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ORIGINAL ARTICLE

Therapeutic potential of spheroids of stem cells from human exfoliated deciduous teeth for chronic liver fibrosis and hemophilia A

Yoshiaki Takahashi[1] · Ratih Yuniartha[1] · Takayoshi Yamaza[2] · Soichiro Sonoda[2] · Haruyoshi Yamaza[3] · Kosuke Kirino[1] · Koichiro Yoshimaru[1] · Toshiharu Matsuura[1] · Tomoaki Taguchi[1]

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Abstract

Purpose Mesenchymal stem cell (MSC)-based cell therapies have emerged as a promising treatment option for various diseases. Due to the superior survival and higher differentiation efficiency, three-dimensional spheroid culture systems have been an important topic of MSC research. Stem cells from human exfoliated deciduous teeth (SHED) have been considered an ideal source of MSCs for regenerative medicine. Thus, in the present study, we introduce our newly developed method for fabricating SHED-based micro-hepatic tissues, and demonstrate the therapeutic effects of SHED-based micro-hepatic tissues in mouse disease models.

Methods SHED-converted hepatocyte-like cells (SHED-HLCs) were used for fabricating spherical micro-hepatic tissues. The SHED-HLC-based spheroids were then transplanted both into the liver of mice with CCl_4-induced chronic liver fibrosis and the kidney of *factor VIII* (*F8*)-knock-out mice. At 4 weeks after transplantation, the therapeutic efficacy was investigated.

Results Intrahepatic transplantation of SHED-HLC-spheroids improved the liver dysfunction in association with anti-fibrosis effects in CCl_4-treated mice. Transplanted SHED-converted cells were successfully engrafted in the recipient liver. Meanwhile, renal capsular transplantation of the SHED-HLC-spheroids significantly extended the bleeding time in *F8*-knock-out mice.

Conclusions These findings suggest that SHED-HLC-based micro-hepatic tissues might be a promising source for treating pediatric refractory diseases, including chronic liver fibrosis and hemophilia A.

Keywords SHED · Spheroids · Transplantation · Chronic liver fibrosis · Hemophilia A

Introduction

Mesenchymal stem cells (MSCs) have been widely investigated for their therapeutic potential due to their homing ability toward damaged tissue and as a source of secreted growth factors and regenerative molecules [1]. Furthermore, recent studies are being conducted to evaluate the immunomodulatory effects of MSCs [2, 3]. MSCs are, therefore, considered a feasible cell source for tissue engineering and cell therapy for treating various diseases.

Stem cells from human exfoliated deciduous teeth (SHED) were isolated from the remnant deciduous dental pulp, and identified as an MSC population with a high proliferation capability, the potential to differentiate into a variety of cell types, including neural cells, endothelial cells, and hepatocytes, as well as mesenchymal stem cell lineage cells [4]. In comparison to other human tissues, exfoliated deciduous teeth offer significant advantages: they are associated

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Yoshiaki Takahashi and Ratih Yuniartha contributed equally to this work.

✉ Takayoshi Yamaza
yamazata@dent.kyushu-u.ac.jp

[1] Department of Pediatric Surgery, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

[2] Division of Oral Biological Sciences, Department of Molecular Cell Biology and Oral Anatomy, Graduate School of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[3] Department of Pediatric Dentistry, Division of Oral Health, Growth, and Development, Graduate School of Dental Science, Kyushu University, Fukuoka, Japan

with fewer ethical issues, can be obtained non-surgically, and they are a readily accessible source. Furthermore, the stem cells retain their high potency with regard to cell proliferation, multipotency, and immunomodulatory functions, even after cryopreservation [4, 5]. Thus, SHED have received a great deal of attention due to their potential applications in the treatment of various human diseases [2]. In the recent report, we successfully generated ex vivo SHED-converted hepatocyte-like cells (SHED-HLCs) expressing hepatocyte-specific genes and functional profiles associated with glycogen storage, the production of albumin and urea, and cytochrome P450 activity [6]. Our recent reports also showed that SHED-based cell therapy improved chronic and fulminant liver failure in mice due to in situ properties, including tissue-integration, trans-differentiation, and tissue-reconstruction in the recipients' damaged liver [6, 7].

On the other hand, cell therapies using MSCs prepared under a regular two-dimensional culture system show low therapeutic efficacy due to their poor survival and low differentiation efficiency of the transplanted cells in vivo [8]. Recently, several groups have reported that the aggregation of MSCs into three-dimensional (3D) spheroids could promote the differentiation of MSCs and enhance their production of trophic and anti-inflammatory factors [9, 10]. The 3D spheroids show beneficial effects in the treatment of treat liver fibrosis and hepatitis in animal models [8, 11]. Thus, the direct intrahepatic implantation of SHED-based or SHED-HLC-based spheroids might be a safe and feasible option for inducing liver regeneration. However, there have been no basic, preclinical, or clinical studies regarding direct transplantation of SHED-HLC-based spheroids (SHED-HLC-spheroids) to damaged liver. Thus, the purpose of this study was to fabricate 3D spherical micro-hepatic tissue using SHED-HLCs, and investigate whether the SHED-HLC-spheroids show a therapeutic effect in mice with CCl_4-induced chronic liver fibrosis and *factor VIII* (*F8*)-knock-out hemophilia A model mice.

Materials and methods

Ethics statement and human subjects

Human deciduous teeth samples were collected as discarded samples from healthy pediatric donors (5–7 years of age) in the Department of Pediatric Dentistry of Kyushu University Hospital. Procedures using human samples were approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (Protocol number: 393-01). We obtained written informed consent from each parent on behalf of the child donors. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Kyushu University (Protocol number: A21-044-1).

Animals

C57BL/6J mice (male, 6–8 weeks of age) were purchased from Kyudo (Tosu, Japan). We also purchased hemophilia A model *F8*-knock-out $F8^{tm1Kaz}$/J mice from Jackson Laboratory (Bar Harbor, ME).

Isolation and culture of SHED

Isolation and culture of SHED were performed according to previous studies [5] as described in the Supplementary Methods.

Generation of SHED-HLCs

The generation of SHED-HLCs was performed according to the methods of a previous study [6], as described in the Supplementary Methods.

Fabrication of SHED-HLC-spheroids

Fabrication of SHED-HLC-spheroids was performed according to a previously reported protocol [12]. Briefly, after generating SHED-HLCs, SHED-HLCs were re-seeded at 0.1×10^6 per well in low cell attachment PrimeSurface 96U multiwell plates (Sumitomo Bakelite, Tokyo, Japan), and cultured in 200 µL of a hepatocyte culture medium containing oncostatin M (20 ng/mL; Pepro Tech, Rocky Hill, NJ), dexamethasone (1 mM; Sigma-Aldrich, St. Louis, MO), ITS premix (1×; Thermo Fisher Scientific, Waltham, MA), and premixed antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Nacalai Tesque, Kyoto, Japan) in Iscove's Modified Dulbecco's Media (IMDM; Thermo Fisher Scientific) for 7 days. Some SHED-HLCs were re-seeded at 1.0×10^6 per tube in a STEMFULL low cell adsorption tube (Sumitomo Bakelite), and were subsequently cultured in 1 mL of the hepatogenic medium for 28 days. The culture medium was changed every 3 days. Finally, some SHED-HLC-spheroids were used for the in vivo transplantation study, and the others and the conditioned medium were used for further analyses.

Transplantation of SHED-HLC-spheroids

To generate chronic liver fibrosis model animals, a mixture of CCl_4 (0.5 mL/kg body weight; Wako Pure Chemicals, Osaka, Japan) and olive oil (1:4 volume/volume; Wako Pure Chemicals) was intraperitoneally injected into C57BL/6J mice (male, 6–8 weeks of age) twice a week during this experimental period. Prepared SHED-HCL-based spheroids

were washed with 100 µL of Hank's balanced salt solution (HBSS; Nacalai Tesque) 3 times just before the transplantation. At 4 weeks after the CCl_4 treatment, SHED-HCL-spheroids (10 spheroids, total 1×10^6 cells per mouse) were implanted into the linear fissure (10 mm in length, 1 mm in depth) on the surface of the left lateral liver lobe. The transplanted mice also subsequently received CCl_4 for 4 weeks. Age-matched and sex-matched C57BL/6 mice that received CCl_4 and a sham operation and sex-matched C57BL/6 mice treated with olive oil (Wako Pure Chemicals) were used as controls. Meanwhile, to avoid excessive surgical procedure-related bleeding, recombinant human factor VIII, Efraloctocog Alfa (0.1 IU/g; Eloctate®, SANOFI, Paris, France) was administered to *F8*-knock-out mice (male, 4–8 weeks old) 1 day before transplantation. SHED-HCL-spheroids (10 spheroids, total 1×10^6 cells per mouse) were implanted under the capsule of the left kidney. Age-matched and sex-matched sham-operated C57BL/6 mice were used as controls in both mouse models. In the present animal study, no immunosuppressant agents were used before or after spheroid transplantation in either mouse model. At 4 weeks after spheroid implantation, all mice were harvested for use in further analyses.

Tail-clip bleeding test

Under systemic anesthesia, the tip (10 mm in length) of the tails of *F8*-knock-out mice that received SHED-HLC-spheroids and a sham operation was cut with scissors, and the mice were returned to their cages, where they were housed separately. The clipped tail of the mice was printed on a filter paper every 1 min. The time to the cessation of bleeding was recorded, and the area of bleeding on the filter paper was measured using the Image J software program (NIH). If the mouse did not stop bleeding at 30 min, we cauterized the wound to save the mouse, according to the methods of a previous study [13].

Histological and immunohistochemical analyses

Histological and immunohistochemical analyses of SHED-HLC-spheroids and mouse liver tissues were performed according to the methods of a previous study [7], as described in the Supplementary Methods. All antibodies used in the present immunohistochemical analysis are summarized in Supplementary Table 1. For the morphometric assays, seven representative images were selected randomly from each group, and the percentage of the fibrous tissue area or primary antibody-positive area was measured using the Image J software program (National Institutes of Health, Bethesda, MD). Images obtained from Sirius red-stained sections were also used for Ishak scoring, which is a scoring system that is widely used for the assessment of liver fibrosis [14].

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

RT-PCR and qRT-PCR assays were performed according to the methods of a previous study [7], as described in the Supplementary Methods. The specific primer pairs and TaqMan probes used in the present RT-PCR assay are summarized in the Supplementary Tables 2 and 3, respectively.

Biochemical and immunological assays of culture supernatants and mouse serum and plasma

Biochemical and immunological assays using the conditioned medium and mouse serum were performed using commercially available kits according to the methods of a previous study [7], as described in the Supplementary Methods. All commercially available kits are listed in Supplementary Table 4.

Statistical analysis

The results of the statistical analyses are expressed as the mean ± standard error of the mean (SEM) or mean ± standard deviation (SD) from at least triplicate determinations. Comparisons between two groups were analyzed by independent two-tailed Student's *t* tests. Multiple groups were compared by a one-way repeated measures analysis of variance (ANOVA) followed by a Tukey post hoc test. *P* values of < 0.05 were considered to indicate statistical significance. All the statistical analyses were performed using the PRISM 6 software program (GraphPad, Software, La Jolla, CA).

Results

Fabrication of 3D spherical micro-liver tissue using SHED-HLCs

SHED-HLCs cultured in low cell attachment wells or tubes formed spherical cell aggregates in the hepatocyte culture medium at 7 days after seeding (Fig. 1a, b). The spherical structures were maintained until 28 days after seeding under the hepatocyte culture condition (Fig. 1a). Hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining revealed that cells in SHED-HLC-spheroids showed morphology and glycogen storage pattern similar to those of cells in human liver tissues (Fig. 1b). RT-PCR showed the gene expression of human albumin (*hALB*) and human F8 (*hF8*) genes in SHED-HLC-spheroids after 28 days of culture, as seen in human liver

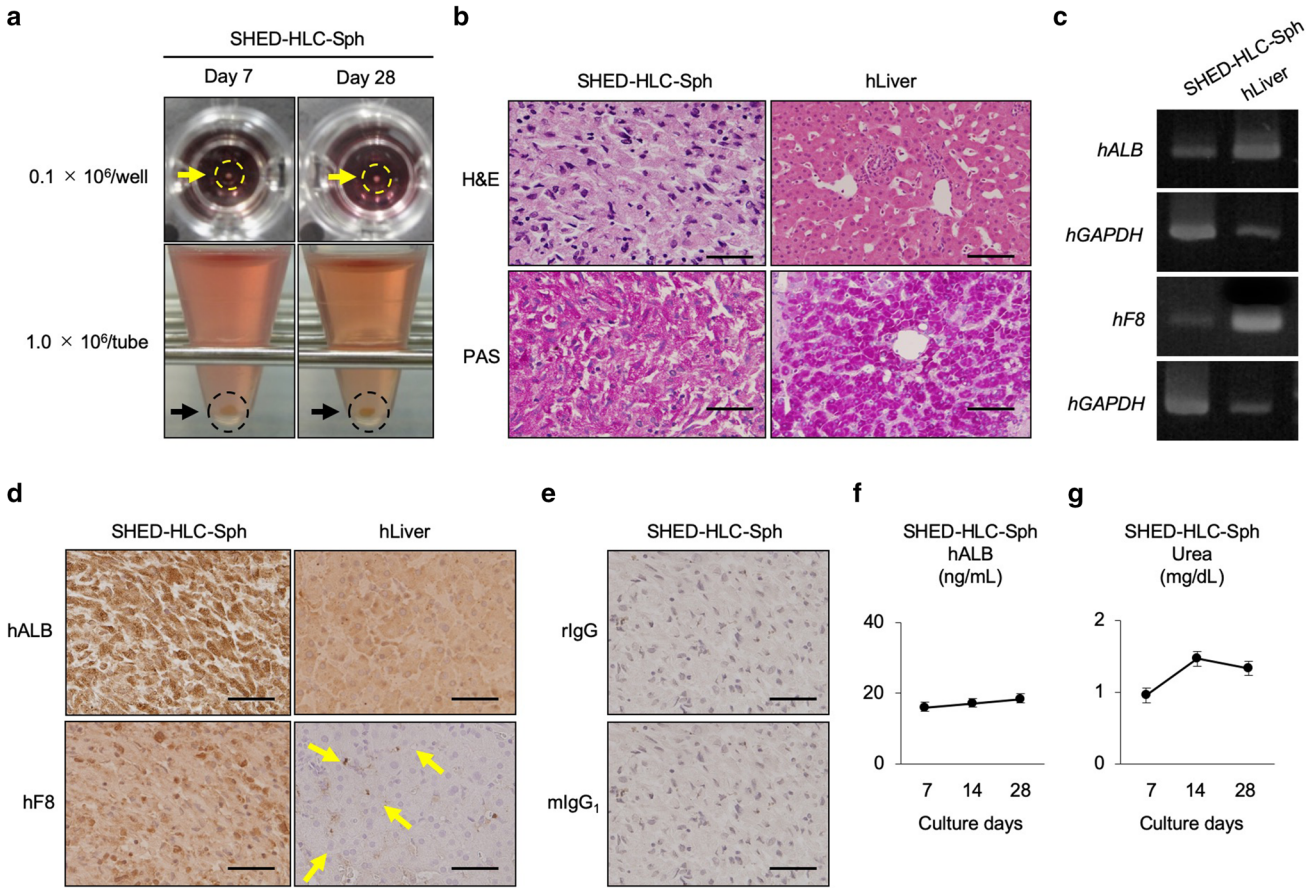

Fig. 1 Fabrication and characterization of spherical human microhepatic tissues using SHED-HLCs. **a** SHED-HLCs were seeded at 0.1×10^6 cells per well and 1.0×10^6 cells per tube in low attachment culture apparatuses, and were cultured for 28 days in a hepatocyte culture medium. Representative images of fabricated SHED-HLC-spheroids seeded at 0.1×10^6 cells per well (upper two panels) or 1.0×10^6 cells per tube (lower two panels) on days 7 and 28 of culture. Fabricated SHED-HLC-spheroids are indicated by dot-lined circles and arrows. **b** The histological features of SHED-HLC-spheroids on day 28 of culture. Representative microscopic images of paraffin sections stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) are shown. Human liver tissues were used for positive controls. **c** The gene expression of human albumin (*hALB*) and human F8 (*hF8*) mRNA, as determined by RT-PCR of SHED-HLC-spheroids on culture day 28. The results show representative images of electrophoresed agarose gels stained with ethidium bromide. *hGAPDH* human glyceraldehyde-3-phosphate dehydrogenase gene. **d**, **e** Localization of hALB and hF8, as determined by an immunohistochemical analysis of SHED-HLC-spheroids on day 28 of culture. The sections were treated with each human-specific antibody against hALB and hF8. Arrows indicate hF8-positive cells in human liver tissues (**d**). Immunohistochemistry of control samples demonstrated staining with non-immune isotype-matched antibodies, including rabbit IgG (rIgG) and mouse IgG_1 ($mIgG_1$) (**e**). All sections were counterstained with hematoxylin. **f**, **g** The secretion of hALB (**f**) and the production of urea (**g**) in the conditioned medium of SHED-HLC-spheroids at the indicated periods, as determined by immunological and biochemical assays, respectively. The results are graphed. The graph bars show the mean ± SD ($n=3$ for all groups). **b**–**e** *hLiver* human liver tissues, *SHED-HLC-Sph* SHED-HLC-spheroids. **b**, **d**, **e** Scale bars 30 μm

tissue (Fig. 1c). Immunohistochemistry also demonstrated that SHED-HLC-spheroids contained hALB-positive and hF8-positive cells, as seen in human liver tissue (Fig. 1d). Immunohistochemical control tests using non-immune isotype-matched antibodies were negative for SHED-HLC-spheroids (Fig. 1e). Further immunological and biochemical assays using culture-conditioned medium were performed to evaluate the secretion of hALB (Fig. 1f) and the production of urea (Fig. 1g).

The therapeutic efficacy of SHED-HLC-spheroid transplantation in the mouse model of CCl_4-induced chronic liver fibrosis

C57BL/6J mice (male, 6–8 weeks of age) received CCl_4 (0.5 mL/kg body weight) twice a week (Fig. 2a). SHED-HLC-spheroids were transplanted into the liver left lateral lobe of mice after 4 weeks of CCl_4-induced damage (Fig. 2b). At 4 weeks after transplantation, the transplanted

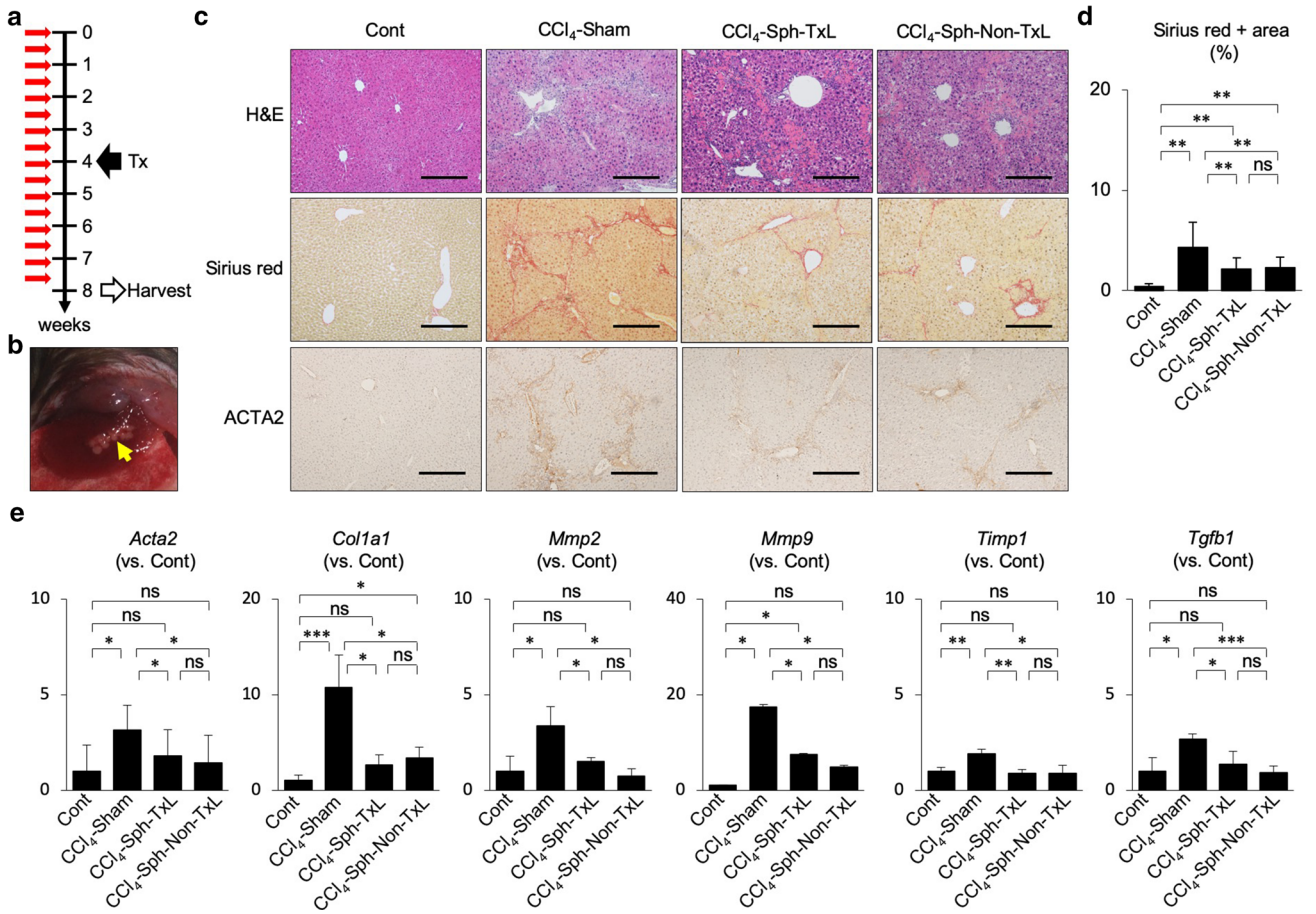

Fig. 2 Therapeutic analyses to investigate the anti-fibrotic efficacy of the intrahepatic transplantation of SHED-HLC-spheroids in a mouse model of CCl_4-induced chronic liver fibrosis. **a**, **b** A schematic illustration demonstrating transplant treatments with spherical human micro-hepatic tissues using SHED-converted hepatocyte-like cells (SHED-HLCs) for chronic liver fibrosis. C57BL/6 mice were intraperitoneally injected with CCl_4 (0.5 mL/kg) or olive oil twice a week, as indicated by the red arrows. SHED-HLC-based spheroids (SHED-HLC-spheroids; 0.1×10^6 cells per spheroids, total 10 spheroids per mice) were transplanted into the liver left lateral lobe of the CCl_4-treated mice 4 weeks after the treatment (Tx, black arrow). The mice were harvested at 4 weeks after transplantation following a total of 8 weeks of treatment of CCl_4 (white arrow). **b** A representative liver image shows the intrahepatic transplantation of SHED-HLC-spheroids into the mouse liver left lateral lobe. Yellow arrow indicates an implanted SHED-HLC-spheroid. **c** The histological analysis of mouse liver tissues. The results show representative microscopic images stained with H&E (H&E, upper three panels), Sirius red staining (Sirius red, middle 3 panels), immunostaining with anti-actin alpha 2, smooth muscle (ACTA2) antibody (ACTA2, lower three panels). Images of the lower three panels were counterstained with hematoxylin. Scale bars 100 µm. **d** A morphometric analysis of fibrous tissue deposition in the mouse liver. The results show the percentage of the Sirius red-positive (Sirius red +) area in mouse liver. **e** A gene expression analysis of *Acta 2*, *collagen type I alpha 1 chain* (*Col1a1*), *matrix metalloproteinase 2* (*Mmp2*), *Mmp9*, *tissue inhibitors of matrix metalloproteinase 1* (*Timp1*), and *transforming growth factor beta 1* (*Tgfb1*) in the mouse liver. The demonstrated the difference in the ratio to the expression in control mice (vs. Cont) by quantitative RT-PCR. **a**–**c** *Control* non-CCl_4-injured and olive oil-treated mouse liver, *CCl_4-Sham* CCl_4-injured and sham-operated mouse liver, *CCl_4-Sph-TxL* CCl_4-injured and SHED-HLC-spheroid-transplanted liver (left lateral lobe), *CCl_4-Sph-Non-TxL* CCl_4-injured and SHED-HLC-spheroid non-transplanted liver (median lobe). **d**, **e** The graph bars show the mean ± SD; $n = 7$ for all groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. *ns* not significant

mice were subsequently used for assays to determine the clinical benefits of SHED-HLC-spheroids. Histological analyses by H&E staining, Sirius red staining, and immunostaining demonstrated that parenchymal disarrangement, severe collagen deposition, and the marked expression of actin alpha 2, smooth muscle (ACTA2) were observed in the liver tissues of CCl_4-treated sham mice in comparison to control olive oil-treated mice (Fig. 2c). Intrahepatic transplantation of SHED-HLC-spheroids markedly inhibited the progression of liver disorders, including liver fibrosis and structural disarrangement induced by chronic CCl_4-treatment (Fig. 2c). Interestingly, Sirius red staining and its associated Ishak score demonstrated significantly reduced CCl_4-induced liver fibrosis in SHED-HLC-spheroid-transplanted mice, not only in the transplanted left lateral lobe, but also in the other non-transplanted lobes in comparison to CCl_4-treated sham mice

Table 1 Ishak scoring of mouse liver tissues

Group	Cont	CCl_4		
		Sham	Sph	
			TxL	Non-TxL
Score	0	3.5* (3–4)	2.0*# (2–3)	3.0*#† (2–3)

The results showed the median (25th–75th percentile)

Control non-CCl_4-injured and olive oil-treated group, *CCl_4* CCl_4-injured group, *Sham* CCl_4-injured and sham-operated group, *Sph* CCl_4-injured and SHED-HLC-spheroid-transplanted group, *TxL* CCl_4-injured and SHED-HLC-spheroid-transplanted liver left lateral lobe group, *Non-TxL* CCl_4-injured and SHED-HLC-spheroid non-transplanted liver median lobe group

*$P < 0.05$ vs. Cont

#$P < 0.05$ vs. Sham

†$P < 0.05$ vs. TxL

(Fig. 2d, Table 1). A gene expression assay by qRT-PCR showed that SHED-HLC-spheroid transplantation reduced the enhanced levels of *Acta 2*, *collagen type I alpha 1 chain* (*Col1a1*), *matrix metalloproteinase 2* (*Mmp2*), *Mmp9*, *tissue inhibitors of matrix metalloproteinase 1* (*Timp1*), and *transforming growth factor beta 1* (*Tgfb1*) in the CCl_4-injured mouse liver (Fig. 2e). Interestingly, the significant anti-fibrotic efficacy of the intrahepatic transplantation of SHED-HLC-spheroids was not only observed in the liver lobe to which spheroids were transplanted (left lateral lobe), but also in the other lobes (caudate, median, and right lobes) without spheroid transplantation (Fig. 2c–e).

Moreover, immunohistochemistry demonstrated that human leukocyte antigen ABC-, hepatocyte paraffin 1-(HepPar1-), and hALB-positive cells were widely distributed in the interlobular and portal areas of the transplanted liver lobe, as well as in the interlobular and portal areas of the other non-transplanted liver lobes of SHED-HLC-spheroid-transplanted mice (Fig. 3a). HepPar1-positive area in the SHED-HLC-spheroid-transplanted and non-transplanted lobes of CCl_4-treated mice was $5.9\% \pm 2.4\%$ and $3.9\% \pm 1.7\%$, respectively, while no HepPar1-positive area was detected in the liver of control and CCl_4-treated sham-operated mice (Fig. 3b). Immunohistochemical control tests using isotype-matched IgG did not detect any immunopositive reaction (data not shown). An immunological assay detected hALB in the serum of SHED-HLC-spheroid-transplanted mice, but not in those of control or CCl_4-treated sham-operated mice (Fig. 3c). Biochemical and immunological serum assays revealed that the transplantation of SHED-HLC-spheroids was associated with the marked recovery of liver dysfunction, indicated by the AST, ALT, and total bilirubin levels, and also reduced the upregulated expression of TGF-β in comparison to CCl_4-treated sham-operated mice (Fig. 3d, e).

The therapeutic efficacy of SHED-HLC-spheroid transplantation in *F8*-knock-out hemophilia A model mice

SHED-HCL-spheroids (10 spheroids, total 1×10^6 cells per mouse) were implanted under capsules of left kidney of *F8*-knock-out mice (male, 4–8 weeks of age), which were treated with a recombinant human factor VIII, Efraloctocog Alfa (0.1 IU/g) 1 day before transplantation (Fig. 4a, b). A tail-clipping test at 4 weeks after transplantation revealed that in control mice, bleeding had not stopped at 30 min after tail clipping. In contrast, in SHED-HLC-spheroid-transplanted mice, bleeding stopped within 30 min after tail clipping (Fig. 4c), and the mice survived after these tests. The transplanted spheroid-derived human cells were not detected in the kidney tissues by immunohistochemistry (data not shown). On the other hand, a small number of HepPar1-positive and hF8-positive cells were found in the recipient liver tissue, especially around the portal veins (Fig. 4d). Unfortunately, hF8 could not be detected in the plasma of SHED-HLC-spheroid-transplanted *F8*-knock-out mice or the control sham-operated *F8*-knock-out mice (data not shown).

Discussion

In this study, we successfully developed unique human 3D-spherical micro-hepatic tissues using SHED-HLCs without a scaffold. The fabricated SHED-HLC-based spherical micro-hepatic tissues contained arranged hepatocyte-like cells that were positive for PAS, hALB, and F8, and which showed hepatic functions, including hALB secretion and urea production in vitro. hALB was expressed in the serum of CCl_4-treated mice with the intrahepatic transplantation of SHED-HLC-spheroids, indicating that the donor cells migrated from the intrahepatically implanted SHED-HLC-spheroids have the capacity of in situ synthesis and to secrete bioactive products, such as hALB in mice. Moreover, intrahepatic and renal sub-capsule transplantation of SHED-HLC-spheroids improved chronically CCl_4-treated and *F8*-knock-out mice, suggesting that the developed SHED-HLC-based 3D micro-hepatic tissues developed in the present study could be a feasible tool for treating chronic liver fibrosis and hemophilia A.

Recent intrasplenic transplantation studies using SHED and SHED-HLCs have demonstrated the anti-inflammatory and anti-fibrotic efficacy, as well as the liver regeneration capacity in mouse models of fulminant hepatitis and chronic liver fibrosis [6, 7]. Meanwhile, the administration of MSC via the intrasplenic route is associated with an increased risk of embolization and hypertension in the portal vein [15, 16]. Thus, the direct intrahepatic implantation of SHED or

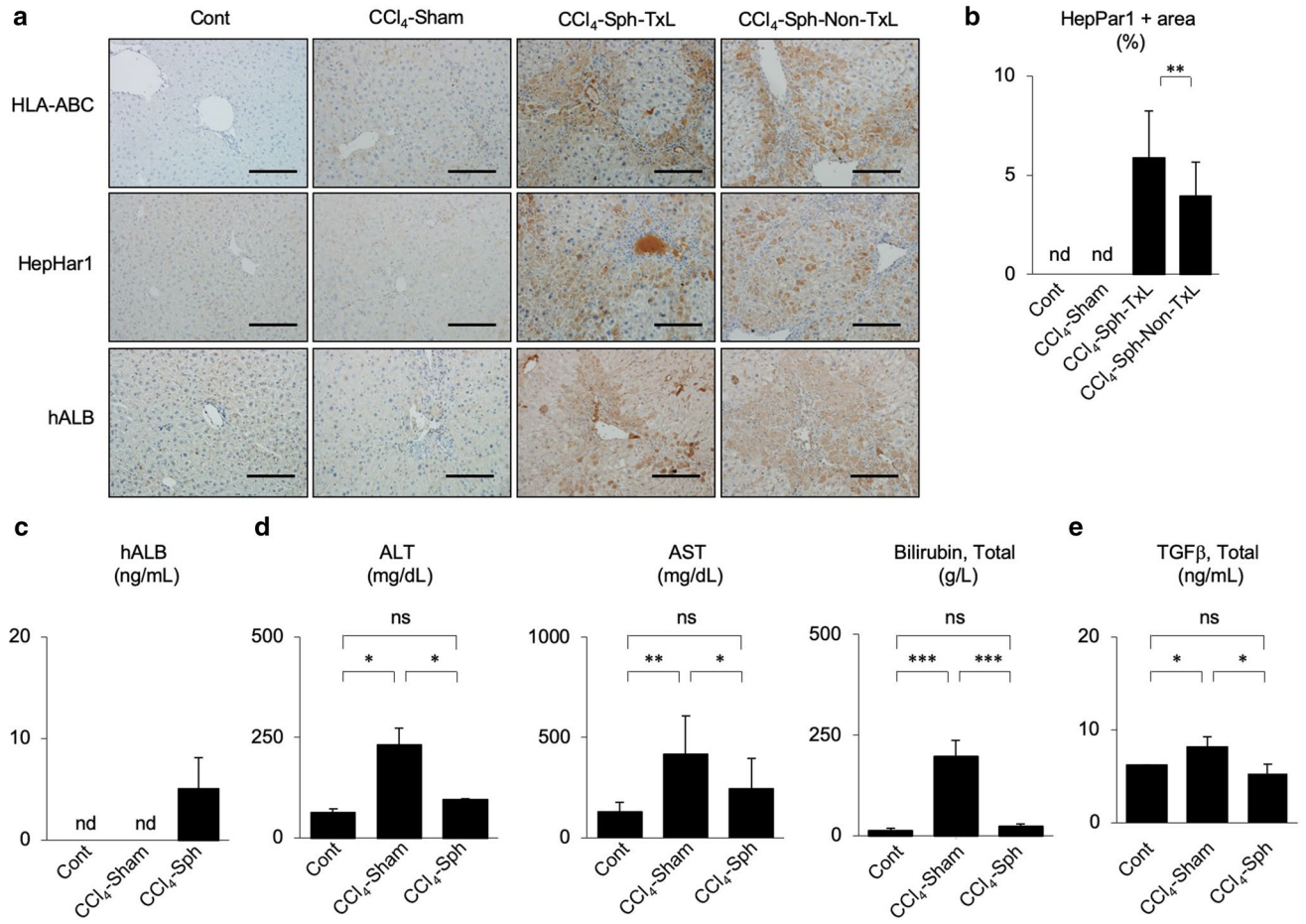

Fig. 3 Therapeutic analyses of the potential for the intrahepatic transplantation of SHED-HLC-spheroids to induce liver regeneration in a mouse model of CCl_4-induced chronic liver fibrosis. CCl_4-treated mice that received the intrahepatic transplantation of SHED-HLC-spheroids (0.1×10^6 cells per spheroids, total 10 spheroids per mice) were harvested at 4 weeks after transplantation under a total of 8 weeks of CCl_4 treatment. **a** Liver regeneration induced by the intrahepatic transplantation of SHED-HLC-spheroids in CCl_4-injured mice. An immunohistochemical analysis of mouse liver tissues from the SHED-HLC-spheroid-transplanted left lateral lobe (transplant site) and the non-transplanted median lobe (non-transplant site). Representative microscopic images by immunostaining with human-specific antibodies against human leukocyte antigen ABC (HLA-ABC), human hepatocyte-specific antigen hepatocyte paraffin 1 (HepPar1), and hALB. The nuclei are counterstained with hematoxylin. Scale bars 100 μm. **b** A morphometric analysis of the HepPar1-positive (HepPar1 +) area in the mouse liver. The results showed the percentage of the HepPar1-positive area in the mouse liver. **c**–**e** Immunological and biochemical assays of the mouse liver function of SHED-HLC-spheroid-transplanted mice. The graphs show the serum levels of hALB (**c**), alanine aminotransferase (ALT, **d**), aspartate aminotransferase (AST, **d**), and mouse total TGF β (**e**). The graph bars showed the mean ± SD; $n = 7$ for all groups. $^*P < 0.05$, $^{**}P < 0.01$. *nd* not detected, *ns* not significant. **a**, **b** *Control* non-CCl_4-injured and olive oil-treated mouse liver, *CCl_4-Sham* CCl_4-injured and sham-operated mouse liver, *CCl_4-Sph-TxL* CCl_4-injured and SHED-HLC-spheroid-transplanted (liver left lateral lobe), *CCl_4-Sph-Non-TxL* CCl_4-injured and SHED-HLC-spheroid non-transplanted liver (median lobe). **c**–**e** *Control* non-CCl_4-injured and olive oil-treated group, *CCl_4-Sham* CCl_4-injured sham-operated group, *CCl_4-Sph* CCl_4-injured SHED-HLC-spheroid-transplanted group

SHED-HLCs might be safer option; however, but no studies on direct transplantation in the damaged liver have been reported. In the present study, we performed intrahepatic transplantation of SHED-HLC-spheroids for the first time, and showed the significant therapeutic efficacy in a mouse model of chronic liver fibrosis. Recent studies have demonstrated that 3D culture systems promoted the differentiation of MSCs and contribute to the therapeutic potential [8, 12, 17, 18]. These findings suggest that the present study might have established functional SHED-HLC-based spherical micro-hepatic tissues with therapeutic potential.

Given the present transplant experiment, the intrahepatically implanted SHED-HLC-based spheroid-migrated functional human hepatocyte-like cells were contained widely not only in the directly transplanted lobe, but also in the other non-transplanted lobes of the mouse liver. Recent studies have shown that the stimulation of inflammatory cytokines, including transforming growth factor β (TGF β)

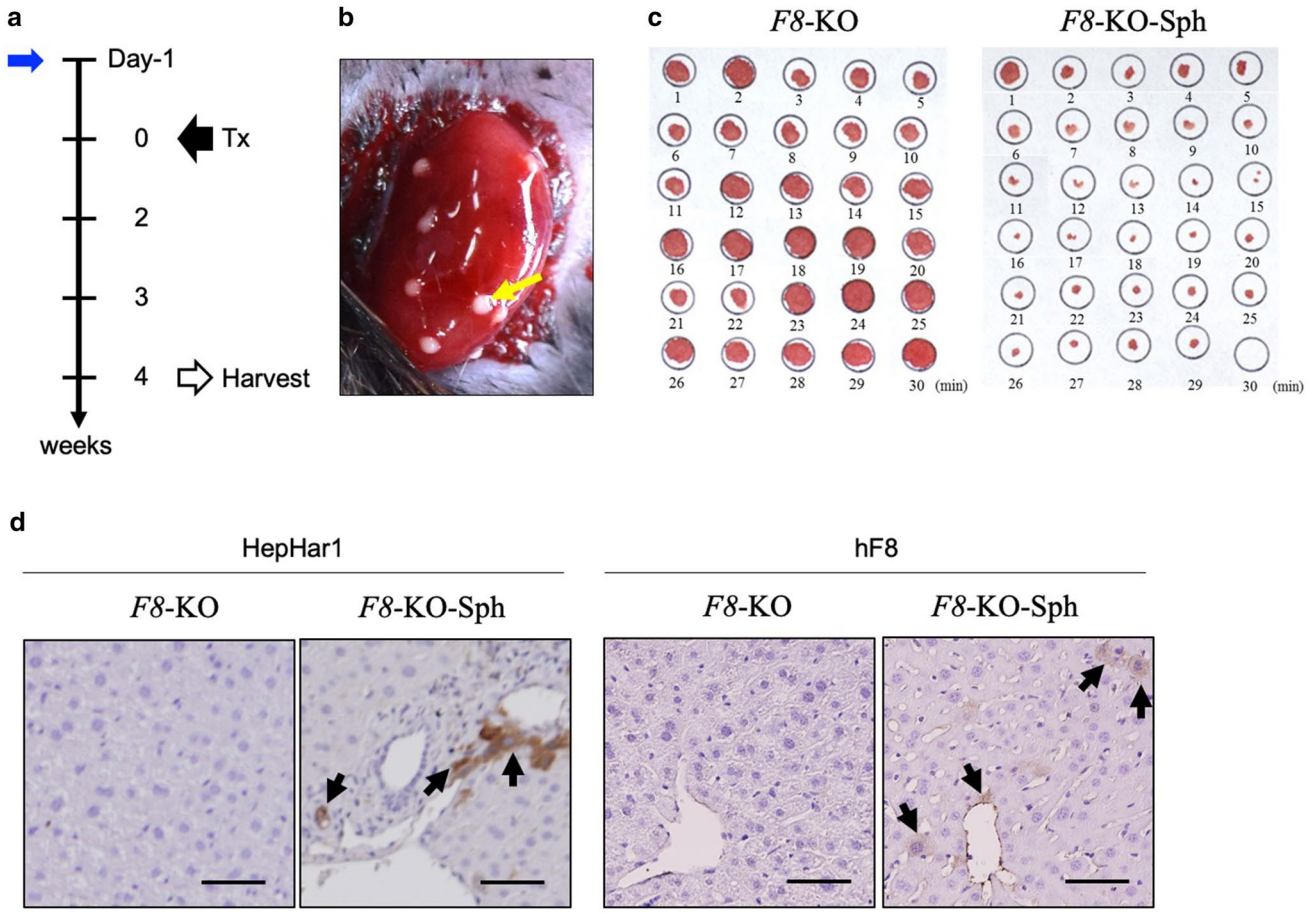

Fig. 4 Therapeutic analyses to investigate the anti-fibrotic efficacy of intrahepatic transplantation of SHED-HLC-spheroids in a mouse model of CCl_4-induced chronic liver fibrosis. **a** A schematic illustration of transplant treatment with spherical human micro-hepatic tissues using SHED-converted hepatocyte-like cells (SHED-HLCs) for a mouse model of hemophilia A. Renal capsule transplantation of SHED-HLC-spheroids (0.1×10^6 cells per spheroid [Tx], a total 10 spheroids per mice) was performed in *F8* knock-out mice, which received recombinant hF8 Fc (0.1 IU/g) 1 day before transplantation (Day-1; blue arrow). The mice were harvested at 4 weeks after transplantation (white arrow). **b** A representative kidney image shows the intrahepatic transplantation of SHED-HLC-spheroids into the left lateral lobe of the mouse liver. Yellow arrow indicates an implanted SHED-HLC-spheroid. **c** Bleeding maps determined by a tail-clipping test in mice. A clipped tail was stamped every 1 min on a filter paper. **d** Localization of the donor cells in the mouse liver tissue. The immunohistochemical analysis of mouse liver tissues of the control and SHED-HLC-spheroid-transplanted *F8*-knock-out mice. Representative microscopic images with immunostaining of HepPar and hF8. The nuclei are counterstained with hematoxylin. Arrows, HepPar- and hF8-positive cells. Scale bars 30 µm. **c**, **d** *F8-KO* non-transplanted *F8*-knock-out group, *F8-KO-Sph* SHED-HLC-spheroid-transplanted *F8*-knock-out group

and tumor necrosis factor α (TNF α) enhances cell migration and the trans-endothelial migration of hepatocytes and hepatocellular carcinoma cells [19–21]. The present SHED-HLC-spheroids are only constructed by a moderate cell–cell integration, because of the scaffold-free and machinery-free manufacturing process. These findings suggest that TGF β and TNF α were overexpressed in the inflammatory conditions in the liver of mice with CCl_4-induced chronic liver fibrosis [7], which might stimulate donor SHED-HLCs released from the locally implanted spheroids to undergo intralobular migration and interlobular transportation via trans-endothelial migration to recruit again into the damaged liver.

Previous tissue engineering approaches using primary hepatocytes successfully improved the therapeutic effects in a mouse model of hemophilia A [22]. The present renal capsule transplantation study using SHED-HLC-spheroids showed significant anti-hemorrhagic efficacy in *F8*-knock-out mice, as indicated by the tail-cutting test, suggesting that SHED-HLC-spheroids might be a novel alternative option to the regular weekly prophylactic factor infusion. Unfortunately, hF8 was not detected in the plasma of the transplanted *F8* knock-out mice, even though SHED-HLC-spheroids expressed hF8. Meanwhile, hALB was detectable in the peripheral serum of CCl_4-treated mice with the intrahepatic transplantation of SHED-HLC-spheroids. The

in vivo discrepancy between the detection of plasma hF8 and hALB might have been due to the significantly low amount of hF8 produced by the transplanted SHED-HLC-spheroids. Further studies should be performed to examine the production and the release of hF8 in *F8*-knock-out mice after SHED-HLC-spheroid transplantation, to confirm the therapeutic efficacy of SHED-HLC-spheroids in hemophilia A.

The present spheroid culture system using SHED-HLCs may provide further novel strategies in stem cell-based tissue engineering and medicine. Recent 3D bioprinter technology has demonstrated scaffold-free, highly designable, and automatic fabrication using cell spheroids [23, 24]. Given the direct multi-differentiation potency of SHED into hepatic and endothelial lineage cells [8, 25], SHED-based spheroids are suggested to be a feasible functional 3D liver tissue-buds to fabricate scalable organ-like tissues using 3D bioprinters for treating human liver disorders [26].

Conclusion

The present findings indicated that 3D-spheroidal micro-human liver microtissues fabricated using SHED-HLCs improved liver dysfunction and potentially life-threatening bleeding in mouse models of chronic liver fibrosis and hemophilia A, respectively, suggesting that 3D-spheroidal micro-human liver microtissues could be a feasible and novel alternative option in the treatment of refractory pediatric diseases, such as biliary atresia and hemophilia.

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Compliance with ethical standards

Conflict of interest All the authors declare no competing financial interests.

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