Am J Transl Res 2021;13(4):2710-2716
www.ajtr.org /ISSN:1943-8141/AJTR0120551

Original Article

Treatment of α-1 antitrypsin deficiency using hepatic-specified cells derived from human-induced pluripotent stem cells

Yingqiang Chen[1*], Ruoqing Li[3*], Lin Zhang[2], Linlin Gan[1], Jianqiang Ding[1]

[1]*Department of Infectious Diseases,* [2]*The Center Laboratory, The Third Affiliated Hospital of Guizhou Medical University, Duyun, Guizhou Province, China;* [3]*Department of General Medicine, Chongqing University Central Hospital, Chongqing Emergency Medical Center, Chongqing, China.* [*]*Equal contributors and co-first authors.*

Received August 17, 2020; Accepted November 24, 2020; Epub April 15, 2021; Published April 30, 2021

Abstract: Objective: α-1 antitrypsin deficiency (AATD) is an inherited liver disease characterized by the "Z" mutations, which can cause pulmonary emphysema and liver fibrosis. Transplantation of the organ (i.e., the lung/liver) is the best treatment method, however, the scarcity of suitable donors limits its application. The cell transplantation technique poses an alternative way of combating liver failure. Methods: Hepatic specific differentiation of the human induced pluripotent stem cells (iPSCs) was initiated with 100 ng/mL activin A, followed by 20 ng/mL of BMP-4 and 10 ng/mL of FGF-2. The cells were transplanted into the livers of AATD transgenic mice using intra-splenic injections. FK506 was used as an immunosuppressor. At 1, 3, and 6 months post-transplantation, the human serum albumin (HSA) levels and its DNA contents, and the mice serum and liver tissues were measured using enzyme-linked immunosorbent assays (ELISA), polymerase chain reactions (PCR), and immunohistochemistry to estimate the repopulation of the hepatic-specified cells. Results: Post transplantation, the hepatic-specified cells were found to be successfully and progressively repopulated in the transgenic mice livers. Additionally, the hepatic-specified cells did not display any carcinogenicity, as confirmed by the absence of any tumors on the animals. Conclusion: We provide a time saving and low cost method of transplanting hepatic-specified cells into the livers of AATD mice without any risk of carcinogenicity, a method that may be a potential option for the treatment of AATD.

Keywords: α-1 antitrypsin deficiency, induced pluripotent stem cell, differentiation, cell transplantation

Introduction

The lack of suitable allografts is a long-standing problem for late-stage liver failure patients. Currently, α-1 antitrypsin deficiency (AATD) is one of the most life-threatening liver diseases [1-3]. Scientists are now trying to exploit cell transplantation to combat liver diseases. Human-induced pluripotent stem cell (hiPSC) derived hepatocyte-like cells (HLCs) are promising alternatives to primary hepatocytes. They have the advantages of renewable sources and no ethical requirements [4-6]. Recent studies have reported a number of hepatocyte transplantation experiments that have been successfully carried out in AATD transgenic mice models [7, 8]. Wild-type murine hepatocytes have been shown to competitively repopulate the livers of AATD transgenic mice as mild to moderate levels of the repopulation of hiPSC derived HLCs (hiPSC-HLCs) have been observed in the livers of AATD transgenic recipients after transplantation [9, 10]. In this study, hiPSC-HLCs were transplanted into the livers of AATD transgenic mice using intra-splenic injections to assess whether the hiPSC-HLCs have a repopulation capability and maturity in the AATD background.

Materials and methods

Generation of the AATD transgenic mice

The transgenic mice were generated following the standard nuclear micro-injection method

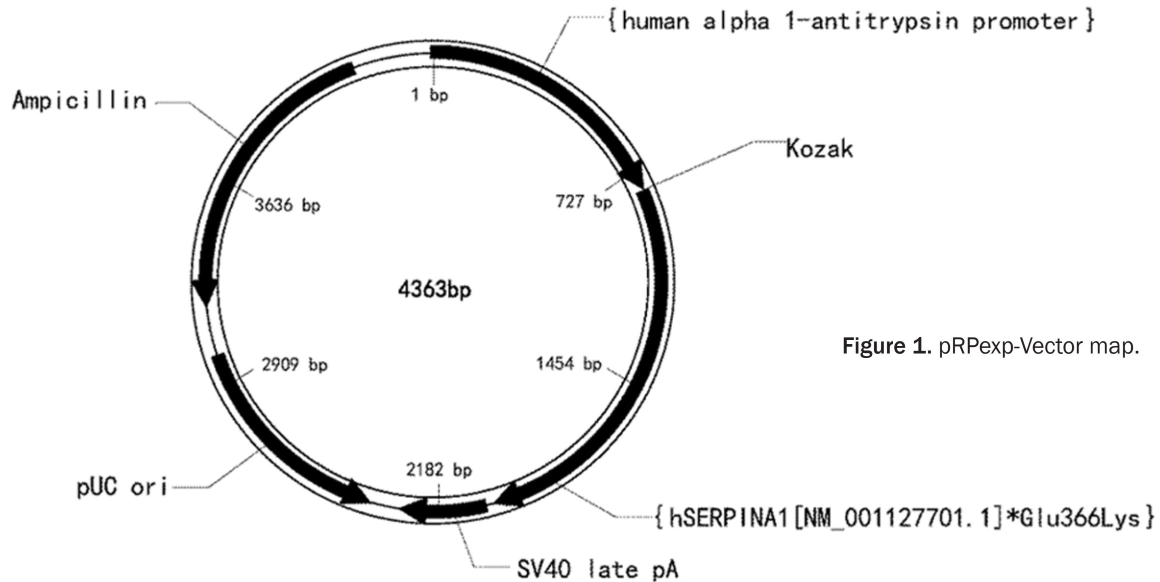

Figure 1. pRPexp-Vector map.

[11, 12]. Briefly, human α-1 antitrypsin containing "Z" mutations and a native gene promoter was cloned into the pRPexp-Vector (Cyagen Bioscience, Santa Clara, CA, USA) (**Figure 1**). This construct was then micro-injected into the mice. Both SERPINA1 cDNA with the "Z" mutation and native promoter were synthesized by General Biosystems (Morrisville, NC, USA). The successful construction of AATD transgenic mice was confirmed by Diastase/PAS (D/PAS) staining, PCR and sequencing. All the mice were maintained in the SPF barrier animal facilities located at the Third Military Medical University. All the animal experiments were performed with the approval of the Animal Care and Use Committees of Third Military Medical University.

Cell line and culture

Human iPS cell line (DYR0100) was purchased from ATCC and cultured in the mTeSRTM1 medium (Stemcell, Vancouver, Canada). The cells were grown in 6-welled plates coated with Matrigel (Corning, NY, USA) and incubated at 4% O_2, 5% CO_2, 37°C. No informed consent was applicable in this experiment.

Hepatic-specified cellular differentiation

The hepatic-differentiation of the hiPSC was initiated by treating the cells with 100 ng/mL activin A (R&D systems, Minneapolis, MN, USA) diluted in RPMI+B27 culture media (Invitrogen, Carlsbad, CA, USA), and maintained at 20% O_2 for 5 days. The cells were then cultured in RPMI+B27 media containing 20 ng/mL BMP-4 (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL FGF-2 (Invitrogen, Carlsbad, CA, USA) and continuously cultured under 4% O_2 for 5 days. The hepatic-specified cells were collected using Accutase (Gibco, Grand Island, NY, USA) for transplantation [13, 14].

Cell transplantation

Thirty-five, male AATD mice (6-8 weeks old) were selected for transplantation since the hepatocytes in the livers of the female transgenic mice were at comparatively lower levels. 1×10^6 human hepatic-specified cells were transplanted into the livers of the transgenic mice using intra-splenic injections. Normal saline was used as a control. One week prior to the transplantation, the mice were provided with drinking water containing FK506 (1 μg/g per day, Sigma-Aldrich, St. Louis, MO, USA).

Co-immunostaining for HSA and hAAT in the mouse liver sections

Frozen liver sections were fixed with 100% ethanol for 15 minutes and permeabilized using 0.05% Tween20 twice, 2 minutes each time. The blocking was performed using 3% bovine serum albumin for 30 minutes at room temperature. After they were washed with PBS, the sections were incubated with 100 ul primary

antibodies, mouse anti-human serum albumin (MAB1455, R&D) and rabbit anti-hAAT (H-203, sc-30121, Santa Cruz) at 1:00 dilution (blocking buffer with 0.5% Triton × 100) at 4°C overnight. The sections were then incubated with 100 ul fluorescent secondary antibody (Donkey anti-rabbit 555 and Donkey anti-mouse 488) at 1:500 (Alexa Fluor) in 1 × PBS for one hour at room temperature. The sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI). Finally, the coverslips were mounted with an anti-fade mounting medium, and the immunofluorescent signals were visualized and recorded using an Olympus DP72 microscope and the cellSens Standard 1.5 software.

Measuring the HSA in the mouse serum using competitive enzyme-linked immunosorbent assays (ELISA)

We collected mouse serum from six mice which had a representative progressive repopulation of donor cells for the test. We used HSA ELISA kits (E01S0014) purchased from Shanghai BlueGene Biotech, China to measure the HSA in the mouse serum [15, 16].

Measuring the HSA gene content in the mouse livers using reverse transcription polymerase chain reaction (RT-PCR)

The HSA gene content in the liver tissues was measured using One Step qDNA PCR kits as follows: 2x SYBR green PCR master Mix 12.5 ul, 90 uM P1 0.25 ul, 90 uM P2 0.25 ul, DNA Template 5 ul, dH_2O 7 ul. PCR reaction program: 95°C × 10 min, 95°C × 15 sec, 60°C × 1 min, for 40 cycles+dissociation on a 7500 real-time PCR machine (Applied Biosystems). We used mouse GAPDH as a control. The PCR product length for the HSA gene was 97 bp (GenBank accession number NC_000004). The PCR primer was designed using NCBI pick primers. The sequence of the forward primer: TAAGGAGACCTGCTTTGCCG; the sequence of the reverse primer: CCCTAAGCCCTAGCCTAACC. The QIAamp® DNA Mini Kits and the PCR kits were purchased from Qiagen (Hilden, Germany) and Promega (Madison, WI, USA), respectively.

Statistical analysis

The HSA levels in the individual mice were measured three times. The donor cell repopulation was estimated by measuring the HAS gene content and expressed as a percentage. The data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). The measurement data were expressed as the mean ± SD and the differences among groups were analyzed using one-way ANOVA and LSD post hoc tests. The count data were expressed as a number or percentage and the comparisons between groups adopted chi square tests. $P<0.05$ indicated that a difference was significant.

Results

AATD transgenic mice

The presence of both the Human SERPINA1 gene and the "Z" mutations were confirmed by PCR and sequencing. The RT-PCR showed positive results in the liver tissue only but not in the others. The D/PAS staining showed the red globules in the hepatocytes (**Figure 2**). These tests proved that we successfully generated transgenic mice.

Co-immunostaining for HSA and hAAT in the mouse liver sections

One month after the transplantation, the immunofluorescence staining of the liver sections from the transgenic mice showed 50-100 clusters of human hepatocytes. This constituted around 5-10% of the total hepatocyte mass. Interestingly, 3 and 6 months after the transplantation, cell proliferation increased progressively and reached 10-15% and 15-20% of the host livers, respectively. The donor cells (green for HSA) showed no red globules (red for hAAT) as they expressed and secreted hAAT at normal levels (**Figure 3**).

The HSA serum levels in the mice

The HSA serum content in the blood at 1, 3, and 6 months after the cell transplantation was measured, and the values were 18.11±1.47, 82.00±5.85, and 464.22±29.90 ng/mL, respectively (**Figure 4**). The HSA content was significantly higher at 3 months and 6 months after the cell transplantation than it was at 1 month after the cell transplantation ($P<0.05$).

The HSA DNA content in the mouse livers

The amount of the HSA gene content was used to estimate the extent of the liver repopulation

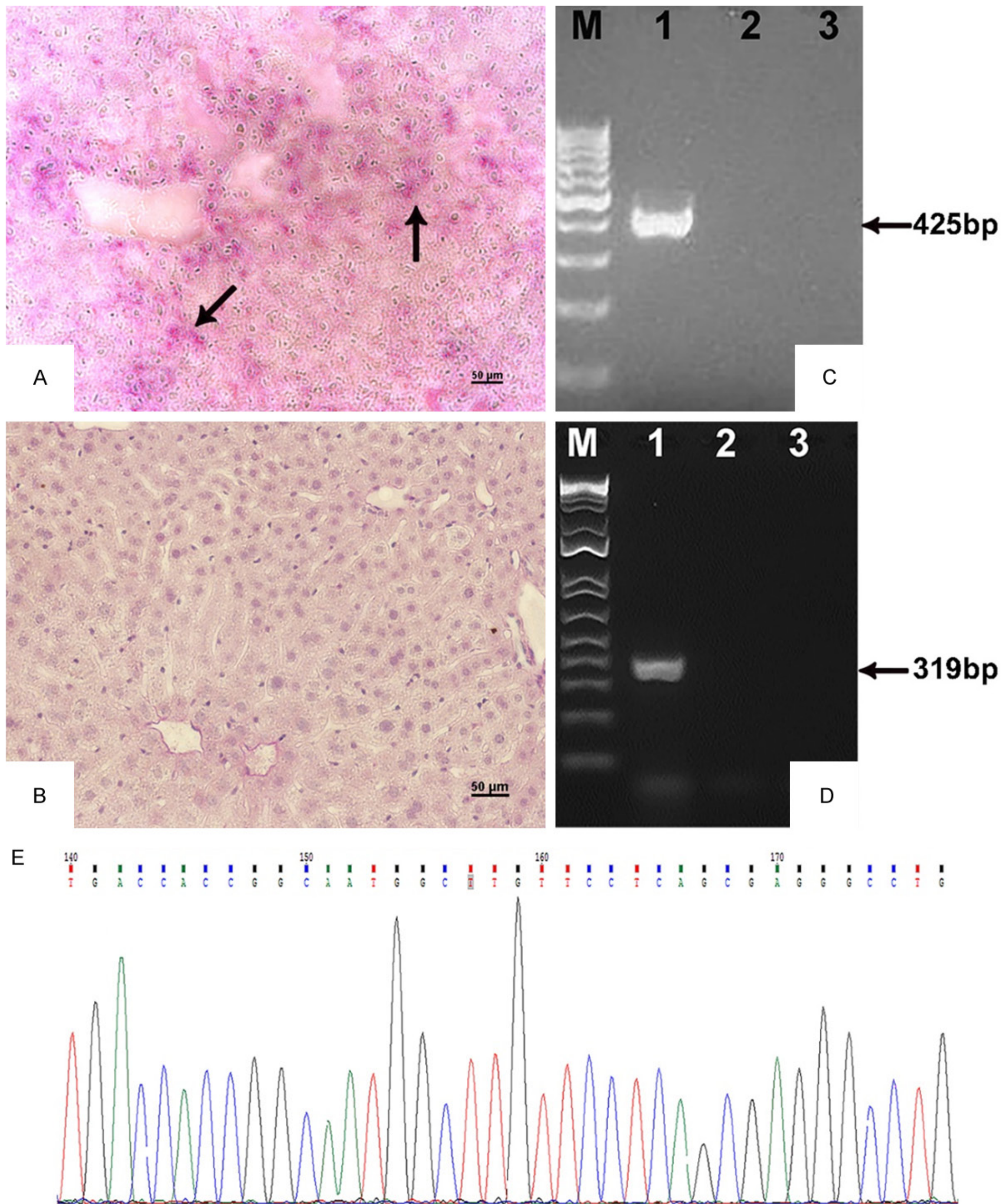

Figure 2. Generation of the transgenic mice. D/PAS staining on the transgenic mouse liver tissue (A) and B6 control (B). PCR (C) and RT-PCR (D) products for transgenic genes running in 0.8% agarose gels, lane 1: transgenic mice DNA, lane 2: B6 mouse DNA, lane3 : water control. Gene sequencing result (E). The arrows in figure A indicate the clusters of globules in the hepatocytes in the liver section.

by the donor cells. The results showed that the donor cells engrafted in the transgenic mice livers and proliferated spontaneously over time. Meanwhile, the HSA DNA contents at 1, 3, and 6 months after the cell transplantation were measured, and the values were 6.74±0.71%, 10.58±0.29%, and 17.19±0.49% of the total DNA, respectively (**Figure 5**). The HAS DNA con-

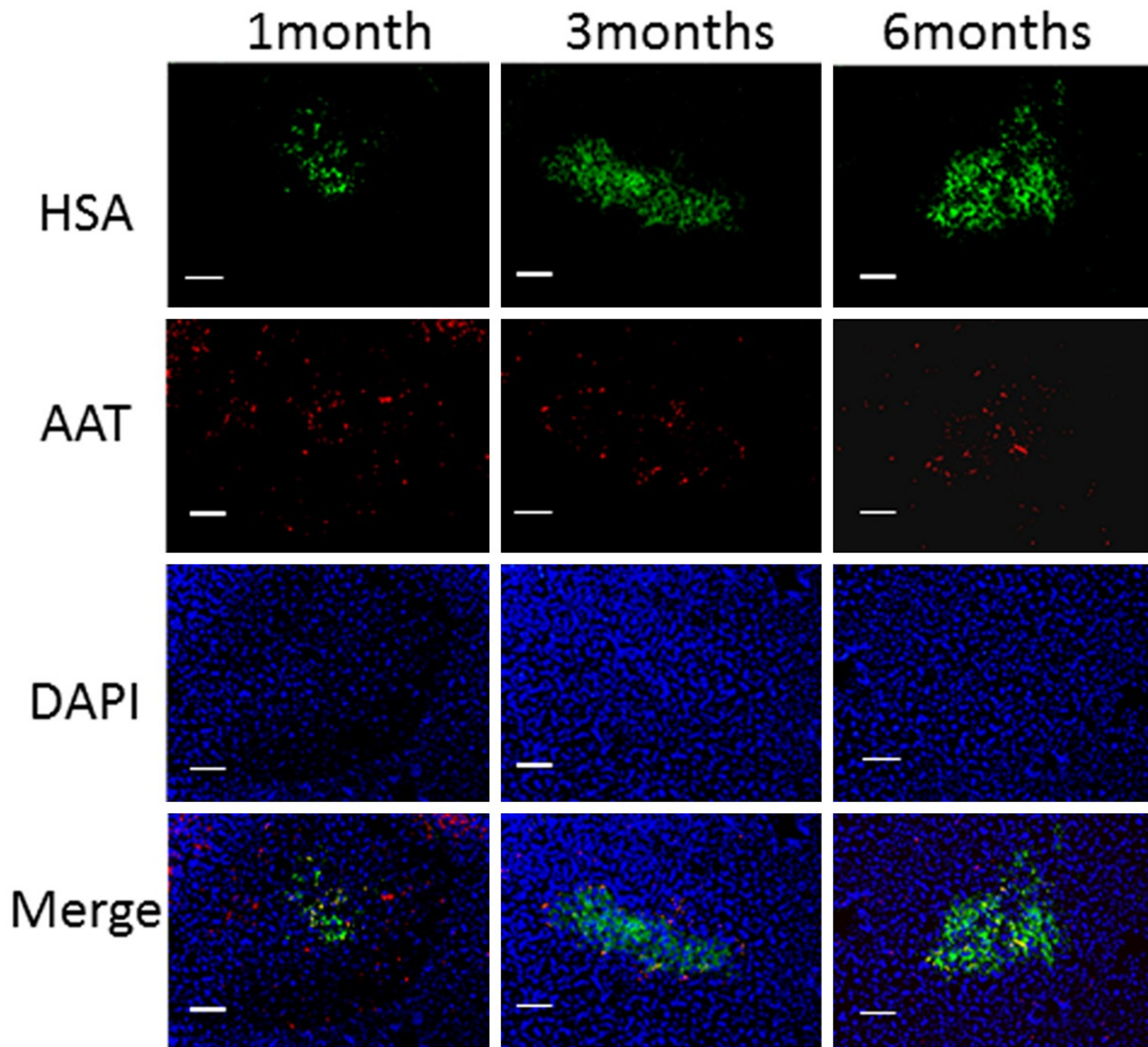

Figure 3. Repopulation of the hiPSC derived hepatic-specific cells in the transgenic mice livers. The transplanted cells proliferated in the transgenic mouse livers as assessed by the immunofluorescence staining for HSA and co-staining with the hAAT antibodies. Scale bar: 100 μm. 20 × microscope magnification.

tent was significantly higher at 3 months and 6 months after the cell transplantation than it was at 1 month after the cell transplantation ($P<0.05$).

Discussion

Stem cell derived hepatocytes are potential sources for cell transplantation [17-19]. However, they have the disadvantages of cell immaturity, lower levels of proliferation, and potential carcinogenicity [20-22]. In AATD mice, their AAT-Z secreting is inefficient, which causes AAT-Z accumulation in the hepatocytes and the inhibition of protease inhibitor activity, further leading to liver injury and emphysema [23]. Rudnick et al. reported higher levels of AAT-Z expression and a greater number of AAT-Z globule–containing hepatocytes in male PiZ AATD model mice when compared with the female and wildtype ones [24]. Therefore, we chose male mice for the experiments after the modeling.

Ding et al. compared the repopulation of the transplanted wild-type AAT expressing hepatocytes expressing and AAT-Z-expressing hepatocytes in the AATD mice, and they found that wild-type hepatocytes proliferated preferentially over the recipient PiZ mouse hepatocytes [9]. In our study, we developed hepatic-specific cells and used them as the donor cells for cell

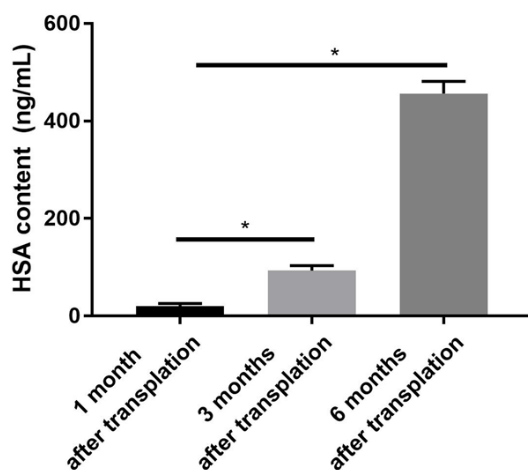

Figure 4. The HSA content in the mouse blood (n=6). All values are given as the means + SEM. *P<0.05.

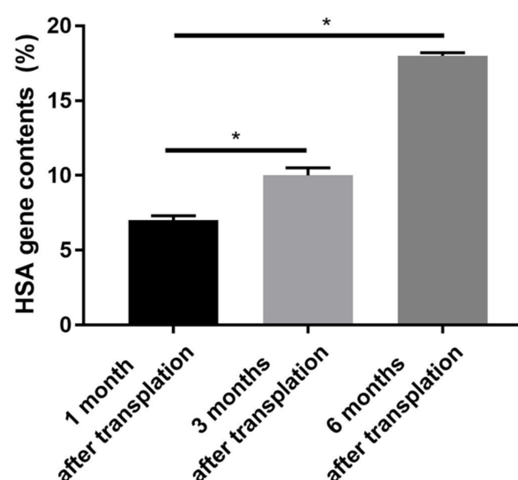

Figure 5. The percentage of the HSA gene contents in the liver tissues (n=6). All the values are given as the means + SEM. *P<0.05.

transplantation. Our experiments provide evidence that the hiPSC-derived hepatic-specific cells can be successfully transplanted into and repopulate in the xenogenic mouse livers, without the unwanted development of liver tumors. Remarkably, the HSA DNA and protein were measured in the mouse serum and liver tissues, and their expressions increased progressively over time. Therefore, the transplanted hepatic-specific cells mature in vivo and synthesize and secrete HSA into the blood in the recipient animals.

However, in the study, only partial donor cells repopulated in the livers of the transgenic mice, which may be related to the donor cell's maturity, host immunity, or the transplantation processing. Stem cell differentiated hepatic cells may have a lower repopulation ability compared to primary hepatocytes. The host body could still have some host versus graft reaction even though FK506 was used at the same time. Whether there are better cell transplantation routes remains to be explored in future research.

Our findings elucidate that the in vitro generated stem cell-derived specific hepatic cells can successfully replace the primary hepatocytes, which may be helpful as novel cell replacement therapy for patients with α-1 antitrypsin deficiency as it is greatly time-saving and cost effective.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (81660090).

Disclosure of conflict of interest

None.

Address correspondence to: Jianqiang Ding, Department of Infectious Diseases, The Third Affiliated Hospital of Guizhou Medical University, No.7 Qixing Road, Duyun 558000, Guizhou Province, China. Tel: +86-15823153198; E-mail: jding18@foxmail.com

References

[1] Doshi SD, Wood L, Abt PL, Olthoff KM, Shaked A, Goldberg DS and Bittermann T. Outcomes of living-donor liver transplantation using grafts heterozygous for α-1 antitrypsin gene mutations. Transplantation 2019; 103: 1175-1180.

[2] Strnad P, McElvaney NG and Lomas DA. Alpha(1)-antitrypsin deficiency. N Engl J Med 2020; 382: 1443-1455.

[3] Köhnlein T and Welte T. Alpha-1 antitrypsin deficiency: pathogenesis, clinical presentation, diagnosis, and treatment. Am J Med 2008; 121: 3-9.

[4] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861-872.

[5] Berger DR, Ware BR, Davidson MD, Allsup SR and Khetani SR. Enhancing the functional maturity of induced pluripotent stem cell-derived human hepatocytes by controlled presentation of cell-cell interactions in vitro. Hepatology 2015; 61: 1370-1381.

[6] Chen Y, Li Y, Wang X, Zhang W, Sauer V, Chang CJ, Han B, Tchaikovskaya T, Avsar Y, Tafaleng E,

Madhusudana Girija S, Tar K, Polgar Z, Strom S, Bouhassira EE, Guha C, Fox IJ, Roy-Chowdhury J and Roy-Chowdhury N. Amelioration of hyperbilirubinemia in gunn rats after transplantation of human induced pluripotent stem cell-derived hepatocytes. Stem Cell Rep 2015; 5: 22-30.

[7] Borel F, Tang Q, Gernoux G, Greer C, Wang Z, Barzel A, Kay MA, Shultz LD, Greiner DL, Flotte TR, Brehm MA and Mueller C. Survival advantage of both human hepatocyte xenografts and genome-edited hepatocytes for treatment of α-1 antitrypsin deficiency. Mol Ther 2017; 25: 2477-2489.

[8] Baligar P, Kochat V, Arindkar SK, Equbal Z, Mukherjee S, Patel S, Nagarajan P, Mohanty S, Teckman JH and Mukhopadhyay A. Bone marrow stem cell therapy partially ameliorates pathological consequences in livers of mice expressing mutant human α1-antitrypsin. Hepatology 2017; 65: 1319-1335.

[9] Ding J, Yannam GR, Roy-Chowdhury N, Hidvegi T, Basma H, Rennard SI, Wong RJ, Avsar Y, Guha C, Perlmutter DH, Fox IJ and Roy-Chowdhury J. Spontaneous hepatic repopulation in transgenic mice expressing mutant human α1-antitrypsin by wild-type donor hepatocytes. J Clin Invest 2011; 121: 1930-1934.

[10] Sauer V, Tchaikovskaya T, Wang X, Li Y, Zhang W, Tar K, Polgar Z, Ding J, Guha C, Fox IJ, Roy-Chowdhury N and Roy-Chowdhury J. Human urinary epithelial cells as a source of engraftable hepatocyte-like cells using stem cell technology. Cell Transplant 2016; 25: 2221-2243.

[11] Sifers RN, Carlson JA, Clift SM, DeMayo FJ, Bullock DW and Woo SL. Tissue specific expression of the human alpha-1-antitrypsin gene in transgenic mice. Nucleic Acids Res 1987; 15: 1459-1475.

[12] Perlino E, Cortese R and Ciliberto G. The human alpha 1-antitrypsin gene is transcribed from two different promoters in macrophages and hepatocytes. EMBO J 1987; 6: 2767-2771.

[13] Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S and Duncan SA. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 2010; 51: 297-305.

[14] Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H and Yamanaka S. A more efficient method to generate integration-free human iPS cells. Nat Methods 2011; 8: 409-412.

[15] Sheng J, Wang Y, Turesky RJ, Kluetzman K, Zhang QY and Ding X. Novel transgenic mouse model for studying human serum albumin as a biomarker of carcinogenic exposure. Chem Res Toxicol 2016; 29: 797-809.

[16] Kohl TO and Ascoli CA. Direct Competitive Enzyme-Linked Immunosorbent Assay (ELISA). Cold Spring Harb Protoc 2017; 2017: pdb.prot093740.

[17] Liu QW, Liu QY, Li JY, Wei L, Ren KK, Zhang XC, Ding T, Xiao L, Zhang WJ, Wu HY and Xin HB. Therapeutic efficiency of human amniotic epithelial stem cell-derived functional hepatocyte-like cells in mice with acute hepatic failure. Stem Cell Res Ther 2018; 9: 321.

[18] Davidson MD, Ware BR and Khetani SR. Stem cell-derived liver cells for drug testing and disease modeling. Discov Med 2015; 19: 349-358.

[19] Jin J. Stem cell treatments. JAMA 2017; 317: 330.

[20] Roy-Chowdhury N, Wang X, Guha C and Roy-Chowdhury J. Hepatocyte-like cells derived from induced pluripotent stem cells. Hepatol Int 2017; 11: 54-69.

[21] Tolosa L, Pareja E and Gómez-Lechón MJ. Clinical application of pluripotent stem cells: an alternative cell-based therapy for treating liver diseases? Transplantation 2016; 100: 2548-2557.

[22] Trounson A and McDonald C. Stem cell therapies in clinical trials: progress and challenges. Cell Stem Cell 2015; 17: 11-22.

[23] Attanasio S, Ferriero R, Gernoux G, De Cegli R, Carissimo A, Nusco E, Campione S, Teckman J, Mueller C, Piccolo P and Brunetti-Pierri N. CHOP and c-JUN up-regulate the mutant Z α1-antitrypsin, exacerbating its aggregation and liver proteotoxicity. J Biol Chem 2020; 295: 13213-13223.

[24] Rudnick DA, Liao Y, An JK, Muglia LJ, Perlmutter DH and Teckman JH. Analyses of hepatocellular proliferation in a mouse model of alpha-1-antitrypsin deficiency. Hepatology 2004; 39: 1048-1055.