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A small molecule Hedgehog agonist HhAg1.5 mediated reprogramming breaks the quiescence of non-injured liver stem cells for rescuing liver failure

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Abstract

Liver is the second most transplanted organ according to united network for organ sharing. Due to shortage of compatible donors, surgical difficulties, immunological hindrance and high post-operative cost, stem cell therapy is an attractive substitute of liver transplant for millions of patients suffering from hepatic failure. Due to several technical limitations such as viral integration, inefficient differentiation and adult phenotypes and epigenetic memory of fibroblasts, iPSCs, MSCs or i-Heps may not present a great clinical substitute for liver transplant. We pioneered a novel technology for robust expansion of quiescent liver stem cells (LSCs) from mice via utilizing of hedgehog agonist HhAg1.5 for 3 weeks. These expanded LSCs retained stem like properties after multiple passaging and differentiated to hepatocytes and cholangiocytes. Grafting of ex vivo expanded LSCs in FRG KO mice, significantly increased life span compared to control group ($p < 0.001$). Thus in this study, we provide a promising viable substitute for primary hepatocytes for regenerative medicine and for life-threatening metabolic liver diseases.

Introduction

Transplantation of livers has been ranked as the second after kidney in clinics as per United network for organ sharing. Liver transplant (LT) is recognized the only proven treatment for patients suffering from therapeutically refractory diseases including end-stage and inherited hepatic diseases. Approximately 17,000 people are waiting for LT in the USA as per the data provided by American liver foundation. Due to shortage of compatible donors, surgical difficulties, immunological hindrance and high post-operative cost, stem cell therapy has been emerged as an effective alternative approach to LT. The adult liver harbors a rich reservoir of stem cells but is unable to expand without any significant injury.

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Conflict of Interest

All authors have no conflict of interest and agreed with journal's policy of conflict of interest.

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To overcome the issue associated with self-renewal, researchers focused on non-hepatic stem cells or genomic modification of fibroblast to derive induced hepatocytes (i-Heps) [1–3]. Together these studies established the generation of highly functional i-Heps or trans-differentiation to hepatocytes in large quantities to pursue drug development, regenerative medicine and clinical studies. With the promising results in preclinical models, there are more than 200 clinical trials ongoing to use non-hepatic stem cells to treat liver failure patients (as per data from [ClinicalTrials.gov](https://clinicaltrials.gov)). The biggest difficulty is to generate reliable and reproducible differentiation of non-hepatic stem cells into expandable, functional and engraftable hepatocytes. Also, the phenotypic stability of non-hepatic stem cells for a prolonged period could be questionable during chronic liver injury.

Method

Mice

Fourteen- to Sixteen-week-old wild-type (WT) mice were provided by Dr. Lopa Mishra's laboratory. Also we purchased GFP transgenic C57bl6 from the Jackson Laboratory. *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice (FRG Knockout) mice were purchased from Yecuris Corporation. FRG KO mice were maintained in 2-(2-nitro-4-trifluoro-methyl-benzoyl)-1,3 cyclohexanedione (NTBC)-containing drinking water at a concentration of 16 mg/l. They were maintained in a specific-pathogen-free facility in accordance with guidelines approved by the Institutional Animal Care and Use Committee of M.D. Anderson Cancer Center.

Chemical reagents and antibodies

Collagenase type I, DNase I, and Pronase were purchased from Sigma Aldrich. The anti- α CD133 antibody was purchased from Miltenyi Biotec. HhAg1.5 was purchased from Cellagen Technology. Cell culture grade dexamethasone and nicotinamide were purchased from Sigma-Aldrich. Recombinant HGF (rHGF), rIL6, rEGF and rFGF2 were purchased from Cell Signaling Technology. 100x Insulin-Tranferrin-Selenium-Ethanolamine (ITS-X) was purchase from GIBCO. The α -Cyclin D1, α -Cyclin D3, α -CDK4, α -CDK6, α -Nanog and α -GAPDH antibodies were purchased from Cell Signaling Technology. The α -Nanog and α -Sox2 antibodies were purchased from Proteintech and Millipore respectively for western blot purpose. The α -HNF3 and α -TAT antibodies were purchased from R&D systems and Bioworld respectively. The α -FAH was purchased from Sigma Aldrich. For cell sorting, α -CD45 and α -CD133 MACS beads were purchased from Miltenyi Biotec. The anti- α CD133 and its isotype was purchased from ebioscience. Cell proliferation was measured using CyQuant cell proliferation assay kit (Thermo Fisher Scientific). Urea assay kit was purchased from Abnova. Matrigel and Poly-D-Lysine/Laminin coated plates and coverslips were purchased from BD Biosciences.

Isolation and expansion of CD133⁺ liver stem cells (Hh3A)

Fourteen- to Sixteen-week-old mice were humanely killed and single cell suspension from liver was made as described previously [4, 5]. CD133⁺ CD45⁻ liver stem cells were enriched using MACS based enrichment technology as per the state procedure [4]. These stem cells were plated on Poly-D-Lysine/ Laminin coated plates with liver specific conditioned media. The liver specific conditioned media contained rHGF (hepatocyte growth factor) (50ng/ml),

rIL6 (interleukin 6) (25ng/ml), rEGF (epidermal growth factor) (20ng/ml), insulin-transferrin-selenium (1x), rFGF (fibroblast growth factor) (20 ng/ml), dexamethasone (1×10^{-7} mol/l) and nicotinamide (10 mmol/l) in Serum-free Dulbecco modified Eagle medium (DMEM)–F12 media with heat inactivated serum. After two weeks of culture, 5 μ M HhAg1.5 (the concentration was based on manufacture's protocol) was added in liver specific conditioned media thrice per week for 3 weeks. Upon expansion of CD133⁺ CD45[–] liver stem cells, these were maintained in liver specific conditioned media without HhAg1.5.

Intrasplenic inoculation of CD133⁺ liver stem cells (Hh3A)

5×10^6 Hh3A cells per 40 μ l PBS or only 40 μ l PBS were injected intrasplenically into FRG KO mice respectively. The grafted and non-grafted FRG KO mice were maintained in NTBC (16 mg/l) containing drinking water for 12 days after grafting. After 10 days mice were provided with normal water without NTBC. Random serial sections from left, middle, and right liver lobes were examined by IHC analysis to determine the donor cell repopulation.

Western blotting

Cell lysis and Western blotting with antibodies against Cyclin D1, Cyclin D3, CDK4, CDK6 and GAPDH were performed as described previously [6]. For all samples, the total protein concentrations were determined with a bicinchoninic protein assay kit (Pierce). Western blots were detected with an enhanced chemiluminescence kit (Cell Signaling Technologies).

Cell cycle analysis

Freshly purified CD133⁺ CD45[–] liver stem cells and expanded Hh3A cells were fixed with ice-cold 70% ethanol overnight. The next day, the samples were centrifuged and the fixative was removed. The cells were resuspended in a propidium iodide working solution (50 μ g/ml propidium iodide plus 20 μ g/ml RNase in phosphate-buffered saline) for 10 minutes prior to flow cytometry.

Sphere Assays

Matrigel was coated onto each well of a chamber slide and incubated for 2 hrs at 37 C to allow solidification. Ten thousand freshly sorted cells were mixed with 2% Matrigel and plated on top of Matrigel-coated chamber slides. Serum-free Dulbecco modified Eagle medium (DMEM)–F12 containing rFGF2 (20 ng/ml) and rEGF (20 ng/ml) was overlaid and then changed twice per week. Spheres were stained with stem cell marker Sox2 at a 1:50 dilution.

Differentiation Assays

Freshly sorted expanded Hh3A cells were maintained in (DMEM)–F12 containing rEGF (20 ng/ml), rFGF2 (20 ng/ml) and 1x Insulin-Transferrin-Selenium-Ethanolamine complete medium. To differentiate into hepatocyte like cells, Hh3A cells were maintained in hepatocyte specific medium as described previously [7, 8] for 3 weeks. Similarly, Hh3A cells were also reprogrammed to cholangiocytes as per the following protocol [7]. We

performed PAS staining to show the increase of glycogen content upon culturing in hepatocyte specific media.

Alanine aminotransferase (ALT) and Bilirubin

Serum ALT and Bilirubin was measured by the standard colorimetric method at 510 nm and 550nm respectively using a kit purchased from Bioo Scientific.

Statistical Analysis

Animals were randomly selected for control and treatment groups. All data were expressed as the mean $\pm$ the standard error of the mean. All statistical analyses were performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software). One-way analysis of variance with Tukey's post-hoc test was used to analyze the data. Statistical significance at $p < 0.05$ was considered significant. Chi-square test was performed to determine the significance of survival curve.

Results

HhAg1.5 breaks the quiescence of CD133⁺ CD45⁻ liver stem cells

There is a clear need to provide non-tumorigenic, engraftable and long term phenotypic stability of cellular therapy. This implies that liver resident stem cells (LSCs) could be a better alternative for uniformity, genetic quality and safer transplantability in clinical environment. Mitotically quiescent organ livers have a rich reservoir of stem cells that have a remarkable capacity to expand only upon acute or chronic injuries in vivo. This spontaneous in vivo expansion property is lost in patients having liver failure or end-stage liver diseases. To overcome this problem we developed an in vitro expansion strategy using a small molecular Hedgehog agonist HhAg1.5 (Fig. 1 a). Periodic culturing with HhAg1.5 for 3 weeks in liver specific conditioned media facilitates dramatic expansion of quiescent CD133⁺ CD45⁻ LSCs (Hh3A cells) enriched from non-injured liver in vitro. Under optimal in vitro culture conditions, Hh3A cells formed crypt shaped structure (Fig. 1 b). To investigate proliferative status of Hh3A cells, cell cycle progression were performed to analyze G1-S-G2 phase. Interestingly, a very high percentage (70%) of Hh3A cells were in S phase as compared to freshly sorted CD133⁺ CD45⁻ LSCs (5%) (Fig. 1 c). The choice of freshly sorted CD133⁺ CD45⁻ LSCs is due to its inability to grow in in vitro culture condition. Also proliferation assays and Ki67 staining of Hh3A cells further substantiated cell cycle analysis data (Supplementary Fig. 1). In accordance with this, we compared different cell cycle markers by western blotting on lysates from expanded LSCs (Hh3A cells) and hepatocytes. High level expressions of several cell-cycle associated proteins (cyclin D1, D3, CDK4 and 6) were detected in Hh3A cells in comparison to terminally differentiated hepatocytes (Fig. 1 d). We then assessed the stem-like properties of Hh3A by surface staining with CD133 antibody. A large number of CD133⁺ population is present in the expanded Hh3A cells (Fig. 1 e). To elucidate the stem like properties in Hh3A cells, sphere formation assays and staining spheres with stem cell-associated marker Nanog were studied. Consistent with flow cytometric data, Hh3A cells formed successful spheres after culturing for 10 days in matrigel based system (Fig. 1 f). These spheres were positively stained with stem cell-associated transcription factor Nanog. Also, western blot analysis of

transcription factors Nanog and Sox2 showed that Hh3A cells maintained stem cell status after in vitro expansion (Fig. 1 h). Taken together, these data indicated that Hh3A cells are highly proliferative and maintain stem cell-like properties.

Expanded Hh3A cells (CDD133⁺ CD45⁻) are capable to differentiated in hepatocytes and cholangiocytes

We next investigated the bipotency of Hh3A cells to differentiate into hepatocytes or cholangiocytes. Hh3A cells were cultured in a hepatocyte- or cholangiocyte-specific culture condition. In hepatocyte specific media, Hh3A cells expressed inducible levels of hepatocyte maturation markers HNF3 (hepatocyte nuclear factor 3) and TAT (tyrosine amino transferase) (Fig. 2 a). Also immunofluorescence analysis revealed that DHh3A cells showed higher accumulation of HNF3 in nucleus compared to expanded Hh3A cells (Fig. 2 b). To determine DHh3A cells showed hepatocyte like function, we quantitated urea production and demonstrated glycogen storage. Upon quantitation of urea in cell culture lysate we found that DHh3A cells produced more urea compared to undifferentiated Hh3A cells (Fig. 2 c). Supporting to this, accumulating more glycogen (PAS-positive staining) in DHh3A cells induces hepatocyte-like phenotype (Fig. 2 d). To correlate our in vitro with in vivo study, Hh3A cells transplanted liver from FRG KO mice was stained with FAH and hepatocyte marker Albumin. Grafted liver showed a strong co-localization of FAH and Albumin that can conclude that Hh3A cells are capable to differentiate into mature hepatocyte like cells. Apart from differentiating into hepatocyte like phenotype, Hh3A cells were able to form lumen or tubular like structures as cholangiocytic phenotypes (Fig. 2 f). These findings demonstrate that Hh3A cells are capable to differentiate into hepatocytes and cholangiocytes.

In vivo grafting of Hh3A cells (CDD133⁺ CD45⁻) restore normal liver functions of FRG KO mice

To show the regenerative capacity of Hh3A cells, we used FRG KO mice as our in vivo model system. FRG KO mice were defective in tyrosine metabolism and immunodeficient[9]. These mice need 2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione (NTBC) supply for prolonged survival[9]. Hh3A cells were injected intrasplenically into FRG KO mice to effectively repopulate the FAH-deficient liver and restored normal tyrosine metabolic function (Fig. 3 a). These mice were maintained with normal drinking water for 10 days after grafting. Our study showed that non-grafted FRG KO mice undergo liver failure and death ~ 8–12 days after removal of NTBC from drinking water (Fig. 3 b). Upon grafting of Hh3A cells in FRG KO mice, there was a dramatic increase of survivability of about 3 to ~4.5 weeks in the absence of NTBC. Evaluation of liver sections of FRG KO mice that had been drinking water with no NTBC revealed massive death of hepatocytes at about 8 to 10 days (Fig. 3 c). In contrast, Hh3A cells restored the normal liver architecture with minimal death of hepatocytes even after the mice drank NTBC-free water for 3 weeks.

To determine whether Hh3A cells are capable of restoring normal liver function, we quantitated serum levels of alanine aminotransferase (ALT) and bilirubin. Notably, transplanted Hh3A cells significantly reduced ALT ($p < 0.001$) and bilirubin ($p < 0.0001$)

levels in the serum of FRG KO mice that had been drinking NTBC-free water for 3 weeks (Fig. 3 d). In accordance with our liver function tests, we investigated the status of damaged hepatocytes and the activation of stress pathways by Western blot analysis of high mobility group box protein 1 (HMGB1)[10], respectively. Grafting of Hh3A cells in FRG KO mice dramatically attenuated the release of HMGB1 compared to ungrafted mice that had been drinking NTBC-free water (Fig. 3 e). Moreover, successful reconstitution of hepatic tissue was detected by identifying FAH-positive hepatocytes in FRG KO mice after transplantation of Hh3A cells (Fig. 3 f). These data revealed that Hh3A transplantation substantially improves liver function in FRG KO mice in absence of NTBC. Also, Hh3A cells seems to be morphologically and functionally simulating the phenotypes of mature hepatocytes and cholangiocytes and can be utilized to repair hepatic defects upon transplantation.

Discussion

In this study, we developed a novel protocol with use of small-molecule agonist HhAg1.5, which efficiently generated expandable LSCs in vitro. Phenotypic characterization of Hh3A cells showed expression of cell cycle markers such as CDK4, CDK6, cyclin D1, and cyclin D3. These Hh3A cells retained stem cell-like properties in Matrigel culture and expressed stem cell-associated transcription factor Nanog. Our in vivo grafting study of Hh3A cells in FRG KO mice showed significant alleviation of liver injury markers ALT and bilirubin compared with the effects on these markers in nongrafted FRG KO mice that drank NTBC-free water. Supporting these findings, transplanted Hh3A cells increased the survivability of FRG KO mice by about 3 to ~4.5 weeks in absence of life saver NTBC. Our success in generating expandable LSCs can be used for autologous cell therapy in hepatic failure and should enable correction of genetic defects linked with inherited liver dysfunction.

The advent of induced pluripotent stem cell (iPSC) technology, bone marrow mesenchymal stem cells (MSCs), and transdifferentiation of fibroblasts to i-Hep cells has brought next-generation treatment in the field of liver transplantation[11]. These personalized cellular therapies might overcome immune rejection and the requirement of large quantities of donor hepatocytes ($\sim 1-5 \times 10^9$ cells)[12]. Also, the shortcomings of rapid dedifferentiation and loss of hepatic functions in these transplanted hepatocytes would be circumvented[13]. Espejel et al.[9] showed that the grafting of iPSCs could generate a functional liver with proliferative hepatocytes in an FAH-deficient mouse model that survived for 10 months with NTBC-free drinking water. Indeed, the transplantation of iPSC-derived hepatocyte-like cells produced beneficial effects in numerous liver disorders such as α_1 -antitrypsin deficiency, hereditary tyrosinemia, and liver injury[14].

Similarly, MSCs are considered another promising autologous source for liver regeneration [15, 16]. Certain preclinical studies have shown that transplantation of MSCs could ameliorate liver fibrosis by multiple mechanisms that prevent the progressive distortion of hepatic tissue architecture[17].

Presently, 244 ongoing clinical trials are investigating the therapeutic potential of stem cell therapy for liver diseases ([ClinicalTrials.gov](https://clinicaltrials.gov)). However, there are no definitive answers about whether these clinical trials will be effective in terms of liver regeneration. There are

several technical limitations to be addressed such as viral integration, inefficient differentiation and adult phenotypes, heterogeneous population, risk of teratoma formation, and epigenetic memory of fibroblasts.[18, 19]

Our in vitro expansion platform of quiescent LSCs brings a new treatment option for patients with end-stage liver diseases. Due to the resident nature of stem cells, these cells can be highly differentiable to hepatocytes and cholangiocytes. Notably, these expandable LSCs can maintain genetically mature hepatocyte-like phenotypes without any time restriction. Thus in this study, we provide a promising viable substitute for primary hepatocytes for regenerative medicine and for life-threatening metabolic liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations in the order of appearance

FRG	((fumarylacetoacetate hydrolase) <i>Fah</i> ^{-/-} <i>Rag2</i> ^{-/-} <i>Il2rg</i> ^{-/-})
IPSCs	Induced Pluripotent Stem Cells
MSCs	Mesenchymal Stem Cells
LSCs	Liver Stem Cells
LT	Liver Transplant
HhAg1.5	Hedgehog Agonist 1.5

References:

1. Huang P, et al., Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature*, 2011 475(7356): p. 386–9. [PubMed: 21562492]
2. Yi F, Liu GH, and Izpisua Belmonte JC, Rejuvenating liver and pancreas through cell transdifferentiation. *Cell Res*, 2012 22(4): p. 616–9. [PubMed: 22373548]
3. Zhang Z and Wang FS, Stem cell therapies for liver failure and cirrhosis. *J Hepatol*, 2013 59(1): p. 183–5. [PubMed: 23353868]
4. Rountree CB, et al., Expansion of CD133-expressing liver cancer stem cells in liver-specific phosphatase and tensin homolog deleted on chromosome 10-deleted mice. *Stem Cells*, 2009 27(2): p. 290–9. [PubMed: 19008348]
5. Mitra A, et al., Cell-surface Vimentin: A mislocalized protein for isolating csVimentin(+) CD133(-) novel stem-like hepatocellular carcinoma cells expressing EMT markers. *Int J Cancer*, 2015 137(2): p. 491–6. [PubMed: 25487874]
6. Mitra A, et al., Signal transducer and activator of transcription 5b (Stat5b) serine 193 is a novel cytokine-induced phospho-regulatory site that is constitutively activated in primary hematopoietic malignancies. *J Biol Chem*, 2012 287(20): p. 16596–608. [PubMed: 22442148]

7. Yu B, et al., Reprogramming fibroblasts into bipotential hepatic stem cells by defined factors. *Cell Stem Cell*, 2013 13(3): p. 328–40. [PubMed: 23871605]
8. Lee KD, et al., In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology*, 2004 40(6): p. 1275–84. [PubMed: 15562440]
9. Espejel S, et al., Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. *J Clin Invest*, 2010 120(9): p. 3120–6. [PubMed: 20739754]
10. Huebener P, et al., The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest*, 2015 125(2): p. 539–50. [PubMed: 25562324]
11. Dianat N, et al., Human pluripotent stem cells for modelling human liver diseases and cell therapy. *Curr Gene Ther*, 2013 13(2): p. 120–32. [PubMed: 23444872]
12. Si-Tayeb K, et al., Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology*, 2010 51(1): p. 297–305. [PubMed: 19998274]
13. Strain AJ, Isolated hepatocytes: use in experimental and clinical hepatology. *Gut*, 1994 35(4): p. 433–6. [PubMed: 8174975]
14. Huebert RC and Rakela J, Cellular therapy for liver disease. *Mayo Clin Proc*, 2014 89(3): p. 414–24. [PubMed: 24582199]
15. van Poll D, et al., Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology*, 2008 47(5): p. 1634–43. [PubMed: 18395843]
16. Itaba N, et al., Human mesenchymal stem cell-engineered hepatic cell sheets accelerate liver regeneration in mice. *Sci Rep*, 2015 5: p. 16169. [PubMed: 26553591]
17. Jang YO, et al., Effect of bone marrow-derived mesenchymal stem cells on hepatic fibrosis in a thioacetamide-induced cirrhotic rat model. *BMC Gastroenterol*, 2014 14: p. 198. [PubMed: 25425284]
18. Sanal MG, Cell therapy from bench to bedside: Hepatocytes from fibroblasts - the truth and myth of transdifferentiation. *World J Gastroenterol*, 2015 21(21): p. 6427–33. [PubMed: 26074681]
19. Subba Rao M, Sasikala M, and Nageshwar Reddy D, Thinking outside the liver: induced pluripotent stem cells for hepatic applications. *World J Gastroenterol*, 2013 19(22): p. 3385–96. [PubMed: 23801830]

Figure 1. In vitro expansion and characterization of CD133⁺ CD45⁻ Hh3A cells from normal murine liver.

(a) Schematic of our strategy to expand quiescent CD133⁺CD45⁻ liver stem cells (LSCs). (b) Representative image of a crypt-shaped colony from expanded Hh3A. (c) Percentages of freshly sorted CD133⁺CD45⁻ LSCs and expanded Hh3A population in G1, S, and G2 phases, determined by flow cytometry. (d) Western blot analysis of cell cycle markers in hepatocytes and expanded Hh3A cells. (e) Expanded Hh3A cells were stained with α-CD133 antibody to determine surface expression (red line) and immunoglobulin G (IgG) isotype control used to determine antibody specificity (blue line). (f) Images of spheres formed in Matrigel by Hh3A cells on day 10. (g) Stem cell-associated transcription factor Nanog was detected with use of fluorescence microscopy in Matrigel culture. (h) Western Blot analysis of Nanog and Sox2 in hepatocytes and expanded Hh3A cells. Data are shown from two independent experiments.

Figure 2. Expanded Hh3A cells show differentiated phenotypes of hepatocytes and cholangiocyte.

Hh3A cells were cultured in normal conditions (nontreated) and in hepatocyte-specific medium. (a) Western blot analysis of hepatocyte maturation markers TAT and HNF3. (b) Hh3A and DHh3A (differentiated Hh3A) cells were fluorescently labeled using α-HNF3 (Alexa-647) and Sytox-green (pseudocolor blue) and visualized by confocal microscopy (LSM 510). (c) Concentration of Urea in cell lysate was shown.

(d) Glycogen storage content of Hh3A cells in nontreated and hepatocyte-specific media, determined by periodic acid-Schiff staining. (e) In vivo differentiation ability of Hh3A cells was shown by colocalization (α-Albumin (green); α-FAH (red) and DAPI (blue)) in liver of FRG KO mice. (f) Hh3A cells formed lumen- and tubular-shaped structures in cholangiocyte-specific culture conditions. Representative data were shown from two independent experiments.

Figure 3. Grafting of Hh3A cells in FRG KO mice significantly improves survivability and restores liver function.

(a) Schematic of Hh3A cell transplantation into the spleen of FRG KO mice. (b) Kaplan-Meier survival curve of FRG KO mice given drinking water containing NTBC ($n=6$ mice) (blue line), FRG KO mice given NTBC-free drinking water ($n=8$ mice) (red line), and FRG KO mice with transplanted Hh3A cells and given NTBC-free drinking water ($n=7$ mice) (green line). (c) Hematoxylin and eosin-stained sections of hepatic tissues (original magnification x400). (d) Serum levels of ALT and bilirubin from FRG KO mice given drinking water containing NTBC (day 30), FRG KO mice given NTBC-free drinking water (~day 8 to 10), and FRG KO mice with transplanted Hh3A cells and given NTBC-free drinking water (day 21). (e) Western blot analysis of HMGB1 a marker for damaged hepatocytes, Albumin and GAPDH as a loading control in hepatic tissue lysates. (f) Representative photomicrographs of FAH immunohistochemical staining in normal, No NTBC, and Hh3A grafted + No NTBC groups of FRG KO mice. Representative data were shown as mean $\pm$ SEM from two independent experiments. One-way Anova analyses or Chi-square test: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.