

Original Article

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

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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 hepato-

cytes 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

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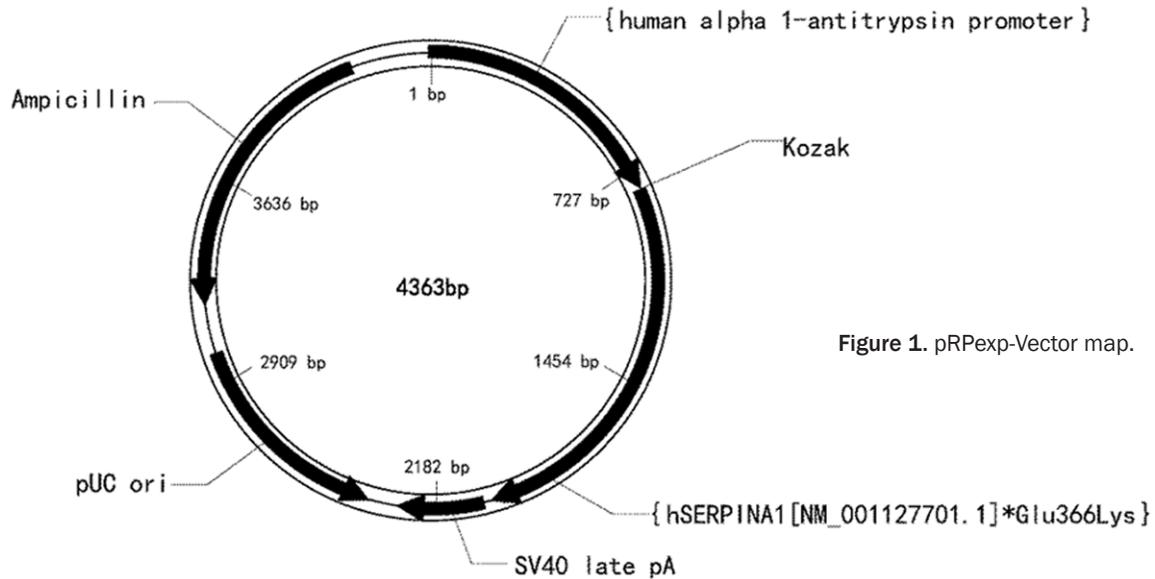


Figure 1. pRPexp-Vector map.

[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 (DYRO100) was purchased from ATCC and cultured in the mTeSR™1 medium (Stemcell, Vancouver, Canada). The cells were grown in 6-welled plates coated with Matrigel (Corning, NY, USA) and incubated at 4% O₂, 5% CO₂, 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% O₂ 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% O₂ 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 μ g/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 μ l primary

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antibodies, mouse anti-human serum albumin (MAB1455, R&D) and rabbit anti-hAAT (H-203, sc-30121, Santa Cruz) at 1:100 dilution (blocking buffer with 0.5% Triton \times 100) at 4°C overnight. The sections were then incubated with 100 μ l fluorescent secondary antibody (Donkey anti-rabbit 555 and Donkey anti-mouse 488) at 1:500 (Alexa Fluor) in 1 \times 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 μ l, 90 μ M P1 0.25 μ l, 90 μ M P2 0.25 μ l, DNA Template 5 μ l, dH₂O 7 μ l. PCR reaction program: 95°C \times 10 min, 95°C \times 15 sec, 60°C \times 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: TAA-GGAGACCTGCTTTGCCG; the sequence of the reverse primer: CCCTAAGCCCTAGCCTAAC. 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 HSA gene con-

tent and expressed as a percentage. The data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). The measurement data were expressed as the mean \pm 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 \pm 1.47, 82.00 \pm 5.85, and 464.22 \pm 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

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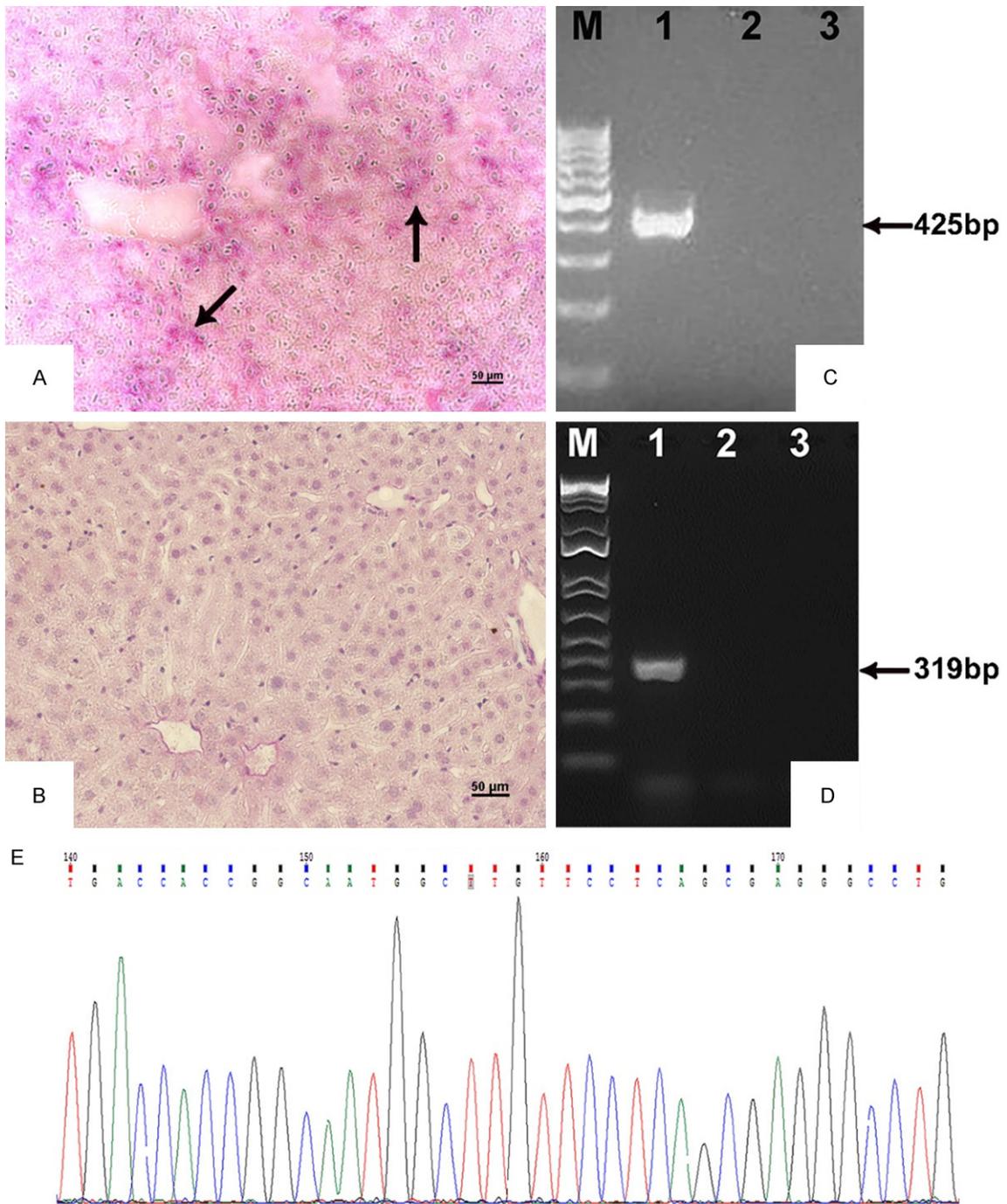


Figure 2. Generation of the transgenic mice. D/PAS staining on the transgenic mouse liver tissue (A) and B6 control (B). PCR (C) and RT-PCR (D) products for transgenic genes running in 0.8% agarose gels, lane 1: transgenic mice DNA, lane 2: B6 mouse DNA, lane3 : water control. Gene sequencing result (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 $6.74 \pm 0.71\%$, $10.58 \pm 0.29\%$, and $17.19 \pm 0.49\%$ of the total DNA, respectively (Figure 5). The HAS DNA con-

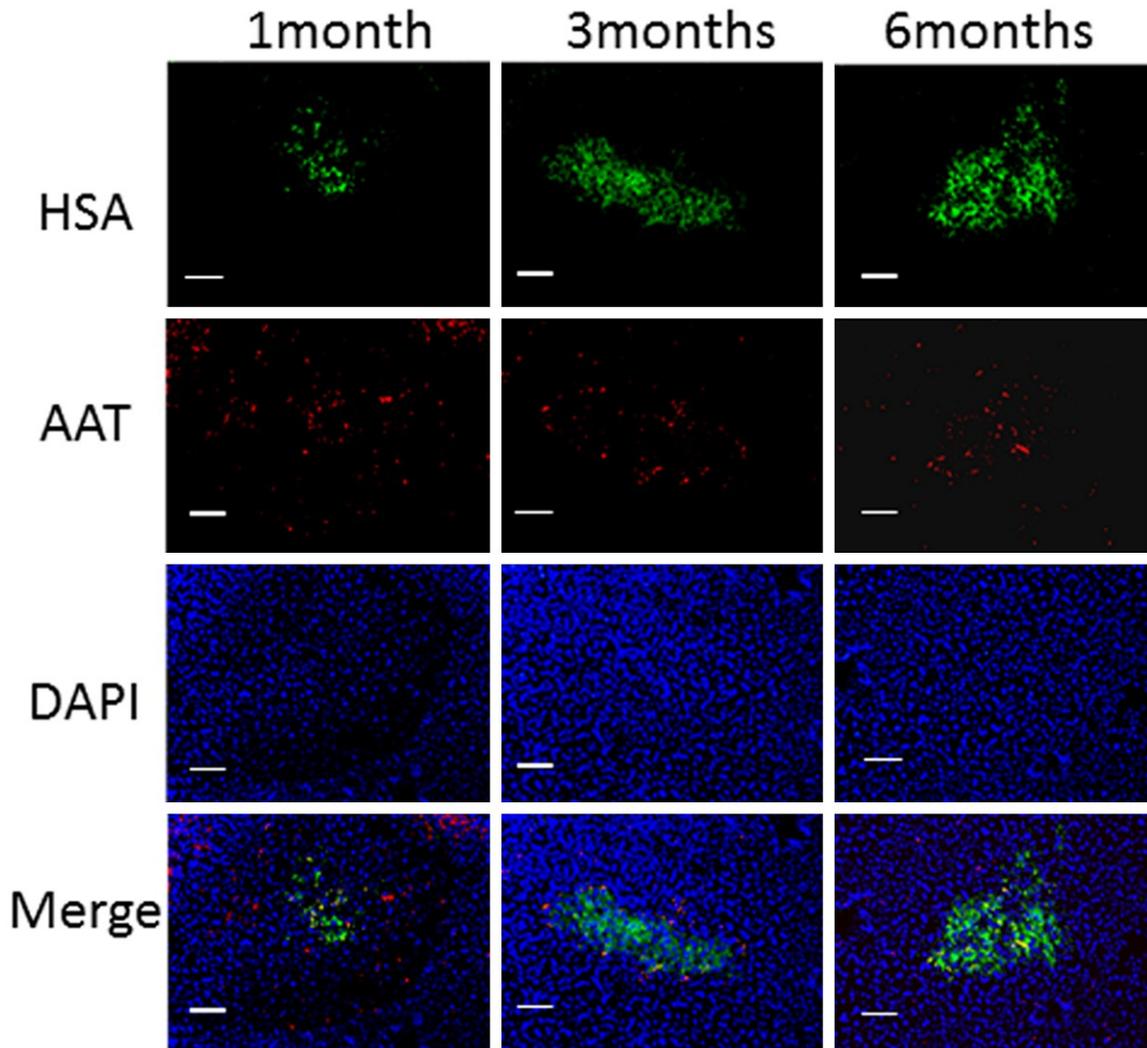


Figure 3. Repopulation of the hiPSC derived hepatic-specific cells in the transgenic mice livers. The transplanted cells proliferated in the transgenic mouse livers as assessed by the immunofluorescence staining for HSA and co-staining with the hAAT antibodies. Scale bar: 100 μ m. 20 \times microscope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

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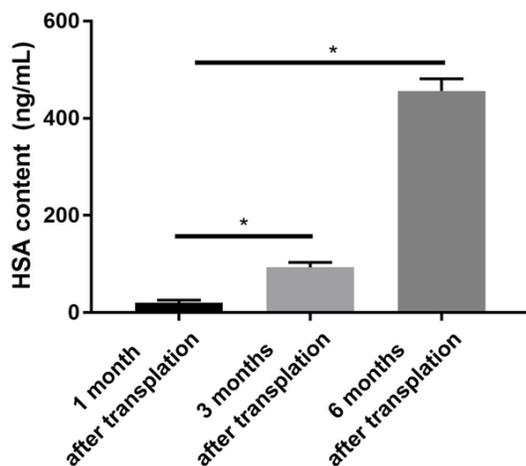


Figure 4. The HSA content in the mouse blood (n=6). All values are given as the means + SEM. *P<0.05.

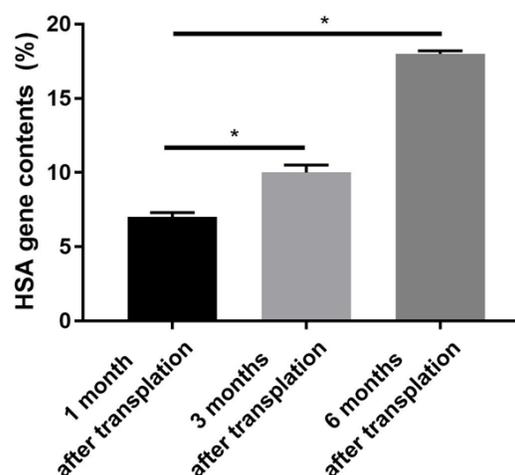


Figure 5. The percentage of the HSA gene contents in the liver tissues (n=6). All the values are given as the means + SEM. *P<0.05.

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.

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Disclosure of conflict of interest

None.

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