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The Efficacy of the Hepatocyte Spheroids for Hepatocyte Transplantation

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Abstract

The safety and short-term efficacy of hepatocyte transplantation (HCTx) have been widely proven. However, issues such as reduced viability and/or function of hepatocytes, insufficient engraftment, and lack of a long-term effect have to be overcome for widespread application of HCTx. In this study, we evaluated hepatocyte spheroids (HSs), formed by self-aggregation of hepatocytes, as an alternative to hepatocytes in single-cell suspension. Hepatocytes were isolated from C57BL/6 J mice liver using a three-step collagenase perfusion technique and HSs were formed by the hanging drop method. After the spheroids formation, the HSs showed significantly higher mRNA expression of albumin, ornithine transcarbamylase, glucose-6-phosphate, alpha-1-antitrypsin, low density lipoprotein receptor, coagulation factors, and apolipoprotein E (ApoE) than 2 dimensional (2D)-cultured hepatocytes ($p < 0.05$). Albumin production by HSs was significantly higher than that by 2D-cultured hepatocytes (9.5 $\pm$ 2.5 vs 3.5 $\pm$ 1.8 μg/dL, $p < 0.05$). The HSs, but not single hepatocytes, maintained viability and albumin mRNA expression in suspension (92.0 $\pm$ 2.8% and 1.03 $\pm$ 0.09 at 6 h). HSs (3.6×10^6 cells) or isolated hepatocytes (fSH, 3.6×10^6 cells) were transplanted into the liver of ApoE knockout (KO-/-) mice via the portal vein. Following transplantation, serum ApoE concentration (ng/mL) of HS-transplanted mice (1w: 63.1 $\pm$ 56.7, 4w: 17.0 $\pm$ 10.9) was higher than that of fSH-transplanted mice (1 w: 33.4 $\pm$ 13.0, 4w: 13.7 $\pm$ 9.6). In both groups, the mRNA levels of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, MCP-1, and MIP-1β) were upregulated in the liver following transplantation; however, no significant differences were observed. Pathologically, transplanted HSs were observed as flat cell clusters in contact with the portal vein wall on day 7. Additionally, ApoE positive cells were observed in the liver parenchyma distant from the portal vein on day 28. Our results indicate that HS is a promising alternative to single hepatocytes and can be applied for HCTx.

Keywords

hepatocyte spheroids, apolipoprotein E knockout mouse, hepatocyte transplantation

Introduction

Hepatocyte transplantation (HCTx) is a promising minimally invasive treatment for patients with acute liver failure or metabolic liver diseases, especially for those who are intolerable of whole liver transplantation. The basic techniques for hepatocyte isolation and transplantation, which involve injection of the isolated single hepatocytes into the liver via the portal vein, have been well established[1]. In addition, the clinical safety and short-term efficacy of HCTx have been widely proven[2,3]. However, issues such as reduction in the viability and/or functions of isolated hepatocytes, insufficient cell engraftment[4], and lack of a long-term effect[5] have to be addressed to enable wider clinical application of HCTx. Hence, a novel and robust strategy that can maintain the viability and function of isolated hepatocytes, and prevent graft damage following transplantation is needed for future use of primary and/or stem cell derived hepatocytes for HCTx.

To isolate hepatocytes from the liver, a standardized three-step collagenase perfusion technique, originally developed by Berry and Friend, has been widely used[6]. However, during the isolation process, hepatocytes are separated from

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each other and are thus deprived of their original environment. This disruption of cell-to-cell and cell-to-matrix contact leads to subsequent hepatocyte dysfunction. Smets et al. showed that the loss of cell anchorage induced apoptosis in primary hepatocytes, which was detected as early as 15 min after the development of detached culture conditions[7]. To maintain specific functions in a culture system, co-culturing the hepatocytes with an extracellular matrix component, such as type IV collagen, perlecan, nidogens, and Matrigel, could be a promising approach[8]. Indeed, primary human hepatocytes cultured on human recombinant laminins showed similar liver-specific functions when compared with those cultured on collagen[9]. However, owing to the diversity of biological material, these strategies would be difficult to apply to clinical HCTx protocols.

The innate immune reaction and inflammation play critical roles after HCTx. It has been reported that up to 70% of transplanted hepatocytes are eliminated by early phagocytic immune response[10]. When cells or tissues are transplanted into the portal vein and come into contact with blood, platelet aggregation and activation of coagulation/complement cascades are immediately induced[11]. In a study by Gustafson et al., a rapid loss of platelets, extensive generation of thrombin-antithrombin complex, and concomitant increase in C3a were observed in a heparinized loop system, in which freshly isolated hepatocytes were exposed to human blood[12]. These reactions can in turn lead to inflammatory responses driven by mediators such as monocyte chemoattractant protein (MCP)-1, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor-necrosis factor-α (TNF-α), and interferon gamma (IFN-γ)[13,14], and subsequently result in the activation of neutrophils and macrophages. Furthermore, these proinflammatory cytokines can directly injure the transplanted cells[15,16]. Thus, it is important to develop a method that can enable engraftment of a higher number of hepatocytes. Transplanting hepatocytes at an extrahepatic site is an attractive strategy because it would not only avoid contact with blood, but also provide additional space to maintain a higher number of functioning cells. Researchers have transplanted hepatocytes at several different extrahepatic sites, including into the omentum[17], mesentery[18], under the kidney capsule[19], and into the subcutaneous space[20]. However, regardless of the transplantation site used, studies have reported inefficient engraftment and short-term survival when single hepatocytes were transplanted.

Spheroidal cells can be formed under special culture conditions, such as under extremely low-adhesion environment[21], micro molding technique[22], rotary culture[23], or hanging drop method[24]. Spheroidal cells help in maintaining cell-to-cell contact and intercellular signals[25], enhancing gene expression associated with apoptosis, survival, and angiogenesis[26]. Taking advantage of these characteristics, spheroidal cells have been used in drug metabolism studies, cancer research[27], and tissue engineering[28]. Hepatocyte spheroids (HSs) were first reported by Landry et al.[29] and were formed by self-aggregation of a single hepatocyte in rats. It has been reported that HSs can be transplanted technically in animal models[30,31]. The structure of HSs closely resembles the original liver tissue environment and it has been demonstrated that HSs can maintain liver-specific functions. The viability and albumin production of HSs were found to be better than normal cultured singe hepatocytes[32]. In addition, the spheroidal structure of HSs protects the hepatocytes from apoptosis[33]. These characteristics suggest that HSs can reduce cell damage due to cell isolation and may improve engraftment and/or survival after HCTx.

In this study, we examined a hypothesis that HSs could tolerate cell damage and inflammatory responses following HCTx, and thus, prevent subsequent loss of transplanted cells. We assessed the feasibility of intraportal transplantation of HSs as an alternative cell form to hepatocytes in single-cell suspension, using a mouse HCTx model.

Materials and Methods

Animals

Male apolipoprotein E-deficient mice (apo E -/-; B6.129P2-Apoetm1 Unc N11) and C57BL/6 J mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA) and Japan SLC, Inc. (Shizuoka, Japan), respectively. The mice were maintained in a specific pathogen-free facility and were used for experiments at 9 to 14 weeks of age. All experiments were approved by the Institutional Animal Care Committee. The study was approved by the local ethics committee and conducted according to the guidelines for the Care and Use of Laboratory Animal of Hokkaido University (approved number: 17-0032).

Hepatocyte Isolation

Hepatocytes were isolated using a three-step collagenase perfusion technique, as described previously[34]. Briefly, the abdomen of the mouse was opened through upper abdominal incision and the liver was perfused sequentially by the following solutions at a rate of 10 mL/min: Hank's balanced salt solution (HBSS, Thermo Fisher Scientific, Waltham, MA, USA) without calcium or magnesium, containing 0.5 mmol/L methylene glycol tetraacetic acid and 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Thermo Fisher Scientific), HBSS with 25 mmol/L HEPES, and William's medium E (WME, Sigma-Aldrich Co. LLC, St. Louis, MO, USA), containing 0.4 mg/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ, USA). All perfusion solutions were preheated and maintained at 38 °C. Digested liver tissue was mechanically disrupted in cold WME. Hepatocytes were pelleted by centrifugation at 50× *g* for 2 min at 4 °C. The final hepatocyte pellet was re-suspended in WME, and the cell count and viability were assessed by standard Trypan blue (Sigma-Aldrich) exclusion test. Hepatocytes with greater than 70% viability were resuspended (3.0×10^5 cells/mL) in perfect medium (WME supplemented with

100 nM dexamethasone, 2 mM glutamine, 5 μg/mL insulin, 20 mM HEPES, 0.1 mM gentamicin, and 10% fetal bovine serum [FBS]).

Formation of Hepatocyte Spheroids

Hepatocyte spheroids were formed by hanging drop method as previously described[35]. Isolated hepatocytes from C57BL/6 J mice were seeded on multiple pore plate (MPc 350_6, Kuraray, Co., Ltd., Tokyo, Japan) at a density of 3.0 $\times$ 10^5 cells/mL in WME solution (supplemented with 100 nM dexamethasone, 2 mM glutamine, 5 μg/mL insulin, 20 mM HEPES, 0.1 mM gentamicin, and 10% FBS) at 37°C in a humidified atmosphere with 5% CO_2. Isolated hepatocytes were also cultured on 6-well plates (Corning, New York, NY, USA), pre-coated with collagen (0.3 mg/mL, Cellmatrix type I-C, Nitta Geratin Inc., Osaka, Japan), at a density of 3.0 $\times$ 10^5 cells/mL. Seventy-two hours after seeding, single or hepatocyte spheroids were collected and the supernatants of the culture media were used for further analysis.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium (MTS) Assay

Twenty microliters of MTS solution (CellTiter 96® AQueous One Solution, Promega Corporation, Madison, WI, USA) was added to each well of the 96-well assay plate, containing hepatocytes in 100 μL of culture medium (at a final concentration of 3.0 $\times$ 10^5 cells/mL), at 0, 3, and 6 h after the isolation of hepatocytes. After incubation at 37°C for 1 h in a humidified atmosphere with 5% CO_2, the absorbance was measured at 490 nm using a 96-well plate reader (BZ-9000, KEYENCE, Osaka, Japan). All measurements were performed in triplicate.

Live Cell Assay

One mM Calcein-AM (Invitrogen, Carlsbad, CA, USA) solution with dimethyl sulfoxide was diluted to 10 μM Calcein-AM solution with phosphate buffered saline (PBS). Calcein-AM solution with 1/10th the volume of the cell culture medium was added to the hepatocyte culture and incubated for 15 min at 37 °C. After washing with PBS, the cells were observed using a fluorescence microscope at an excitation wavelength of 490 nm and emission wavelength of 515 nm (BZ-9000, KEYENCE).

Measurement of Albumin and Urea Concentration

The concentrations of albumin and urea were measured using Serotec TIA-ALBG (Serotec Co., Ltd., Sapporo, Japan) and Serotec UN-L (Serotec Co., Ltd.), respectively, and a Hitachi 7020 automatic biochemical analyzer (Hitachi, Tokyo, Japan).

Hepatocyte Transplantation

The isolated hepatocytes (3.6 $\times$ 10^6 cells) or HSs (3.6 $\times$ 10^6 cells in total) were transplanted into the liver of apolipoprotein E KO-/- mice via the portal vein. In some cases, hepatocytes or HSs were transplanted under the kidney capsule or under the skin. Following transplantation, blood samples were collected from the tail vein under isoflurane anesthesia, and serum were preserved at −80 °C until use. Liver samples were obtained from the recipient animals and used for further analysis.

Enzyme-linked Immunosorbent Assay

ApoE concentrations were analyzed by the sandwich enzyme-linked immunosorbent assay (ELISA) using Mouse Apolipoprotein E ELISA Kit (ab215086, abcam, Cambridge, UK). Serum samples were diluted 1:30 in attached Sample Diluent NS. Next, 50 μL of the diluted sample was added to each well, containing 50 μL of antibody cocktail. The plates were sealed and incubated for 1 h at room temperature on a plate shaker at 400 rpm. The plates were then washed three times with Wash buffer. After the last wash, any traces of liquid were removed completely and 100 μL of TMB substrate was added to each well. After incubation at room temperature on a plate shaker at 400 rpm for 10 min in the dark, 100 μL of Stop solution was added to each well and shaking continued for 1 min to allow mixing. Finally, the optical density of the samples was recorded at 450 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific). A standard curve was constructed for each plate, using serial dilutions of C57BL/6 J serum with apolipoprotein E knockout mouse serum.

Real Time PCR

Total RNA was extracted from tissue samples using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 1 kg total RNA using the Omniscript RT Kit (Qiagen) with an oligo(dT)-20 primer (TOYOBO, Osaka, Japan). For relative quantification by PCR, each cDNA product was analyzed in a LightCycler (version 1.4), using a QuantiTect SYBR Green PCR Kit (Qiagen) along with 0.5 KM of specific primers (Invitrogen) in a 20 KL reaction volume. The PCR parameters were as follows: 94 °C for 15 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. The following mRNA primers, targeting specific mRNA, were used: albumin (Alb), ornithine transcarbamylase (Otc), coagulation factor 7 (F7), coagulation factor 9 (F9), glucose-6-phosphate (G6P), apolipoprotein E (Apoe), alpha-1-antitrypsin (A1AT), IL-1β, IL-6, TNF-α, macrophage inflammatory protein 1β (MIP-1β), and MCP-1 (Table. 1). The relative levels of the target gene mRNAs were normalized to the expression level of GAPDH and to the corresponding mRNA levels in native livers.

Table 1. The primers targeting specific mRNAs used for mRNA quantification.

Gene	Forward	Reverse
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG
Albumin	AAGCCACTCTGGAAAAGTGC	TGGAATCCATATTCTCCAAGC
Ornithine transcarbamylase	AGGGTCACACTTCTGTGGTTC	CAGAGAGCCATAGCATGTACTG
Glucose 6 phosphate	CTGAGCGCGGGCATCATAAT	GATTCTTAGGATCGCCCAGAAAG
Alpha1antitrypsyn	AGGAGGCTCTTCTCCACCTC	TGCCCAGAAATAGGAGCATC
Low density lipoprotein receptor	GAATCTACTGGTCCGACCTGTC	CTGTCCAGTAGATGTTGCGGTG
Coagulation factor VII	GGAACAGTGCTCCTTTGAGGAG	GACTTGAGATGATCCTGGCAGG
Coagulation factor IX	TGTAACTGCTGCCCACTGTC	TGCATTGTACTGGTGATGAGG
Apolipoprotein E	CTCCCAAGTCACACAAGAACTG	CCAGCTCCTTTTTGTAAGCCTTT
IL-1β	GGATGAGGACATGAGCACCT	AGCTCATATGGGTCCGACAG
IL-6	CAAAGCCAGAGTCCTTCAGAG	GCCACTCCTTCTGTGACTCC
TNF-α	ACCCTCACACTCAGATCATC	GAGTAGACAAGGTACAACCC
MCP-1	TCCCAATGAGTAGGCTGGAG	TCTGGACCCATTCCTTCTTG
MIP-1β	CCCACTTCCTGCTGTTTCTC	GTCTGCCTCTTTTGGTCAGG

Pathological Study

HSs or the recipient liver samples were stained with hematoxylin and eosin (H&E), anti-albumin antibody (ab192603, abcam), anti-apolipoprotein E antibody (ab183597, abcam), and anti-cytokeratin 18 (ab181597, abcam). Elastica-Masson staining was performed with Maeda modified resorcin-fuchsin solution (Muto Pure Chemical Co., Ltd., Tokyo, Japan), 0.75% orange G solution (Muto Pure Chemical Co., Ltd.), Masson B stain solution (Muto Pure Chemical Co., Ltd.), phosphotungstic acid (FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan) and aniline blue liquid (FUJIFILM Wako Pure Chemical Co., Ltd.).

Statistical Analysis

Quantitative results are presented as mean values ± standard deviation (SD). Comparison of two groups was performed using the Mann-Whitney U test. In case of three groups, Kruskal-Wallis tests with Dunn's multiple comparison test were used. A p value < 0.05 was considered statistically significant and all calculations were performed using GraphPad Prism® software version 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

The viability and Function of Isolated Hepatocytes Decreases Rapidly in Suspension

For clinical hepatocyte transplantation, good manufacturing practice of hepatocytes that complies with the regulations is needed. However, this requires highly specialized facilities and techniques, which are not always available. Therefore, in some cases, the isolated hepatocytes need to be transported to the recipient patients after isolation. In this study, we first evaluated the functions of freshly isolated single hepatocyte (fSH) and changes over time, with respect to viability and function in suspension. The albumin mRNA expression level of fSH was greater than that of 3-day cultured single hepatocytes (cSH) ($p < 0.001$, $n = 6$; Fig. 1A). However, the viability of fSH in suspension decreased rapidly to 58.4 ± 4.3% (3 h after isolation) and 40.1 ± 17.0% (6 h after isolation), when compared to those at just after isolation, respectively. Likewise, the relative albumin mRNA expression level of fSH decreased to 0.69 ± 0.06 (3 h after isolation) and 0.52 ± 0.09 (6 h after isolation), respectively ($n = 3$, Fig. 1B and 1C).

Hepatocyte Spheroids Formed by Hanging Drop Method Retain Morphology After Collection

After three days of culturing single hepatocytes, approximately 200 μm of HSs were formed using the hanging drop method; these could be collected easily (Fig. 2A). The HSs retained morphology even after collection and were positive for cytokeratin 18 staining (Fig. 2B).

The Viability of the Hepatocyte Spheroids was Maintained During the Three Days of Culture

The viability of HSs was evaluated after the collection and compared with those of fSH and cSH. The positive Calcein red orange staining of the spheroids were better than that of cSH (Fig. 3).

The Functions of Hepatocyte Spheroids Were Better than Those of CSH

The functions of HSs, formed by culturing for three days, were evaluated and compared with those of isolated hepatocytes cultured for three days (cSH) or fSH. To investigate the liver specific function, the mRNA expression levels of Alb, Otc, G6p, A1AT, low density lipoprotein receptor (ldlr), F7, F9, and ApoE were evaluated. The mRNA expression levels of HSs were lower than those of fSH. However, the mRNA expression levels of Alb, Otc, A1AT, ldlr, F7, F9,

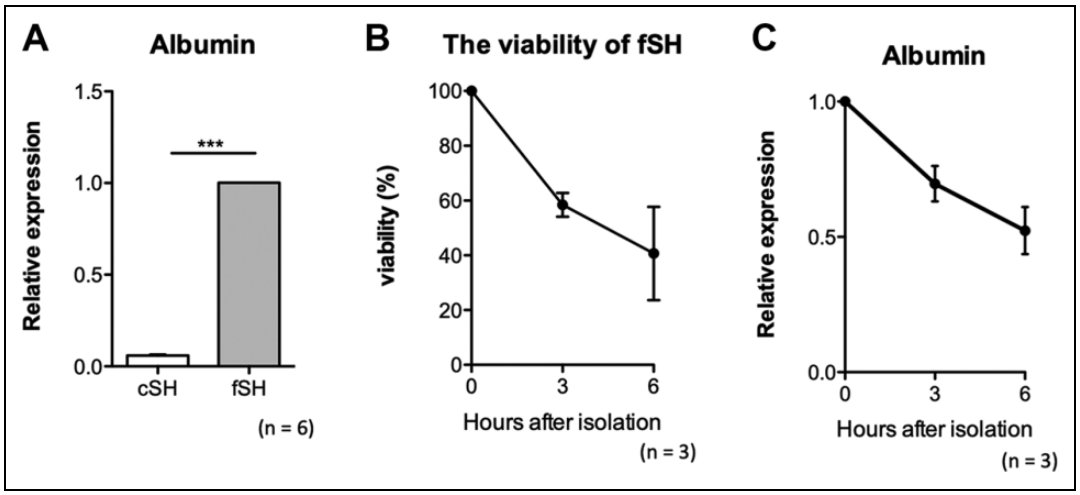

Figure 1. The viability and function of isolated hepatocytes decreases rapidly in suspension. (A) The relative albumin mRNA expression of isolated fresh hepatocyte (fSH, grey box) was higher than that of 3-day cultured single hepatocytes (cSH, white box) ($n = 6$, $^{***}p < 0.001$). (B) In suspension, the viability of fSH decreased rapidly to 58.4 $\pm$ 4.3% (3 h after isolation) and 40.1 $\pm$ 17.0% (6 h after isolation), and the relative albumin mRNA expression of fSH decreased to 0.69 $\pm$ 0.06 (3 h after isolation) and 0.52 $\pm$ 0.09 (6 h after isolation) when compared to those at just after isolation, respectively ($n = 3$).

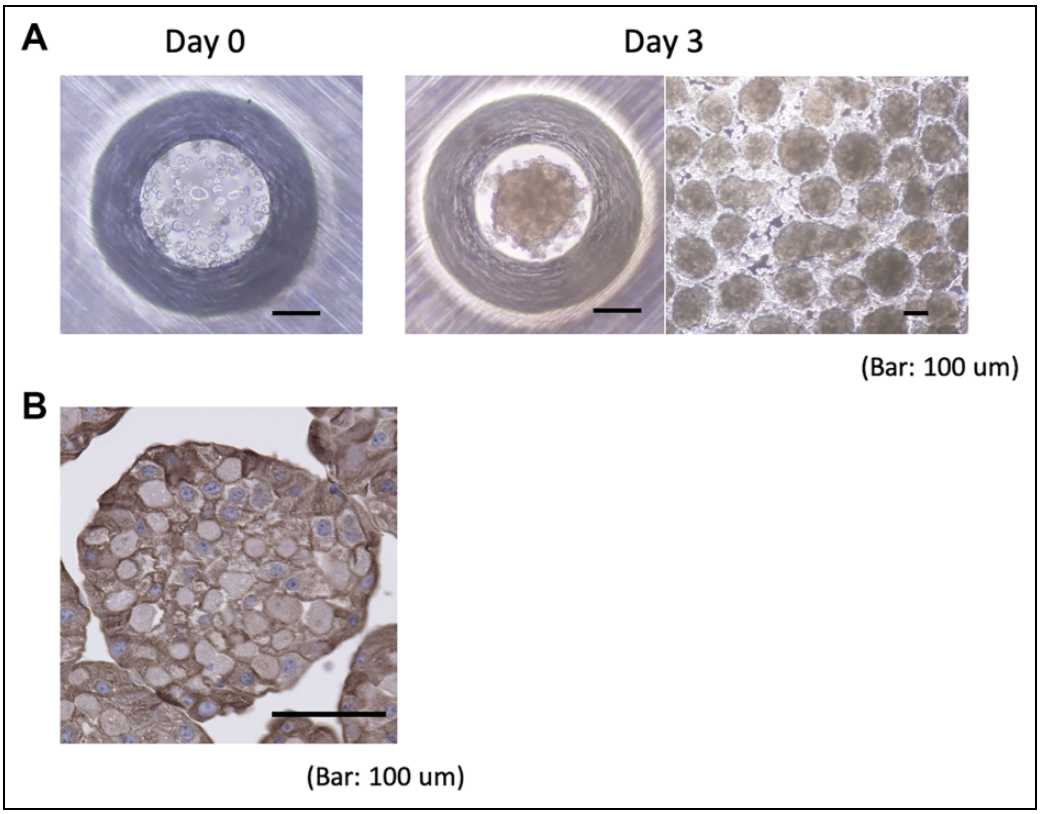

Figure 2. Hepatocyte spheroids formed by hanging drop method retain morphology after collection. (A) After three days of culturing of single hepatocytes by the hanging drop method, approximately 200 μm of hepatocyte spheroids (HSs) were formed. (B) HSs retained morphology even after collection and were positive for cytokeratin-18 staining.

ApoE of HSs were significantly higher than those of cSH ($p < 0.05$, $n = 6$, Fig. 4A). Pathologically, HSs were positive for albumin and ApoE immunostaining (Fig. 4B). The concentration of albumin, urea, and ApoE in the supernatant were also measured after three days of culturing. The albumin and urea concentrations of HS (9.5 $\pm$ 2.5 μg/

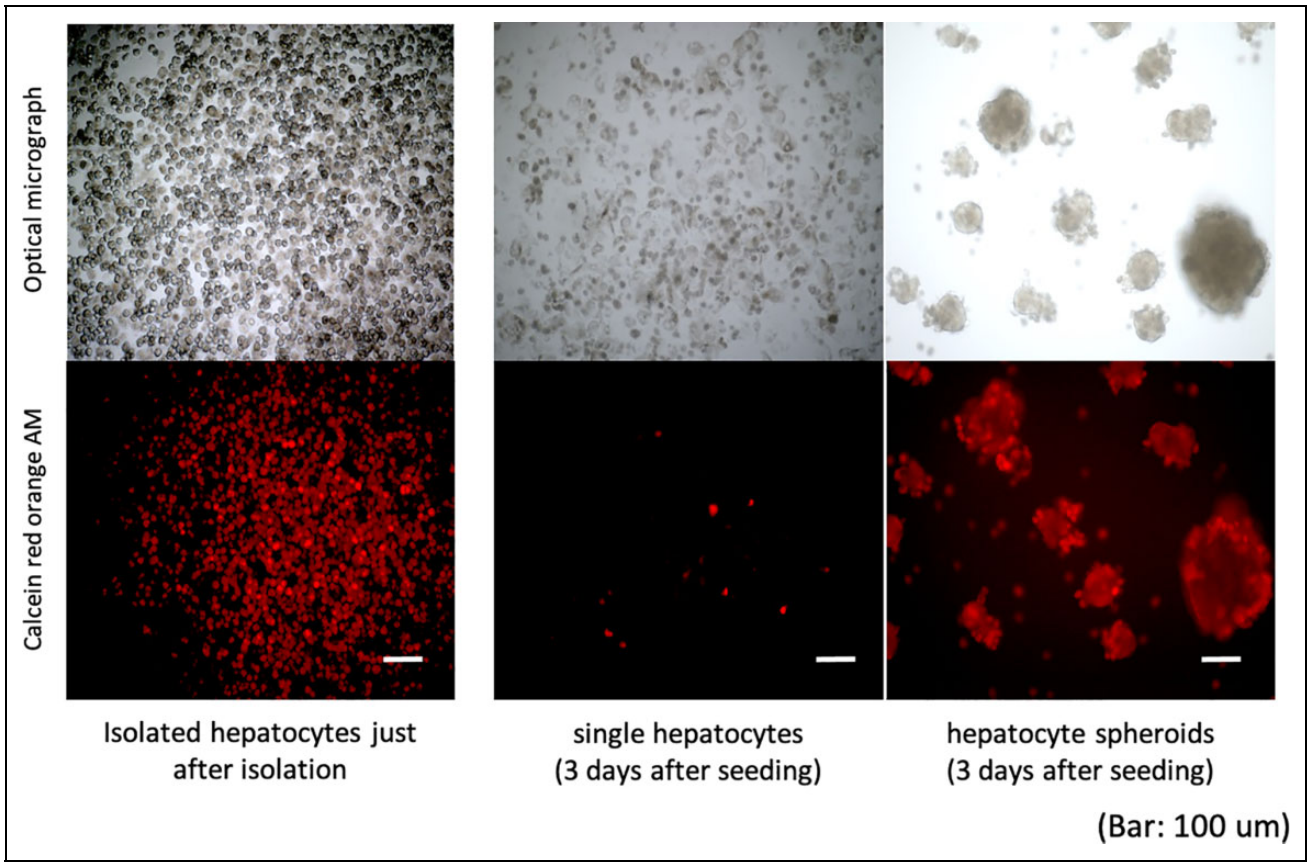

Figure 3. The viability of the hepatocyte spheroids was maintained during culturing for three days. HSs were positive for Calcein red orange staining, and the viability of HSs was superior to cSH.

dL and 6.9 ± 1.2 μg/dL) were significantly higher than those of cSH (3.5 ± 1.8 μg/dL, $p = 0.006$, and 5.9 ± 0.4 μg/dL, $p = 0.022$). The level of ApoE in the culture medium of HS (1.89 ± 1.09 μg/mL) was higher than that in the culture medium of cSH (1.60 ± 0.99 μg/mL, $p = 0.455$) (Fig. 4C).

Hepatocyte Spheroids Retain Viability and Function even in Suspension

The viability and function of HSs in suspension were evaluated over time. In contrast to isolated single hepatocytes, the viability of HSs was maintained even in suspension; 96.3 ± 1.8% (3 h after collection) and 92.0 ± 2.8% (6 h after collection). Likewise, the relative albumin mRNA expression of HS was maintained at 0.91 ± 0.05 (3 h after collection), 1.03 ± 0.09 (6 h after collection) when compared to those at just after collection, respectively (Fig. 5A, B).

Hepatocyte Spheroids Can Be Transplanted Into the Liver Via the Portal Vein and Transplanted Cells Are Observed Pathologically

HSs or isolated single hepatocyte (fSHs) were transplanted into the ApoE KO mouse liver via the portal vein. The liver was obtained from recipient animals and transplanted cells or HSs were investigated pathologically. No recipient animals died prematurely. ApoE immunostaining was performed at 4, 7, and 28 days after transplantation. In the fSHs transplanted animals, ApoE positive cells were scattered in the liver parenchyma and were observed on the 4th day after fSH transplantation. These cells were also observed at 28th day after transplantation (Fig. 6A–C). In contrast, after transplantation of HSs, transplanted HSs were observed as cell clusters in the portal vein on the 4th day after transplantation (Fig. 6D). Seven days after transplantation, transplanted HSs were observed as flat cell clusters in contact with the portal vein wall. ApoE positive cells were found in the liver parenchyma and this was also observed on the 28th day after transplantation (Fig. 6E, F). Immunohistochemistry analysis with Elastica-Masson staining indicated that ApoE positive cells in the portal vein were surrounded by endothelial cells on day 28. In addition, some of these ApoE positive cells were also observed in the liver parenchyma distant from the portal vein on day 28 (Fig. 6G: H.E., 6H: Elastica-Masson, 6I: ApoE).

Transplanted HSs Maintained Their Function (ApoE Production) Even After Transplantation

We evaluated the graft function (fSHs or HSs) before and after transplantation. After the hepatocyte isolation, fSH

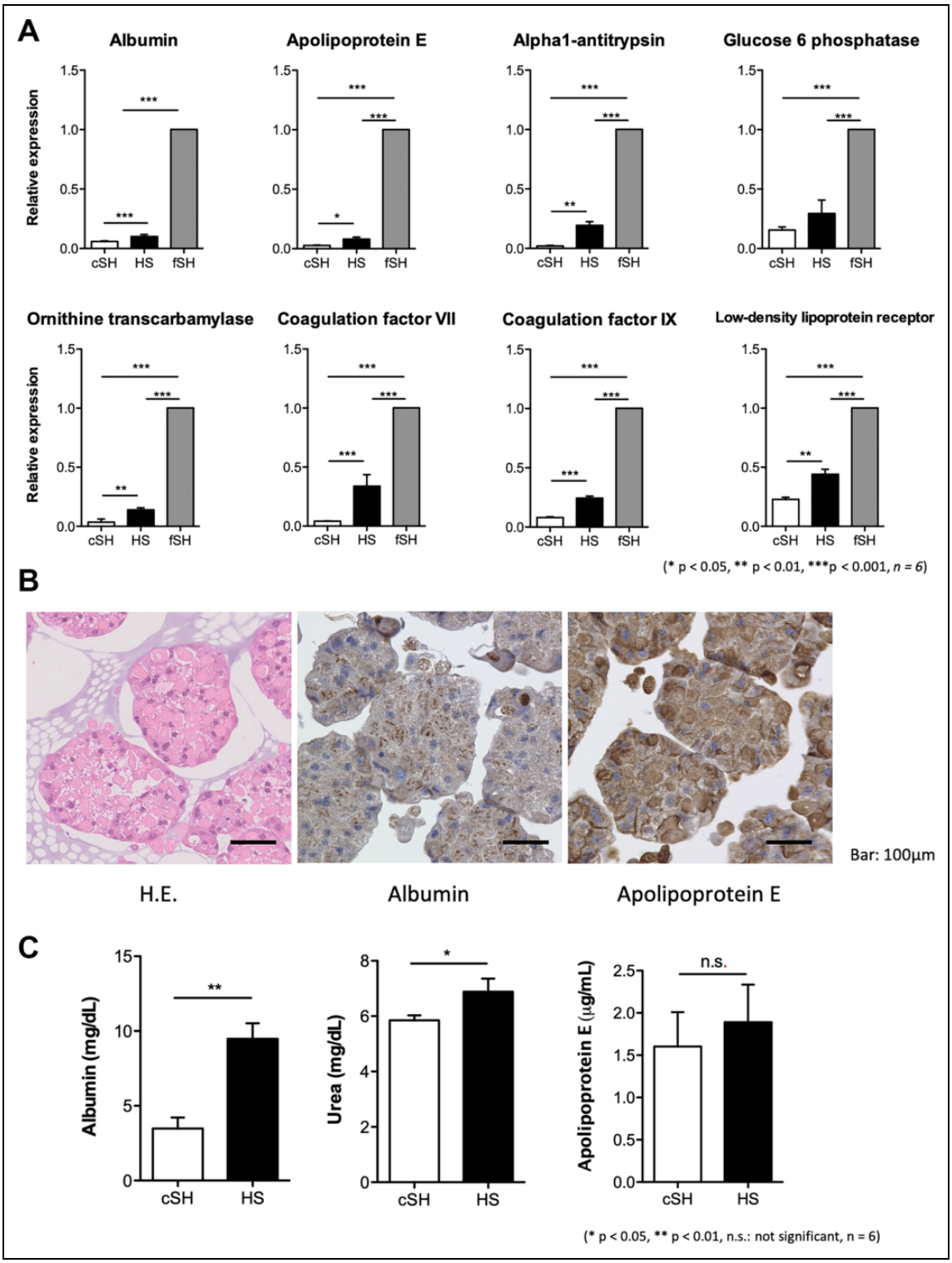

Figure 4. The liver specific functions of hepatocyte spheroids. (A) The relative gene expressions of liver functions of HS (black boxes) were lower than those of fresh isolated single hepatocyte (fSH: grey boxes). However, the relative gene expressions of HS were significantly higher than those of three days cultured single hepatocyte (cSH: white boxes) in *Alb* (1.91 $\pm$ 0.76, $p = 0.032$), *Otc* (16.5 $\pm$ 8.28, $p = 0.005$), *a l at* (8.97 $\pm$ 4.39, $p = 0.006$), *ldlr* (1.96 $\pm$ 0.41, $p = 0.002$), *F7* (6.20 $\pm$ 1.68, $p = 0.0006$), *F9* (3.14 $\pm$ 0.46, $p < 0.0001$), and *Apoe* (3.34 $\pm$ 1.81, $p = 0.025$). (B) Hepatocyte spheroids were positive for albumin and apolipoprotein E immunostaining (left: Hematoxylin and Eosin stain, center: albumin stain, right: apolipoprotein E stain). (C) The albumin and urea concentration in the culture supernatant of HS (9.5 $\pm$ 2.5 μg/dL, and 6.9 $\pm$ 1.2 μg/dL) were significantly higher than those of cSH (3.5 $\pm$ 1.8 μg/dL, $p = 0.006$, and 5.9 $\pm$ 0.4 μg/dL, $p = 0.022$, $n = 6$). Apolipoprotein E in the culture medium of HS was higher than cSH (1.89 $\pm$ 1.09 μg/mL, 1.60 $\pm$ 0.99 μg/mL, $p = 0.455$, $n = 6$).

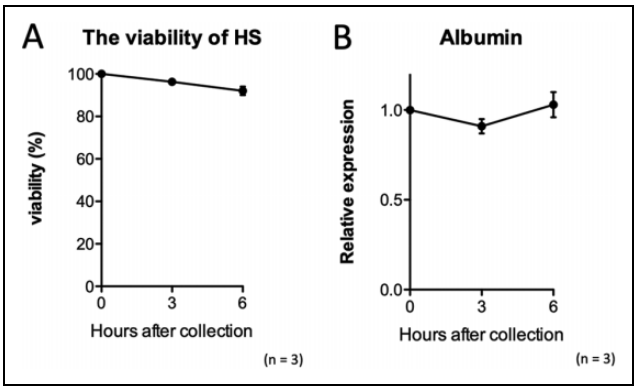

Figure 5. Hepatocyte spheroids retain viability and function even in suspension. (A) The viability of HSs was maintained at 96.3 ± 1.8% (3 h after collection) and 92 ± 2.8% (6 h after collection) of viability at just after collection. (B) Likewise, the relative albumin mRNA expression of HS was maintained at 0.91 ± 0.05 (3 h after collection), 1.03 ± 0.09 (6 h after collection) when compared to those at just after collection, respectively ($n = 3$).

showed significantly higher ApoE mRNA expression compared to collected HSs ($p < 0.001$, $n = 6$, Fig. 7A). Following transplantation, however, serum ApoE concentration of HSs-transplanted animals (1w: 63.1 ± 56.7 ng/mL, 2w: 24.1 ± 16.5 ng/mL, 4w: 17.0 ± 10.9 ng/mL) tended to be higher than that of fSH-transplanted animals (1w: 33.4 ± 13.0 ng/mL, 2 w: 13.1 ± 8.0 ng/mL, 4w: 13.7 ± 9.6 ng/mL, Fig. 7B), but were not statistically significant (1 w; $p = 0.192$, 2 w; $p = 0.151$, 4 w; $p = 0.267$).

Intrahepatic Inflammatory Reaction Following Transplantation

To investigate inflammatory responses at the transplantation site, liver samples were obtained from the recipient animals 6 h after the transplantation and mRNA expression levels of proinflammatory cytokines were assessed. Following transplantation, the mRNA levels of IL-6, IL-1β, TNF-α, MCP-1, and MIP-1β were upregulated in both HSs and fSHs-transplanted animals compared with mRNA levels in the normal liver. However, there were no significant differences between the HSs and fSHs-transplanted groups. (Fig. 8)

HSs Can Be Transplanted Under the Kidney Capsule or the Skin

To evaluate its clinical potential, HSs were transplanted under the kidney capsule or under the skin. Four weeks after the transplantation, transplanted HSs were observed under the kidney capsule and the skin, and were found to be positive for albumin or ApoE immunostaining (Fig. 9A1, 9A2, 9B1, and 9B2). Conversely, transplanted hepatocytes were not observed after fSH transplantation (Fig. 9A3, 9A4, 9B3, and 9B4).

Discussion

In this study, we investigated the efficacy of HSs as an alternative cell form to isolated primary hepatocyte, which is widely used for current clinical HCTx. We demonstrated that, in contrast to single hepatocytes, the viability and function of HSs were maintained even in suspension, and the liver specific functions were retained after intraportal HCTx. Further, pathological analysis detected functioning hepatocytes in the recipient mouse liver after one month of intraportal HCTx. Besides, functioning cells were observed in HSs-transplanted animals after under the kidney capsule or subcutaneous transplantation, but not in single hepatocytes transplanted animals. These results suggest that HSs are a promising cell form for HCTx and can be engrafted following intraportal, under the kidney capsule, or subcutaneous HCTx.

Along with the innovation of biotechnological approaches, spheroids are increasingly used as building tissue-blocks in tissue engineering because they can mimic the physiological three-dimensional environment[36,37]. They maintain wide and tight cell-to-cell contact and viability[38], a possess the ability to steadily different[39]. Therefore, in contrast to isolated or monolayer cultured cells, HSs have been reported to maintain viability and liver-specific functions[40]. In line with these reports, we demonstrated that the mRNA expression levels of Alb, Otc, G6P, A1AT, ldlr, F7, F9, and ApoE of HSs, and albumin production in HSs were significantly higher than those in cultured single hepatocytes. We also showed that HSs retained viability and albumin production even in suspension. Hepatocytes are the major parenchymal cells responsible for most liver-specific functions and the majority of circulating plasma proteins are expressed by hepatocytes[41]. Accordingly, HCTx offers an opportunity to treat patients with urea cycle disorder[3], A1AT deficiency, or hemophilia[42]. Because cell-signaling pathways are tightly coordinated with the surrounding ECM[43], some cell-signaling pathways, such as the RAS/MEK/ERK pathway, are activated when isolating hepatocytes, whereas others are silenced, for example, HNF-4α[44]. The enhancement of cell signaling due to the wide cell-to-cell contact in spheroids can result in an increased protein secretion. It has been shown that the culture of HepG2 cells in spheroids enhanced the nuclear protein content of PPAR-α, PPAR-γ, LXR-α, and RXR-α, and resulted in enhanced albumin and ApoE secretion[45]. The findings in this study that the high mRNA expressions of therapeutic proteins/enzymes, such as Otc, AIAT, and coagulation factors in HSs, indicate that HSs have the ability to make up for the missing proteins/enzymes, if engrafted.

The outcome of HCTx is strictly dependent on the engraftment of the transplanted hepatocytes into the liver of the recipient. After intraportal transplantation, the innate immune reaction, followed by platelet aggregation and coagulation/complement activation, plays a critical role on the early outcome[15,16]. This sequential reaction, also known as instant blood mediated inflammatory reaction (IBMIR), was

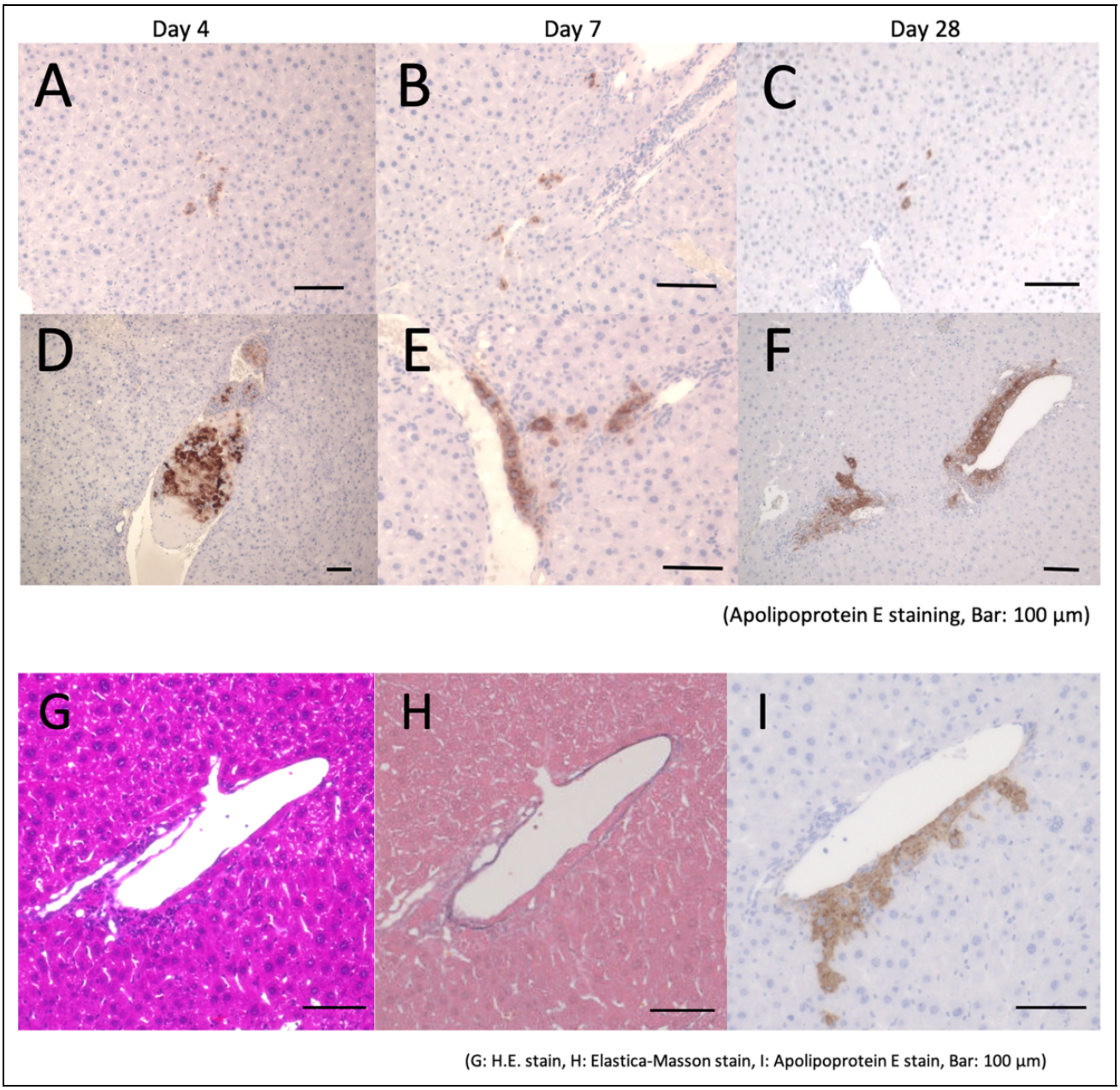

Figure 6. Hepatocyte spheroids can be transplanted into the liver via the portal vein and could maintain its ApoE production even after transplantation Following HSs or fSHs transplantation, the liver was obtained from ApoE KO recipient animals, and transplanted cells or HSs were investigated pathologically. ApoE positive cells were observed in the liver parenchyma after the fSHs transplantation (A, B, C: apolipoprotein E × 20). ApoE positive HSs were also observed in the portal vein 4 days after the transplantation (D: apolipoprotein E × 20). At 7 days after transplantation, the hepatocyte spheroids changed the form to flat shaped mass, and were also observed at 28 days after transplantation (E: apolipoprotein E × 40 and F: apolipoprotein E × 20). Some ApoE positive cells observed in the liver parenchyma. ApoE positive cells were observed in the portal vein and were surrounded by endothelial cells. Some of ApoE positive cells were observed in the liver parenchyma beyond the native portal endothelial cells (G: hematoxylin Eosin × 20, H: Elastica-Masson × 20, I: apolipoprotein E × 20).

originally defined in pancreatic islet transplantation[11] and is also observed following hepatocytes transplantation[12]. In some study, HSs were transplanted in rodent or pig model, and showed higher risks for thrombosis and increased portal pressures following intra-portal transplantation[30,31]. In this study, we assessed inflammatory responses after HS transplantation using a mouse syngeneic intraportal HCTx model. As is the case with pancreatic islet or isolated hepatocytes transplantation, we showed that the mRNA levels of the proinflammatory cytokines IL-6, IL-1β, TNF-α, MCP-1, and MIP-1β were upregulated in the HSs-transplanted recipient liver. Of particular note, although the diameter of

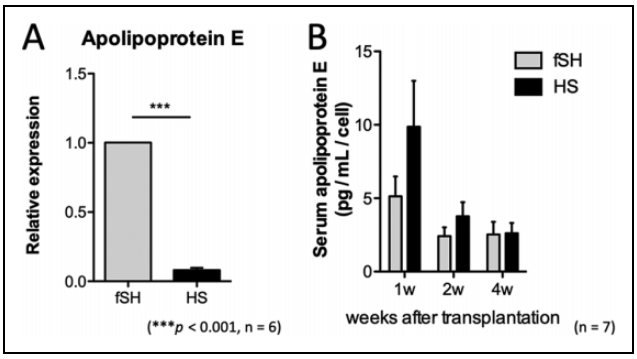

Figure 7. Transplanted HSs produced ApoE even after transplantation. (A) The relative ApoE mRNA expression level of fSH just after isolation (0.101 $\pm$ 0.04) was significantly higher than that of HSs just after collection. ($p < 0.001$, $n = 6$) (B) Following HSs or fSHs transplantation, ApoE was detected by ELISA, both in the serum of HSs (1 w; 63.1 $\pm$ 56.7, 2 w; 24.1 $\pm$ 16.5, and 4 w; 17.0 $\pm$ 10.9) and fSHs (1 w; 33.4 $\pm$ 13.0, 2 w; 13.1 $\pm$ 8.0, and 4 w; 13.7 $\pm$ 9.6) transplanted animals, and there was no significant difference between the groups (1 w; $p = 0.192$, 2 w; $p = 0.151$, 4 w; $p = 0.267$, respectively, $n = 6$, in each group).

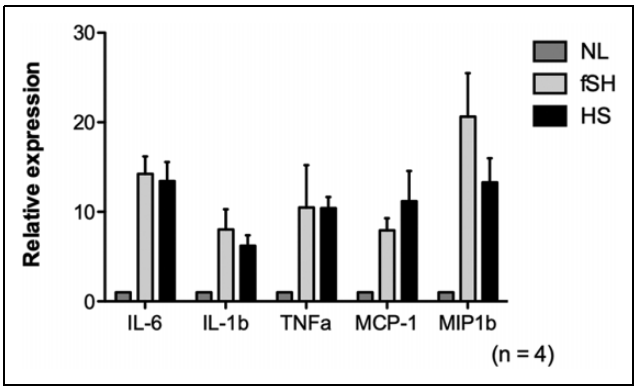

Figure 8. Intrahepatic inflammatory reaction following transplantation. The mRNA expression levels of proinflammatory cytokines (IL-6, IL-1β, TNF-α, MCP-1, MIP-1β) in the recipient liver at 6 h after HSs or fSHs transplantation were evaluated and compared with the normal liver (NL: charcoal boxes). There were no significant differences between fSH (grey boxes) and HS transplantation (black boxes) ($n = 4$, in all groups).

spheroids (around 200 μm) raised a concern that the intraportal transplantation might induce strong inflammatory reactions, there were no statistical difference in the upregulation of proinflammatory cytokines mRNAs between spheroids and single hepatocytes transplanted animals. A rapid loss of platelets and generation of thrombin-antithrombin complex due to the contact of hepatocytes with blood[12] indicate that a binding of activated platelets to the transplanted hepatocytes make clusters with blood clot into the portal system. The fact that hepatocytes infusions have to be extended to control the portal pressure indicates obstruction of portal blood flow. Apart from the effect of hepatocyte size, the occurrence of the IBMIR may explain the observation of no statistical differences in the upregulation of pro-inflammatory cytokines mRNAs between spheroids and single hepatocytes transplants in this study. Regarding the size of spheroids, the oxygen and nutrient demands in spheroids at the transplant site would be different from those of the single hepatocytes. HSs with a size greater than 200 μm resulted a critical O_2 concentration in the core[46]. Besides, pancreatic islet size has also generated interest as a potential factor for improving engraftment or functionality; some studies demonstrated superiority of smaller islets in clinical[47] or animal experiments[48]. While, some reports showed no relationship between islet size and clinical outcome[49]. We selected spheroids comprising around 1,400 hepatocytes due to technical reasons associated with animal transplantation. Further studies are needed to elucidate the mechanism of spheroidal cells engraftment and to find the optimal size of spheroids promoting early engraftment after HCTx.

After the injection of single hepatocytes into the portal vein, transplanted hepatocytes are entrapped in the sinusoids[50,51]. After escaping from the IBMIR, further engraftment requires migration across the endothelial cell barrier, with subsequent integration into the liver parenchyma[52]. In line with previous reports, we found that the transplanted single hepatocytes were observed in the liver parenchyma, but not in the portal vein, at 4 days after HCTx; these may be eliminated by host immune reaction from the portal vein as indicated[53]. On the other hand, transplanted HSs were observed in the portal vein at 24 h after HCTx. Following transplantation, the transplanted spheroids were still observed in the portal vein on day 4 and were found as flat cell clusters in contact with the portal vein wall at day 7. Of note, the ApoE positive cells were also found in the liver parenchyma on days 7 and 28. Interestingly, immunohistochemistry analysis with Elastica-Masson staining indicated that ApoE positive cells in the portal vein were surrounded by endothelial cells. Moreover, some ApoE positive cells were observed in the liver parenchyma distant from the portal vein on day 28. As reported in previous studies, the hepatic sinusoidal endothelium is a strong barrier to the entrance of transplanted cells from the sinusoidal space into the hepatic parenchyma[54]. Vasodilator has been used in an attempt to facilitate the migration of the transplanted hepatocytes into the liver sinusoids, keeping the entry and engraftment window open[51]. Disruption of the sinusoidal endothelium using cyclophosphamide[54] or hepatic irradiation[55] has also been reported to injure the endothelial cells, enhancing the initial engraftment of transplanted hepatocytes[55]. The tight and wide contact with the portal vein wall as flat cell clusters, which was observed following HSs transplantation on day 7, may increase porosity of sinusoids and contribute to further migration into the liver parenchyma. The spheroid structure itself, consisting of thousands or millions of cells, may explain its role in engraftment, even in extrahepatic transplant site (under the kidney capsule or subcutaneously), as demonstrated in this study.

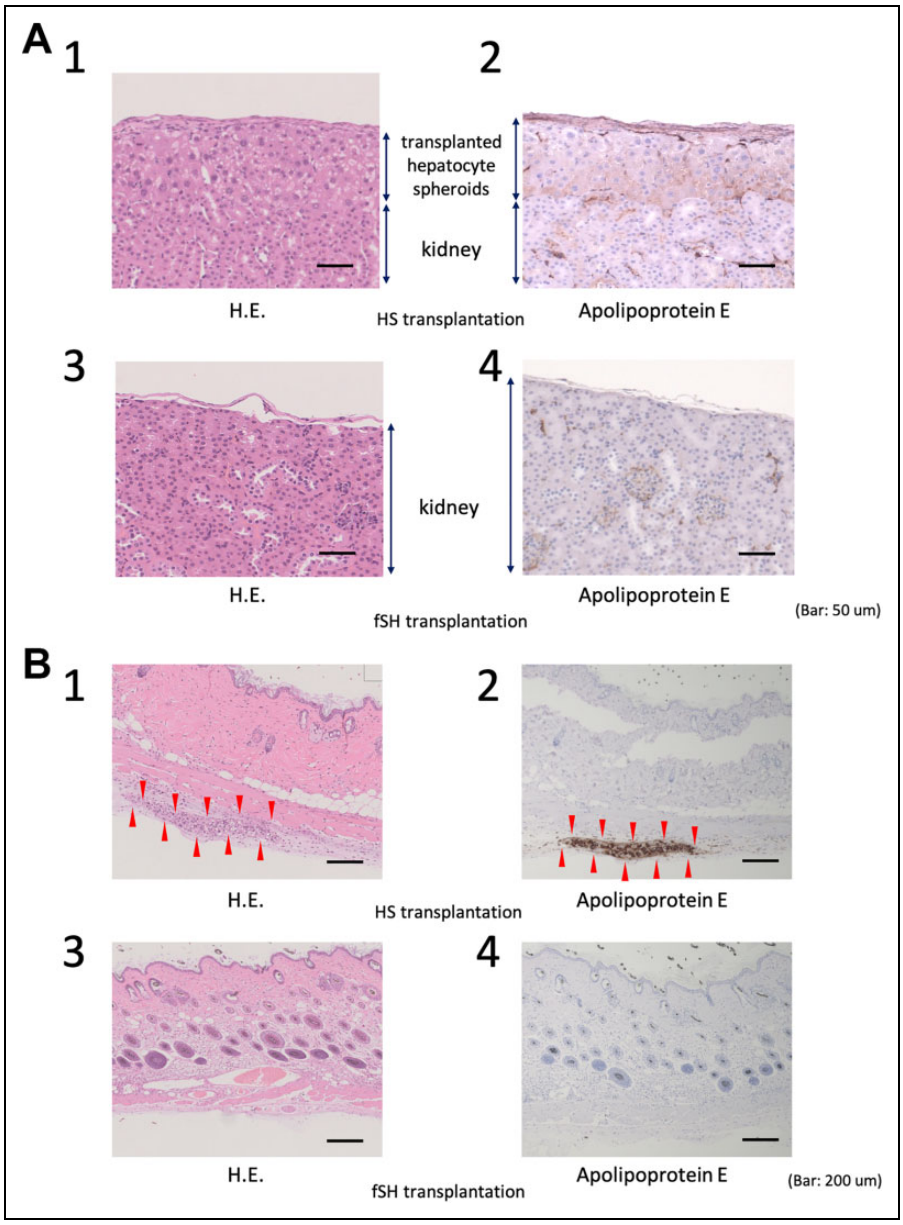

Figure 9. Hepatocyte spheroids could be transplanted under kidney capsule or skin. At 4 weeks after under kidney capsule or subcutaneous transplantation, transplanted-HSs were observed and found positive for albumin staining (kidney capsule; A1: H.E. × 20, A2: albumin × 20, bar: 50 μm) and ApoE staining (arrow heads; skin; B1: H.E. × 5, B2: apolipoprotein E × 5, bar: 200 μm). In contrast, transplanted hepatocytes were not observed pathologically after fSH transplantation (kidney capsule; A3: H.E. × 20, A4: albumin × 20, bar: 50 μm, skin; B3: H.E. × 5, B4: apolipoprotein E × 5, bar: 200 μm).

In terms of the proliferation of transplanted hepatocytes, many attempts have been made to precondition the liver of recipients and offer a selective advantage to the transplanted hepatocytes[56]. Johrns et al. reported that patients with Crigler-Najjar syndrome who underwent partial hepatectomy followed by HCTx showed a decrease of bilirubin for more than six months[2]. Moreover, portal vein embolization of 50% of the liver volume, followed by HCTx, in a nonhuman primate model resulted in replacement of 10% of the organ total volume in the non-embolized lobe[57]. Hepatocyte spheroids transplantation, together with these additional protocols, is technically feasible and would be a promising strategy to achieve long-lasting cell integration allowing stable expression of the missing protein/enzyme over time.

In this study, the isolated hepatocytes showed much better function before HCTx. However, serum Apo E concentration tended to be higher in HSs-transplanted animals after HCTx. This could be explained by the finding that some hepatic gene markers were transiently downregulated during spheroid formation, and it need several days to recover[58]. This discrepancy could also be explained by the findings that the viability and function of isolated hepatocytes decreased

rapidly in suspension and thus the liver functions of single hepatocytes might be lost before and during the HCTx procedure, as previously reported[59]. The structural stability of HS may also contribute to the better engraftment and function after HCTx. As discussed above, transplanted spheroids need to survive the early graft damage due to innate immune responses and engraft into the liver parenchyma thereafter. As demonstrated pathologically, HSs were positive to cytokeratin 18, a liver specific skeletal marker[60], and at least, the core of the spheroids is speculated to be tolerant to the phagocytic activity of Kupffer cells with proinflammatory cytokines.

There are several merits of the clinical usage of HSs. First, cell viability and functions can be examined during the spheroid induction process and damaged cells can be excluded from HCTx. The morphology of hepatocytes, in combination with albumin secretion, is frequently used as proof of hepatocyte functionality. However, these parameters alone do not prove the existence of other hepatocyte specific functions[61]. Therefore, it is difficult to evaluate the quality of the isolated hepatocytes immediately after isolation. Second, as demonstrated in this study, HSs retain their viability and function even in suspension. HSs have a reduced risk of losing viability during transportation or HCTx procedure compared to single hepatocytes. Third, the spheroids formation period would facilitate time for recipient selection. In this study, HSs were formed by the hanging drop method[35]. In clinical settings, however, spheroids need to be prepared efficiently under good manufacturing practice protocols at a larger scale. Such facilities are being made available, and cell producing projects are currently ongoing, aiming for clinical applications[62]. For wide clinical application, inducing spheroids at such a large-scale platform would be a promising method, along with hepatocytes induction from induced pluripotent stem cells[63], artificial organoids[64], or biomaterials[65]. It should also be considered that the current clinical HCTx is performed in allogeneic settings, in which engrafted cells are exposed to subsequent adoptive immune reactions[66]. It is thus necessary to verify the engraftment and proliferation of transplanted-HSs using allogeneic and/or large animal HCTx models. Such studies are currently under progress.

We conclude that HSs are a promising cell form alternative to single hepatocytes for HCTx. While intraportal transplantation with HSs induced initial innate/inflammatory reactions, these were comparable with those induced by single hepatocyte transplantation. In addition, HSs enabled graft acceptance, even when transplanted under the kidney capsule or subcutaneously. The findings of this study may contribute to the establishment of a novel strategy that would enable the successful engraftment of hepatocytes following HCTx.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

All experiments were approved by the Institutional Animal Care Committee, and the study was approved by the local ethics committee and conducted according to the guidelines for the Care and Use of Laboratory Animal oh Hokkaido University, Japan (approved number: 17-0032).

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Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Care and Use of Laboratory Animal of Hokkaido University, Japan (approved number: 17-0032).

Statement of Informed Consent

This study does not contain any human subjects and informed consent is not applicable.

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