [18]. UDCA induces a sustained increase of proliferative miRNAs starting shortly after partial hepatectomy [27]. Our data show that the liver regeneration in the wild-type mice was enhanced substantially by CA feeding and mildly by UDCA treatment. This enhancement was not significant in the Bsep−/− mice, partly because the cytotoxic effects of UDCA on the Bsep−/− mice hepatocytes counteracted its effects on
proliferation. Interestingly, FXR was expressed more highly in the wild-type donor cells, with prominent nuclear localization, in our study. FXR, which is the ‘bile acid sensor’, is a key nuclear receptor that regulates bile acid homeostasis in the liver. FXR plays important roles in liver regeneration, and its activation by bile acids drives homotrophic liver growth [26]. The roles of FXR-mediated signalling in cell therapy and liver repopulation deserve further investigation. Taken together, the above data show that the supplementation of UDCA and CA increased the cell toxicity in the Bsep/C0/C0/C0 hepatocytes and promoted the proliferation of the wild-type hepatocytes. The effect of bile acids on hepatocyte injury/apoptosis should counteract their effects on promoting proliferation in either the donor or recipient hepatocytes, creating a growth advantage in one cell type. The balance of the net effect of hepatocyte death/proliferation between donor and recipient cells determines the success or failure of donor repopulation.

Another notable advantage of using UDCA in cell transplantation is its applicability to clinical settings. The UDCA can be orally administered and used in newborns or young infants. It can also be used for long-term treatment and causes low toxicity. PH or medications such as chemical drugs and growth factors have been commonly used in animal models to provide growth stimuli, but their value in clinical settings is limited due to safety concerns [28]. Our study provides a simple and safe model that may be valuable for further exploration of cellular therapies for human PFIC patients. However, although UDCA may facilitate donor cell repopulation in the context of cell transplantation, a concern remains for the treatment of PFIC patients. Due to possible cholestatic injury, cautions should be taken when using UDCA in patients lacking BSEP activity (PFIC-2).

The future prospects of hepatocyte transplantation in PFIC-2 patients could include early treatment before liver fibrosis develops or a bridging treatment to orthotopic liver transplantation. A relatively small number of the donor cells in the recipient liver may be sufficient to alleviate symptoms in genetic or metabolic liver diseases, especially in young infants and children, as reported in Crigler-Najjar syndrome type I, argininosuccinate lyase deficiency and ornithine transcarbamylase deficiency [21, 29, 30]. One concern is that BSEP-

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**Table 1** Liver regeneration rate after partial hepatectomy (PH) in the wild-type and Bsep−/− mice in response to the control, CA, UDCA or CA + UDCA supplemented diet

|                  | Wild-type | Bsep−/−                  |
|------------------|-----------|--------------------------|
|                  | Control diet | +CA | +UDCA | +CA and UDCA | Control diet | +UDCA |
| Baseline LW/BW ratio* | 5.02 ± 0.40 |   |   |   | 11.16 ± 0.71 | p1 |
| PH response |           |   |   |   |   |   |
| LW/BW ratio | 3.92 ± 0.14 | 4.72 ± 0.49 | 4.34 ± 0.35 | 4.58 ± 0.19 | 8.31 ± 0.36 | 10.83 ± 2.50 |
| Liver regeneration rate (%)** | 76.13 ± 2.73 | 98.07 ± 14.67 | 86.51 ± 7.02 | 89.79 ± 3.67 | 74.42 ± 3.24 | 97.01 ± 22.43 | p5 |

*P1 < 0.01, comparison of the liver weight/body weight (LW/BW) ratio between wild-type and Bsep−/− mice

**Liver regeneration rate: the percentage of LW/BW ratio 24 hrs after 1/3 partial hepatectomy to the baseline LW/BW ratio (as 100%); P2 < 0.01, P3 < 0.001, P4 < 0.001, P5 = 0.087: comparisons between mice fed CA-, UDCA-, or CA and UDCA-supplemented diet with control diet, respectively.

Data on the Bsep−/− mice fed CA or CA and UDCA were not available due to high rate of mortality in these two groups.
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In summary, we have shown the successful repopulation of donor hepatocytes and phenotype correction in the Bsep−/− mice receiving hepatocyte transplantation under bile acid stress. In addition, UDCA, which is used as a choleretic and cytoprotective drug, caused cholestatic injury in the Bsep−/− mice. This finding raises concern about the potential adverse effects in clinical applications of UDCA in PFIC-2 patients. Bile acid supplementation in our model changed the balance of recipient–donor growth capacities and provided a pro-donor growth environment. The bile acid toxicity and growth modulation in hepatocytes can be of great value in liver-based cell therapy.

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Author contributions

Huey-Ling Chen, Hui-Ling Chen and Mei-Hwei Chang designed the research study; Huey-Ling Chen, Ray-Hwang Yuan, Shang-Hsin Wu, Ya-Hui Chen, Chin-Sung Chien and Shi-Ping Chou conducted the study, Huey-Ling Chen, Hui-Ling Chen and Shang-Hsin Wu analysed the data; Renxue Wang and Victor Ling contributed the knockout mice, primers and antibody for the study, and critical review of the manuscript. Huey-Ling Chen wrote the article.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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