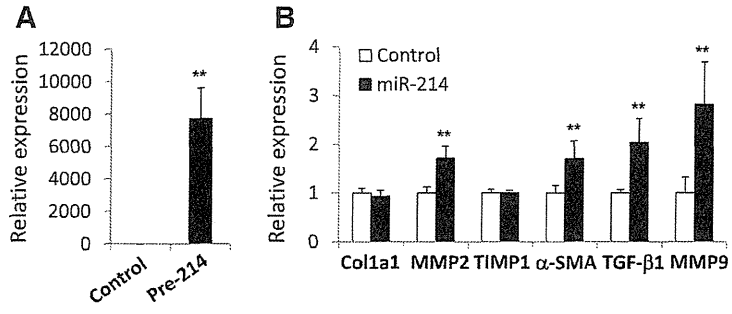

Figure 5 Effect of miR-214 overexpression on mRNA expression in LX-2 cells. (A) LX-2 cells were transfected with a miR-214 precursor or a negative control (control) at a final concentration of 50 nM and incubated for 24 hours. miR-214 expression was quantitated using real-time PCR. **(B)** The expression of fibrosis-related genes in LX-2 cells transfected with miR-214 precursors was analyzed using real-time PCR. The results are expressed as the expression relative to that in cells transfected with the control. $^{**}P < 0.01$.

PTEN: 0.97- to 1.12-fold, Plenxin-B1: 0.99-fold, Ezh2: 0.96-fold, TFAP2C: 0.94-fold, and Quaking: 0.88- to 1.18-fold change compared with cells transfected with control miRNA). The PTEN 3'-UTR did not interact with miR-214-5p in a luciferase reporter assay in LX-2 cells (data not shown). We also found that miR-214-5p overexpression had a negligible effect on LX-2 proliferation and migration. Therefore, the mRNA targets of miR-214-5p in LX-2 cells are not identical to those in previous reports.

Conclusions

We report an increase in miR-214-5p in liver fibrosis in humans and mice and the possible association of miR-214-5p with stellate cell activation. miR-214 expression in

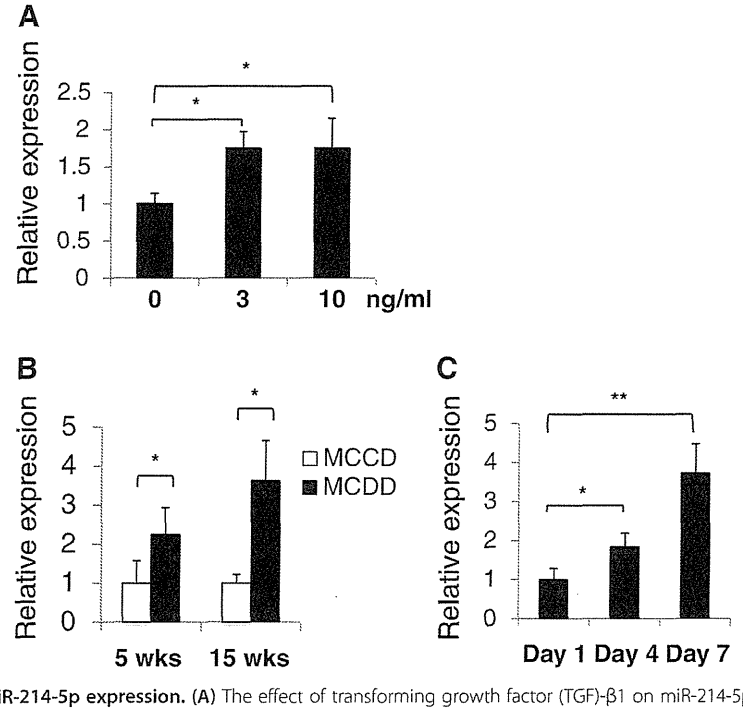

Figure 6 Regulation of miR-214-5p expression. (A) The effect of transforming growth factor (TGF)-β1 on miR-214-5p expression. LX-2 cells were treated with recombinant human TGF-β1 (3 or 10 ng/ml) for 24 hours in DMEM containing 0.1% fetal bovine serum (FBS). The results are expressed relative to miR-214 expression in cells that did not receive TGF-β1 treatment. $^{*}P < 0.05$. **(B)** Twist-1 expression in the fibrotic livers of mice fed a methionine- and choline-deficient diet (MCDD). Twist-1 expression was analyzed using real-time PCR. The results are expressed relative to Twist-1 expression in methionine- and choline-control diet (MCCD) mice. $^{*}P < 0.05$. **(C)** Twist-1 expression in primary-cultured mouse stellate cells. Twist-1 expression was analyzed using real-time PCR. The results are expressed relative to Twist-1 expression in cells on day 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1 List of primers

Gene name		Sequence from 5' to 3'
Mouse GAPDH	F	TGCACCACCAACTGCTTAG
	R	GGATGCAGGGATGATGTTC
Mouse α-SMA	F	TCCCTGGAGAAGAGCTACGAACT
	R	AAGCGTTCGTTTCCAATGGT
Mouse Col1a1	F	CCTGGCAAAGACGGACTCAAC
	R	GCTGAAGTCATAACCGCCACTG
Mouse PDGFR-β	F	GCGTATCTATATCTTTGTGCCAGA
	R	ACAGGTCCTCGGAGTCCAT
Mouse TGF-β1	F	GCAACATGTGGAACTCTACCAGAA
	R	GACGTCAAAAGACAGCCACTC
Mouse FN1	F	GATGCCGATCAGAAGTTTGG
	R	GGTTGTGCAGATCTCCTCGT
Mouse DDR2	F	CGAAAGCTTCCAGAGTTTGC
	R	GCTTCACAACACCACTGCAC
Mouse ITGB1	F	CAACCACAACAGCTGCTTCTAA
	R	TCAGCCCTCTTGAATTTTAATGT
Mouse Twist-1	F	AGCTACGCCTTCTCCGTCT
	R	TCCTTCTCTGGAAACAATGACA
Human GAPDH	F	GCACCGTCAAGGCTGAGAAC
	R	TGGTGAAGACGCCAGTGGA
Human Col1a1	F	CCCGGGTTTCAGAGACAACTTC
	R	TCCACATGCTTTATTCCAGCAATC
Human MMP2	F	TGACATCAAGGGCATTCAGGAG
	R	TCTGAGCGATGCCATCAAATACA
Human TIMP1	F	GGATACTTCCACAGGTCCCACAA
	R	CTGCAGGTAGTGATGTGCAAGAGTC
Human α-SMA	F	GACAATGGCTCTGGGCTCTGTAA
	R	CTGTGCTTCGTCACCCACGTA
Human TGF-β1	F	AGCGACTCGCCAGAGTGGTTA
	R	GCAGTGTGTTATCCCTGCTGTCA
Human MMP9	F	TCGAACTTTGACAGCGACAAGAA
	R	TCAGTGAAGCGGTACATAGGGTACA

α-SMA, α-smooth muscle actin; Col1a1, the type 1 collagen alpha 1 chain; DDR, discoidin domain receptor; F, forward primer; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITGB1, β1 integrin; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; R, reverse primer; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor.

stellate cells is regulated by TGF-β and possibly by the transcription factor Twist-1. These results should be pursued further to identify the role of miR-214-5p in liver fibrogenesis and to develop a biomarker that reflects the stage of liver fibrosis more accurately than a pathological staging score.

Methods

Ethics Statement

The Ethics Committee of the Osaka City University Graduate School of Medicine approved this study (Approval No. 1358), which complied with the principles of the Declaration of Helsinki (2008 revision). All of the patients provided written, informed consent.

Liver biopsy specimens

Liver biopsy specimens were obtained from 35 patients with chronic HCV (genotype 1) infection as described previously [25]. The stage of liver fibrosis was evaluated using the METAVIR scoring system [37]. Normal liver tissues were taken as control samples from four patients who underwent resection for metastatic liver tumors.

Animals

Eight- to 12-week-old male C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animal procedures were performed according to the guidelines of the Osaka City University and Faculty of Medicine Animal Research Committee and were approved by the committee. The mice received either a MCDD (n = 7, MP Biomedicals, Solon, OH, USA) or a MCCD (n = 7, MP Biomedicals) for 5 or 15 weeks, as previously described [26]. A similar protocol was followed in rats purchased from Japan SLC, Inc. Rats received MCCD for 10 weeks, MCDD for 10 weeks, or MCDD for 8 weeks followed by MCCD for the last 2 weeks (the last of the these being the recovery group) [26].

Cells

LX-2 cells (donated by Dr Scott Friedman [38]) and Huh7 cells were maintained in plastic culture plates in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). HepG2 cells (JCRB1054) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in plastic culture plates in Minimum Essential Medium (Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen), and 1% non-essential amino acids (Invitrogen). Primary hepatic stellate cells and hepatocyte-rich and Kupffer cell-rich fractions were prepared from mouse livers according to the previously reported method [39].

Histochemistry and immunohistochemistry

The sections were stained with 0.1% (w/v) Sirius red in a saturated aqueous solution of picric acid (Direct Red 80; Aldrich, Milwaukee, WI, USA) for 1 hour at room temperature to visualize collagen fibers. Immunostaining for α-SMA was performed as previously described [25]. Mouse liver tissue was fixed in 10% formaldehyde, embedded in paraffin, and cut into 4 μm thick sections.

Quantitative real-time PCR

Gene expression was measured by real-time PCR using cDNA, real-time PCR Master Mix Reagents (Toyobo, Osaka, Japan), and gene-specific oligonucleotide primers

(Table 1) in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), as previously described [25].

Transforming growth factor-β1 stimulation of LX-2 cells

LX-2 cells were seeded on 24-well plates in DMEM supplemented with 10% FBS at a density of 2×10^5 cells/ml. The cells were cultured for 14 hours, and the medium was changed to DMEM supplemented with 0.1% FBS plus TGF-β1 (3 or 10 ng/ml). The culture was continued for an additional 24 hours.

Transient transfection of miRNA precursors

miR-214-5p precursors and negative control miRNA were transfected into LX-2 cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nM, as described previously [20,25]. The cells were collected after 24 hours, and total RNA was extracted.

Statistical analysis

The data shown in the bar graphs represent the means ± SD of at least three independent experiments. Statistical analysis was performed using the Student's *t*-test. The Jonckheere-Terpstra test was used to compare differences between the four groups in the progressive stages of liver fibrosis. $P < 0.05$ was considered statistically significant.

Abbreviations

bp: base pair; Col1a1: the type 1 collagen alpha 1 chain; DDR: discoidin domain receptor; DMEM: Dulbecco's modified Eagle's medium; ECM: extracellular matrix; FBS: fetal bovine serum; FN: fibronectin; HCV: hepatitis C virus; ITGB1: β1 integrin; MCCD: methionine- and choline-control diet; MCDD: methionine- and choline-deficient diet; miRNA: microRNA; MMP: matrix metalloproteinase; PCR: polymerase chain reaction; PDGFR: platelet-derived growth factor receptor; α-SMA: α-smooth muscle actin; TGF: transforming growth factor; UTR: untranslated region.

Competing interests

KY is an employee of PhoenixBio Co. Ltd as an academic advisor. There is no direct financial benefit to KY for the publication of this manuscript. All other authors declare that they have no competing interests.

Authors' contributions

Conception and design (MI, TO, KI, NK); data acquisition (TO, ME, YM), data analysis and interpretation (MI, TO, KY); writing and review of the manuscript (MI, KY, KI, NK). All authors read and approved the final manuscript.

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Human Hepatocyte Propagation System in the Mouse Livers: Functional Maintenance of the Production of Coagulation and Anticoagulation Factors

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We previously reported that cell-based therapies using isolated hepatocytes including hepatocyte transplantation and liver tissue engineering approaches provide therapeutic benefits to hemophilia. For clinical application of these approaches, it is important to establish an active hepatocyte proliferation system that enables providing a sufficient number of hepatocytes. We also reported that human hepatocytes, which were transplanted into the liver of urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice, were able to proliferate while retaining their ability to produce coagulation factor IX. The objective of this study was to explore the functionalities of other coagulation and anticoagulation factors of the propagated human hepatocytes in uPA/SCID mice. Human hepatocytes were transplanted into the liver of uPA/SCID mice, and the propagation status of human hepatocytes in the mice was monitored by the increase in serum human albumin levels and immunohistochemical evaluation on the liver sections. Using uPA/SCID livers with various stages of human hepatocyte propagation, we analyzed the gene expression levels of coagulation factors (prothrombin, factor VII, factor X, and factor VIII) and anticoagulation factors (protein C and protein S) by real-time polymerase chain reaction (PCR) using human-specific primers. As a result, the total amount of raw messenger RNA expression levels increased in all genes analyzed according to the progress of hepatocyte propagation and proliferation. Except for factor VIII, the gene expression levels of the highly repopulated uPA/SCID mouse livers with human hepatocyte showed higher levels than those of normal human livers, indicating that propagated human hepatocytes in the uPA/SCID system possess full functions to produce most of the coagulation-related factors. The current work demonstrated that human hepatocytes can be propagated in experimental animals while maintaining normal gene expression levels of coagulation-related factors. It could be speculated that the propagated cells serve as a cell source for the treatment of various types of coagulation factor deficiencies.

Key words: Hepatocyte; Cell therapy; Hepatocyte transplantation; Coagulation factor; Urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mouse; Anticoagulation factor

INTRODUCTION

Production of coagulation and anticoagulation factors is one of the important functions of the liver, and most of these coagulation-related factors are produced by hepatocytes (4,6,30,31,33). There are various types of congenital bleeding disorders that lack a production of coagulation factor in the liver, showing a symptomatic bleeding tendency. Hemophilia A or B is well known as a representative bleeding disorder, which is caused by a failure in the production of functional coagulation factor VIII (FVIII) or factor IX (FIX) from the liver. Although the ultimate cure for hemophilia patients could be obtained by liver transplantation (10,13,14), world-wide donor organ shortage is the most critical obstacle. For patients with hemophilia and other congenital coagulation factor deficiencies, the elevation of the responsible factor level to 1–2% of normal levels can provide a phenotypic change from severe to moderate form, resulting in a marked improvement in the symptomology and the

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quality of life (5). Cell-based approaches using isolated hepatocytes could be a feasible therapeutic option toward these coagulation factor deficiencies (18,20,21,24).

Proof-of-concept studies for hepatocyte-based approaches have been accomplished in both laboratory animals and humans. We recently reported that hepatocyte transplantation provided an increase of 1–2% of coagulation activities in a mouse model of hemophilia B, FIX-knockout mice (31). We also reported that engineering functional liver tissues beneath the kidney capsule were able to provide therapeutic effects in the mouse model of hemophilia A as well as hemophilia B (20,21). As reported by Dhawan et al. (7), hepatocyte transplantation was also successful in the clinic for the treatment of congenital factor VII deficiency. In utero liver cell transplantation was also investigated by Rosen et al. (25). They described phenotypic improvements in the mouse model of factor X deficiency. It is important to note that these hepatocyte-based approaches could be employed with a simple procedure and in a less invasive manner compared with organ transplantation (18,22).

One of the major hurdles in establishing hepatocyte-based approaches is the limited availability of biologically functional hepatocytes. At present, the number of donor livers for hepatocyte isolation remains severely limited. In most of cases, donor livers are of marginal quality that makes it difficult to obtain functional hepatocytes (23). An additional issue is that current technology for hepatocyte primary culture appears to be unable to support extensive cell proliferation (19). Under these circumstances, we previously proposed urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice as a feasible in-mouse hepatocyte propagation tool. uPA/SCID mice have a feature to develop an active damage of their own hepatic parenchymal cell and subsequent occurrence of continuous release of regenerative stimulus. Because of this nature, uPA/SCID mice provide a hepatic environment that is more conducive to the engraftment of human hepatocytes and a selective advantage for transplanted cells to proliferate (29). An important property of uPA/SCID mice was recently reported to allow human hepatocytes transplanted into the liver of uPA/SCID mice to actively propagate while retaining their ability to produce and secrete human FIX (30). From these data, it was reasonably speculated that the propagated human hepatocytes can serve as a cell source for future hepatocyte-based therapies toward hemophilia B. The functional preservation of FIX production of the propagated human hepatocytes encourages us to further assess functionalities for the production of other coagulation or anticoagulation factors.

We hypothesized that propagated human hepatocytes in the uPA/SCID mouse livers retained a normal gene expression of other coagulation and anticoagulation factors including prothrombin, factor VII, factor VIII, factor X, protein C, and protein. This report documents the first comprehensive analyses of coagulation factor-related gene expressions during in-mouse propagation status of human hepatocytes.

MATERIALS AND METHODS

Animals

Recipient uPA/SCID mice were generated at Phoenix Bio (Higashihiroshima, Hiroshima, Japan) as described previously (29). Genotyping for the presence of uPA transgene in SCID mice was confirmed by polymerase chain reaction assay of isolated genomic DNA as described previously (11,29). Experimental protocols were developed in accordance with the guidelines of the local animal committees located at both PhoenixBio and Nara Medical University.

Transplantation of Human Hepatocytes for Propagation in the uPA/SCID Livers

Human hepatocytes, isolated from a 1-year-old white male and a 6-year-old Afro-American female were purchased from In Vitro Technologies (Baltimore, MD). The cryopreserved hepatocytes were thawed and suspended in transplant medium (9,29). The cell viability of the human hepatocytes was determined to be 64.4% and 49.2% by trypan blue exclusion test, respectively. One day prior to the transplantation and 1 week after the transplantation, uPA/SCID mice, 20–30 days old, received intraperitoneal injections of 0.1 mg of anti-asialo GM1 rabbit serum (Wako Pure Chemical Industries, Osaka, Japan) to inhibit recipient natural killer cell activity against the transplanted hepatocytes. Viable human hepatocytes (0.75×10^6) were transplanted using an infusion technique into the inferior splenic pole in which the transplanted cells flow from the spleen into the liver via the portal system ($n = 18$). After the transplantation, uPA/SCID mice were treated with nafamostat mesilate to inhibit complement factors activated by human hepatocytes as described elsewhere (29).

Determination of Replacement Ratio

Blood samples were collected periodically from the tail vein, and the levels of human albumin were determined with a Human Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX) to estimate the status of proliferation and propagation of the transplanted human hepatocytes as previously described (29). For accurate determination of the ratio that transplanted human hepatocytes occupied in the recipient mouse livers (the replacement ratio), the harvested liver section were stained with antibodies against human-specific

cytokeratins 8 and 18 (hCK8/18), as described elsewhere (29). The replacement ratios of the mouse liver that received human hepatocytes were calculated as the ratio of area occupied by hCK8/18-positive hepatocytes to the entire area examined immunohistochemical sections of six lobes.

RNA Isolation and Quality Controls

Total RNA was extracted from the liver of the recipient mice with various stages of the replacement and normal human liver tissue samples by a RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Normal human liver tissue portions were obtained from surgical specimens during liver surgery for metastatic liver tumors after acquiring a written informed consent for the experimental use of harvested liver samples. DNase I was used to eliminate genomic DNA contamination, and the concentration of the RNA was determined by UV spectrometry. All of the RNA samples used in this study had an absorbance ratio (260/280 nm) between 1.9 and 2.1, and the integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

Reverse Transcription Coupled to Quantitative Real-Time PCR (Real-Time RT-PCR)

Total RNA (1 μg) was reverse-transcribed using oligo d(T)16 primers as described by the manufacturer (Omniscript RT Kit; QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using a StepOne Real-time PCR system (Applied Biosystems, Tokyo). For this experiment, we examined the following gene groups: 1) seven housekeeping reference genes, including glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*), transferrin receptor (*Tfrc*), β-glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*); 2) the genes of five vitamin K-dependent coagulation factors, including prothrombin (*F2*), factor VII (*F7*), factor X (*F10*), protein C (*Prosc*), and protein S (*Pros1*); and 3) factor VIII (*F8*) gene. TaqMan probes and primers for these genes were chosen from a TaqMan Gene Expression Assay (Applied Biosystems), and the information regarding these primer sets are listed in Table 1. All PCR analyses were performed using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The specificity of the primers was verified by 2% agarose gel electrophoresis of the amplicons derived from naive human liver cDNAs. The PCR primers used in this study were confirmed to be human specific and to have no cross-reactions with mouse-derived genes. For quantification of gene expression, the cDNAs derived from total RNA extracted from normal human liver tissues were serially diluted and used to generate calibrations.

Statistical Analysis

Correlation coefficients between the repopulation rate and each gene expression were determined using Excel (Microsoft).

RESULTS

Propagation of Human Hepatocytes in uPA/SCID Mouse Liver

Human hepatocytes were transplanted to uPA/SCID mice, which were sacrificed to excise the liver tissues at various time periods after the transplantation with monitoring the levels of blood human albumin. Using the collected liver samples, hCK8/18 immunostaing on the liver sections was performed to accurately assess the replacement ratios with human hepatocytes as described in Materials and Methods. As a result, the repopulation ratios ranged from 0% to 98%, and the number of mice in each repopulation category was 2, 4, 4, 4, and 4 in 0–20%, 21–40%, 41–60%, 61–80%, and 81–100%, respectively.

Selection of an Appropriate Reference Gene

The expressions of seven commonly used housekeeping genes (HKG) specific to human cells were evaluated in the recipient uPA/SCID mouse livers. Figure 1 shows that the raw expression levels of all seven HKGs increased in parallel to the replacement ratios. The correlation coefficiency with the replacement ratios of each gene was 0.68 for h*Gapdh*; 0.86 for h*Actb*; 0.72 for h*Ppia*; 0.82 for h*Rpl4*; 0.68 for h*Tfrc*; 0.75 for h*Gusb*; and 0.78 for h*Hprt1*. When the replacement ratios exceeded 80%, the expression levels of all HKGs but *Hprt1* became higher than those of normal human liver samples. h*Gapdh* expression levels in the repopulated uPA/SCID livers were also observed to be beyond the levels of normal human livers at the repopulation ratio as low as 40%. Eventually, h*Gapdh* expression levels reached approximately sixfold of normal human liver levels when the repopulation ratios exceeded 80%. In contrast, gene expression levels of h*Actb* failed to reach to comparable levels with normal human liver until the repopulation ratios were close to 100%. Under the condition of varied gene expression levels of HKGs, it is important to select the most stably expressed HKG to assess the expression of target human genes in the uPA/SCID livers. For achieving this, *Actb* gene, which demonstrated the best correlation coefficient with the replacement ratios, was selected as a reference normalizing gene in the following gene expression analyses.

Table 1. Primers Used in This Study

Symbol	Gene Name	Assay ID
Housekeeping genes		
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
Actb	actin, beta	Hs99999903_m1
Ppi	peptidylprolyl isomerase A	Hs99999904_m1
Rpl4	ribosomal protein L4	Hs03044647_g1
Tfrc	transferrin receptor	Hs00174608_m1
Gusb	glucuronidase, beta	Hs99999908_m1
Hprt1	hypoxanthine phosphoribosyltransferase	Hs99999909_m1
Target genes		
F2	coagulation factor II (prothrombin)	Hs01011995_g1
F7	coagulation factor VII	Hs00173398_m1
F9	coagulation factor IX	Hs00609168_m1
F10	coagulation factor X	Hs00173450_m1
Prosc	protein C	Hs00165584_m1
Pros1	protein S, alpha	Hs00165590_m1
F8	coagulation factor VIII	Hs00240767_m1

Expression of Coagulation Factor Genes

Human-specific coagulation-related gene expression levels were assessed on human hepatocyte-repopulated uPA/SCID mouse livers. The genes analyzed were: vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X) and anticoagulation factors (protein C and protein S), in addition to factor VIII. Raw expression levels of all vitamin K-dependent coagulation and anticoagulation factor genes showed a positive correlation with the repopulation ratios (Fig. 2). The correlation coefficient between the gene expression levels and the repopulation ratios were 0.78, 0.74, 0.80, 0.80, and 0.82 in *F2*, *F7*, *F10*, *Prosc*, and *Pros1*, respectively. The raw gene expression levels of all but *F8* were beyond the levels of the normal human liver samples (defined as 1.0) as the repopulation ratios increased. In marked contrast, *F8* gene expression levels were less than 40% of normal human liver tissues even though the repopulation ratios reached approximately 100%. The low levels of *F8* gene expression failed to show a significant correlation with the repopulation ratios ($R = 0.66$).

In order to evaluate the gene expression levels per human hepatocytes, the gene expression levels were normalized by *ACTB* gene expression levels in each sample (Fig. 3). As a result, normalized gene expression values of all the analyzed coagulation-related factor genes showed constant expression levels regardless of the repopulation ratios, demonstrating that the human hepatocytes in the uPA/SCID livers stably express coagulation-related factor genes throughout the repopulation stages.

DISCUSSION

Propagation of primary human hepatocytes that possess hepatocyte-specific functionalities including blood clotting factor production has been one of the major paradigms in liver regenerative medicine (18,24). In the present study, we transplanted primary human hepatocytes to the liver of uPA/SCID mice and succeeded in propagating the human hepatocytes in the mouse livers. We then investigated mRNA expression levels of human-specific vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X), anticoagulation factors (protein C and protein S), and factor VIII of the propagated hepatocytes at various stages of propagation. The results showed that mRNA expression levels per human hepatocyte of all the analyzed genes were maintained through the propagation stage, indicating that the uPA/SCID in-mouse hepatocyte propagation system is a viable method to propagate hepatocytes that are intact in coagulation factor productions.

Coagulation factors are produced mainly by hepatocytes, and the long-term synthesis of these factors from primary human hepatocytes in vitro have been recently achieved by plating cells inside a 3D collagen gel matrix together with hormonally enriched culture medium under chemically defined conditions (6), indicating the pivotal role of the extracellular environment for coagulation factor production. However, the current procedure for the culture of primary hepatocytes appears to be difficult to support extensive cell proliferation (19), still remaining the problem of donor cell shortage unresolved. It is true that isolated hepatocytes could obtain proliferating ability and long-term survival in vitro by immortalization (26,34,35) or by selective culture of small hepatocyte population (27), but there is no report for studying the gene expression and production of coagulation factors including factor IX in these cell types. On the other hand, embryonic stem (ES) cells and

induced pluripotent stem (iPS) cells have been intensively investigated as an attractive cell source for liver regenerative medicine, and differentiation technologies of these stem cells into hepatocyte-like cells have been improved (12). In these circumstances, Basma et al. (3) recently succeeded in differentiating human ES cells into hepatocytes-like cells maintaining the ability of human factor VII production; however, the expression of coagulation factors other than factor VII were undocumented. In contrast to these in vitro cell culture systems, there are several in vivo hepatocyate propagating systems, in which transplanted hepatocytes can efficiently proliferate in mouse livers, such as uPA/SCID mice (29) or fumarylacetoacetate hydrolase$^{-/-}$/recombination activating gene$^{-/-}$/gamma chain of the interleukin-2 receptor$^{-/-}$ (Fah$^{-/-}$/Rag2$^{-/-}$/Il2rg$^{-/-}$) mice (2).

In our previous series of experiments, we found that human hepatocytes that were transplanted into the liver of uPA/SCID mouse perform active cell proliferation leading to a nearly total repopulation of the liver (17, 29,30,36) and confirmed that those proliferated hepatocytes maintained their ability to produce and secrete biologically functional human factor IX (30). In addition to human hepatocytes, we also reported that primary canine hepatocytes could proliferate in uPA/SCID mouse liver while retaining functions for canine factor IX production

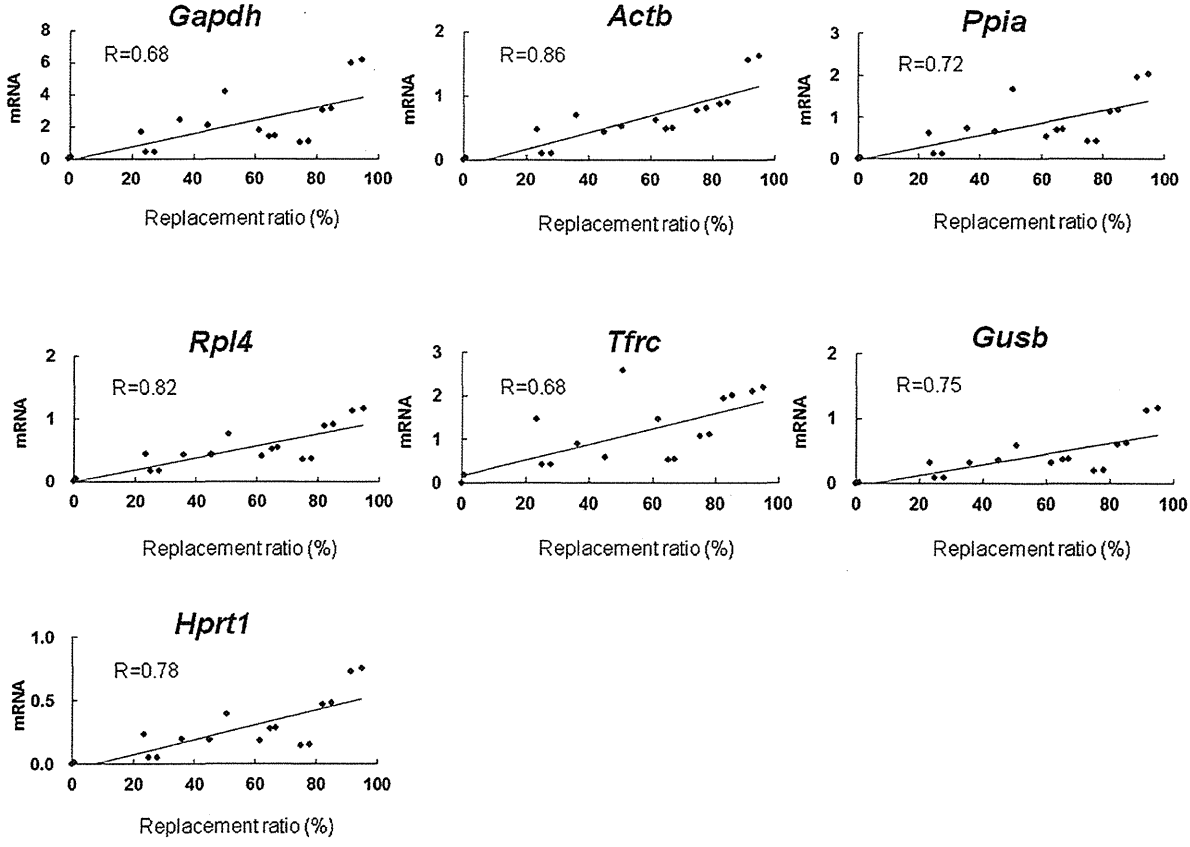

Figure 1. The raw gene expression levels of human housekeeping genes in the human hepatocytes repopulated in human hepatocytes were transplanted into urokinase-type plasminogen activator transgenic sever combined immunodeficient uPA/SCID mouse livers. Isolated uPA/SCID mouse livers (n = 18), and the livers were excised at various points of repopulation ratios determined by blood human albumin levels and human-specific cytokeratins 8 and 18 (hCK8/18) immunohistochemistry on the liver sections. The repopulation ratio ranged from 0% to 98% (0–20%, two mice; 21–40%, four mice; 41–60%, four mice; 61–80%, four mice; and 81–100%, four mice). Gene expression levels of commonly used seven housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*), transferrin receptor (*Tfrc*), β-glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*)] in human–chimeric mouse liver samples were quantified by real-time PCR with human-specific primers and expressed as relative values against the control normal human liver tissue (defined as 1.0). The correlation coefficient of each gene was expressed as an R value.

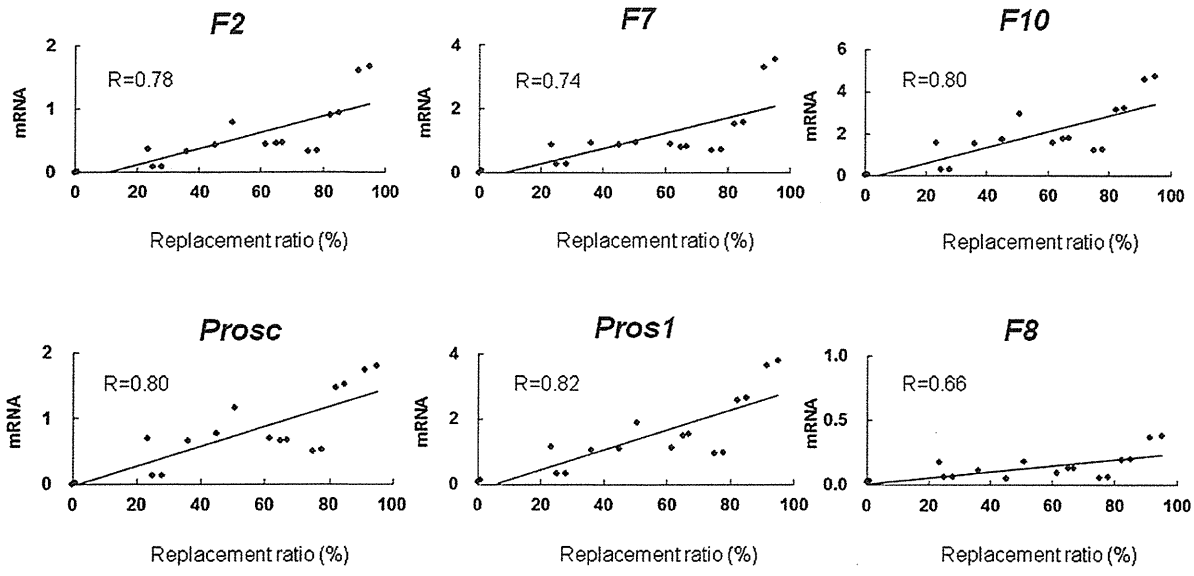

Figure 2. The raw gene expression levels of human coagulation and anticoagulation factors in the human hepatocytes repopulated in the uPA/SCID mouse livers. Isolated human hepatocytes were transplanted into uPA/SCID mouse livers (n = 18), and the livers were excised at various points of repopulation ratios determined by blood human albumin levels and hCK8/18 immunohistochemistry on the liver sections. The repopulation ratio ranged from 0% to 98% (0–20%, two mice; 21–40%, four mice; 41–60%, four mice; 61–80%, four mice; and 81–100%, four mice). Gene expression levels of coagulation factors (prothrombin, *F2*; factor VII, *F7*; factor X, *F10*; and factor VIII, *F8*) and anticoagulation factors (protein C, *Prosc*; protein S, *Pros1*) in the human–chimeric mouse liver samples were quantified by real-time PCR with human-specific primers and expressed as relative values against the control normal human liver tissue (defined as 1.0). The correlation coefficient of each gene was expressed as an R value.

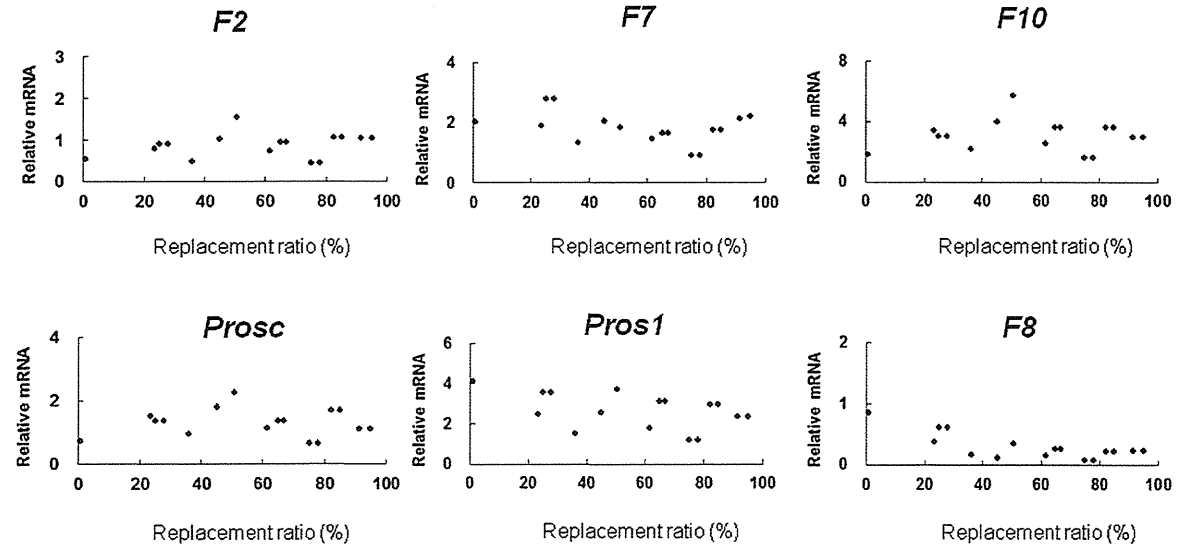

Figure 3. The relative gene expressions levels of human coagulation and anticoagulation factors in the human hepatocytes repopulated in the uPA/SCID mouse livers. The raw gene expression levels of human coagulation and anticoagulation factors show in Figure 2 were normalized with *Actb* gene expression levels in each human–chimeric mouse liver samples and plotted the repopulation ratios.

(30). At least, this in-mouse hepatocyte propagating system is only an available and promising procedure for proliferating factor IX-producing hepatocytes at the present time. In this regard, it is important to clarify gene expression of other coagulation factors as well as factor IX in propagated human hepatocyte in uPA/SCID mouse liver, because to establish a method for hepatocytes proliferation while retaining ability for coagulation factor production is indispensable for clinical cell therapy toward coagulation disorders.

We first assessed the expression levels of seven commonly analyzed HKGs for human genome in the human hepatocyte-repopulated uPA/SCID mouse livers. The gene expression levels of all seven HKGs increased in parallel to the increase of the repopulation ratio with a high correlation coefficient, providing direct evidence that the transplanted human hepatocytes progressively proliferated in uPA/SCID mouse livers (Fig. 1). Interestingly, as for six out of seven HKGs analyzed, the gene expression levels of samples with more than 80% repopulation ratios surpassed the levels of normal human livers (arbitrarily defied as 1.0). It was reported that the upregulation of certain HKGs, especially *Gapdh*, was closely associated with the events of DNA synthesis and cell division (1). Such HKG upregulation profiles associated with the hepatocyte proliferation were also observed in our previous works where hepatocyte proliferation in mouse livers was induced through the mode of compensatory regeneration (32) or direct hyperplasia (28). These findings suggest that human hepatocytes progress their cell cycling events in the uPA/SCID livers while upregulating their structural proteins necessary for cell proliferation. Since HKGs are used as internal reference gene(s) in gene expression analyses, it is essential to identify appropriate HKGs that are stably expressed during examined pathological process. During the human hepatocyte repopulation process in the uPA/SCID livers, we found that the expression levels of *Actb* showed the highest correlation coefficient ($R = 0.86$) with the repopulation ratios among seven HKGs analyzed. Therefore, we concluded that the use of *Actb* for normalization of gene expression levels was appropriate for obtaining accurate gene expression values.

To elucidate gene expression levels of coagulation and anticoagulation factors in propagating human hepatocytes in the liver of uPA/SCID mice was the main objective in this study. Using human-specific PCR primers, we found that raw expression levels of all genes increased in parallel to the increase of repopulation ratios with a high correlation coefficient. As the repopulation ratios increased higher than 80%, the expression levels of all genes except *F8* surpassed the levels of normal human livers (Fig. 2). We also investigated the gene expression levels per human hepatocyte by normalizing the expression levels of each gene by the levels of *Actb*. Results clearly showed that the gene expression levels of all analyzed coagulation and anticoagulation factors were stably maintained throughout the in-mouse repopulation process (Fig. 3). Overall, the present study combined with our previous investigation (30) demonstrated that human primary hepatocytes were able to proliferate in the liver of uPA/SCID mice while retaining the cellular machinery for expressing *F2*, *F7*, *F9*, *F10*, *Prosc*, and *Pros1*. Since human hepatocyte propagated in uPA/SCID mice are able to be isolated and purified by cell-sorting technology (37), the present in-mouse propagated human hepatocytes is a feasible candidate cell source for a future therapeutic use toward coagulation factor deficiencies. Furthermore, it was reported that efficient gene transduction into proliferated human hepatocytes in uPA/SCID mice was possible by using retroviral vector system (9), indicating the capability of obtaining hepatocytes that were genetically modified in vivo for the purpose to achieve higher expression levels of target proteins (15).

In the present study, gene expression levels of *F8* were remarkably low compared with those of other factors assessed. Even the liver samples showing the repopulation ratio more than 80% demonstrated only less than 40% of the control normal human liver tissues in human *F8* gene expression (Fig. 2). uPA/SCID mice have a characteristic to allow their own hepatocytes to be replaced by repopulated human hepatocytes, but all other intravital environment as well as nonparenchymal liver cells remain predominantly host origin. As a result, it is likely that various signal cross-talks between repopulated human hepatocytes and host cells or humoral factors might become dysfunctional. For example, although hepatocytes require growth hormone (GH) for their active cell proliferation, human hepatocytes transplanted into mouse livers failed to be the target for such growth advantage because rodent GH is unable to bind to human GH receptors (17). We have indeed established that this functional deficiency could be fully recovered by administration of human GH to the human hepatocyte-repopulated uPA/SCID mice (17). The fact that human *F8* gene expression level failed to increase may be explained by some mechanism similar to this type of interspecies incompatibility. An alternative possibility is that nonparenchymal cells, but hepatocytes, are the main responsive cells for human factor VIII productions. Although the liver has been shown to be the major site of factor VIII production as evidenced by previous liver transplantation clinical experiences (10,13,33), the precise type of liver cells contributing to factor VIII production has not been fully identified (4,6,8,16). In either the case, the present in-mouse hepatocyte propagation system using uPA/SCID mouse might be a valuable tool

for the elucidation of cellular mechanism of factor VIII synthesis and production.

In conclusion, the present study provides encouraging evidence that uPA/SCID mouse system supported the active proliferation of human hepatocytes while maintaining cellular machinery to produce vitamin K-dependent coagulation (prothrombin, factor VII, and factor X) and anticoagulation factors (protein C and protein S) in addition to factor VIII. The current work thus can serve as a basis to create a hepatocyte propagation system to prepare sufficient amount of cells for the therapeutic purposes for deficiencies of these factors as well as for the research purpose to investigate the hepatocyte-specific production mechanisms of coagulation factors.

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Liver Tissue Engineering Utilizing Hepatocytes Propagated in Mouse Livers In Vivo

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Recent advances in tissue engineering technologies have highlighted the ability to create functional liver systems using isolated hepatocytes in vivo. Considering the serious shortage of donor livers that can be used for hepatocyte isolation, it has remained imperative to establish a hepatocyte propagation protocol to provide highly efficient cell recovery allowing for subsequent tissue engineering procedures. Donor primary hepatocytes were isolated from human α-1 antitrypsin (hA1AT) transgenic mice and were transplanted into the recipient liver of urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) mice. Transplanted donor hepatocytes actively proliferated within the recipient liver of the uPA/SCID mice. At week 8 or later, full repopulation of the uPA/SCID livers with the transplanted hA1AT hepatocytes were confirmed by blood examination and histological assessment. Proliferated hA1AT hepatocytes were recovered from the recipient uPA/SCID mice, and we generated hepatocyte sheets using these recovered hepatocytes for subsequent transplantation into the subcutaneous space of mice. Stable persistency of the subcutaneously engineered liver tissues was confirmed for up to 90 days, which was the length of our present study. These new data demonstrate the feasibility in propagating murine hepatocytes prior to the development of hepatic cells and bioengineered liver systems. The ability to regenerate and expand hepatocytes has potential clinical value whereby procurement of small amounts of tissue could be expanded to sufficient quantities prior to their use in hepatocyte transplantation or other hepatocyte-based therapies.

Key words: Liver tissue engineering; Cell proliferation; Cell sheet; Tissue engineering; Hepatocyte transplantation; Regenerative medicine

INTRODUCTION

Liver tissue engineering using primary hepatocytes presents the opportunity to create a de novo liver system, which can lead to a new therapeutic modality towards the treatment of various liver diseases (4,10, 15,18,28) and may have the potential to obviate the need for organ liver transplantation. In fact, hepatocyte transplantation into the liver of a recipient patient has proven to be effective in some experimental and clinical settings (3,10,14,20,21,24,26).

We have recently developed a novel approach for engineering a functional ectopic liver system within the subcutaneous space in mice (17). The engineered liver systems have been shown to persist for at least 200 days, and the persistency of the de novo engineered tissue has been largely predicated on two key factors. First, the creation of a vascularized platform using a growth factor-releasing device at the target subcutaneous site prior to the transplantation of the donor hepatocytes (30). The prevascularization process plays a pivotal role in the survival of the transplanted hepatocytes by providing an adequate blood supply to these transplanted cells, due to their high metabolic activities that require an active nutrient supply and waste exchanges (13,16). The second factor is the creation of uniform hepatocyte sheets using the isolated individual donor hepatocytes in culture (17). The in vitro created hepatocyte sheets maintain their intercellular communications (e.g., desmosomes and bile canaliculi) and exhibit other essential cellular microstructures to document their continued hepatocyte phenotype and functionality. Recent studies by our

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group has shown that combining these two factors in which the in vitro-generated hepatocyte sheets were transplanted within a prevascularized subcutaneous site potentiated the formation of a fully functional, long-lasting two-dimensional liver systems (17). Moreover, a more spatially complex, three-dimensional liver systems could be successfully achieved by stratifying additional hepatocyte sheets in the subcutaneous space (17). The confirmed functionality of the engineered liver system includes liver-specific protein productions, chemical uptake and subsequent metabolizing enzyme expressions, and regenerative growth (17).

Although this approach has shown considerable promise in recent years, there are several hurdles that need to be overcome for continual advancement to occur using hepatocyte-based technologies. Most notably, the potential lack of donor hepatocytes could become an issue depending on the abundance of the liver mass obtained for this procedure, since isolated hepatocytes lose their ability to actively proliferate following transfer to culture conditions (9,14). For this reason, an efficient method to propagate donor hepatocytes needs to be established. However, we and others have shown that primary donor hepatocytes can be successfully proliferated in vivo following transplantation in the livers of several mouse models, including the urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice (1,6,7,19,22,23).

Towards this end, we have designed the present study to determine whether primary donor mouse hepatocytes transplanted into the livers of uPA/SCID recipient mice can be efficiently propagated in vivo for subsequent downstream utilization as a valuable source for the creation of hepatocyte cell sheets. The efficacy in the generation of hepatocyte cell sheets in the prevascularized subcutaneous space of mice and their persistent biological function(s) will be investigated.

MATERIALS AND METHODS

Animals

Transgenic mice expressing human α-1 antitrypsin (hA1AT) under the hepatocyte-specific promoter (hA1AT-FVB/N, H-2^q; kindly provided by Dr. Bumgardner, Ohio State University, Columbus, OH) (2) at 13 weeks of age were used as donors for hepatocyte isolation. Wild-type female FVB/N mice (11–12 weeks of age), which were syngenic to the hA1AT-FVB/N, were used as the recipient animals. hA1AT transgenic (hA1AT-TG) and FVB/N mice were maintained in the Animal Center at Tokyo Women's Medical University. uPA/SCID mice used in the hepatocyte propagation experiments were generated at Hiroshima Prefectural Institute of Industrial Science and Technology (HPIIST) and PhoenixBio, Co. Ltd. as described previously (22,23,31). Presence of the uPA transgene in each SCID mouse was genotypically confirmed by PCR as described previously (23). Experimental protocols were developed in accordance with the guidelines of the local animal committees at HPIIST, PhoenixBio, Co., Ltd., and Tokyo Women's Medical University. Mice were placed in cages within a temperature-controlled room with a 12-h light/12-h dark cycle as well as ad libitum access to food and water.

Hepatocyte Isolation and Purification

Hepatocytes were isolated from hA1AT-TG mice or hA1AT hepatocyte-repopulated uPA/SCID mice using a modified two-step collagenase perfusion method as previously described (5,11,12,16,17,30). Briefly, the livers were primarily perfused with Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO) containing 0.09% EGTA followed by a second perfusion using HBSS containing 0.03% collagenase (Sigma) and 5 mM $CaCl_2$. Isolated cells were filtered through a nylon mesh membrane and hepatocytes were then purified by slow speed centrifugation at $50 \times g$ for 5 min followed by Percoll (GE Healthcare, Buckinghamshire, UK) isodensity purification. The viabilities of the isolated mouse hepatocytes were determined by trypan blue exclusion test. In studies of hA1AT hepatocyte transplantation to the uPA/SCID mice, experiments were conducted only when the hA1AT hepatocyte viabilities exceeded 95%.

Transplantation of hA1AT Hepatocytes for Propagation in the uPA/SCID Livers

Isolated hA1AT hepatocytes were resuspended with serum-free Dulbecco's modified Eagle medium (DMEM) (Sigma) to a final ratio of 1.5×10^7 hepatocytes/ml. A total of 5×10^5 viable hepatocytes were transplanted into the liver of uPA/SCID mice using an infusion technique into the inferior splenic pole as described previously (22,23,31).

Creation of Two-Dimensional Hepatic Tissue Sheets

The creation of hepatocyte sheets was performed as described previously (17). Briefly, hA1AT hepatocytes were isolated from hA1AT mouse livers and hA1AT hepatocyte-repopulated uPA/SCID livers were plated on the temperature-responsive polymer [poly(*N*-isopropylacrylamide)]-coated (PIPAAm) dishes (UpCell, 35 mm, CellSeed, Tokyo, Japan) at a density of 8×10^5 cells/dish. Cell culture was performed at 37°C. Three days later, when the plated hepatocytes reached confluency, the cultured hepatocytes were detached from the culture dish and harvested as a uniformly connected tissue sheet by lowering the culture temperature to 20°C for 15 min. The harvested hepatocyte tissue sheets were attached to a support membrane (CellShifter, CellSeed) for subsequent transplantation.

Hepatocyte Sheet-Based Liver Tissue Engineering Procedure

Prior to the liver tissue engineering procedure, the basic fibroblast growth factor (bFGF)-releasing device was inserted into the subcutaneous space on the back of FVB/N mice as described previously (17,30). Ten days after the device insertion, a highly vascularized subcutaneous platform was developed. Right before the hepatocyte sheet transplantation, an L-shaped skin incision was made to open the vascularized platform followed by removal of the device. The harvested hepatocyte sheet was transplanted onto the vascularized platform with the support membrane. Approximately 5 min later, the support membrane was removed while leaving the hepatocyte sheet in vivo and procedure was finalized by closing the skin wound.

Enzyme-Linked Immunosorbent Assay (ELISA)

Propagation status of the hA1AT hepatocytes in the uPA/SCID livers and functional volume of the liver tissues engineered in the FVB/N mice were assessed by measuring the recipient serum hA1AT concentrations by ELISA. The ELISA used an antibody against hA1AT (DiaSorin, Stillwater, MN) coupled with a secondary goat IgG antibody conjugated with horseradish peroxidase (Resewarch Diagnostics Inc., Flander, NJ) as previously described (10).

Histological Analyses

In some recipient uPA/SCID mice, livers were harvested at 8 weeks after hA1AT hepatocyte transplantation. Portions of subcutaneous tissues of FVB/N mice containing engineered liver tissues were harvested 90 days after hepatocyte sheet transplantation. Liver specimens and subcutaneous tissues were fixed in 10% buffered formalin and embedded in paraffin. The specimens were sliced into 5-μm-thick sections, which were processed for hematoxylin & eosin (H&E) staining or immunohistochemical staining. Immunohistochemical staining was performed using the avidin-biotin complex method with the rabbit polyclonal hA1AT-specific antibody (1:200, YLEM, Roma, Italy). For the cellular glycogen detection, Periodic Acid Schiff (PAS) histochemical staining was performed as described previously (16). To confirm the staining specificity of the cellular glycogen, serial sections were pretreated with salivary amylase for 60 min followed by the same PAS staining procedures.

Statistical Analysis

All of the values calculated in the present study were provided as mean ± SD. Statistical differences in the values were determined by a Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

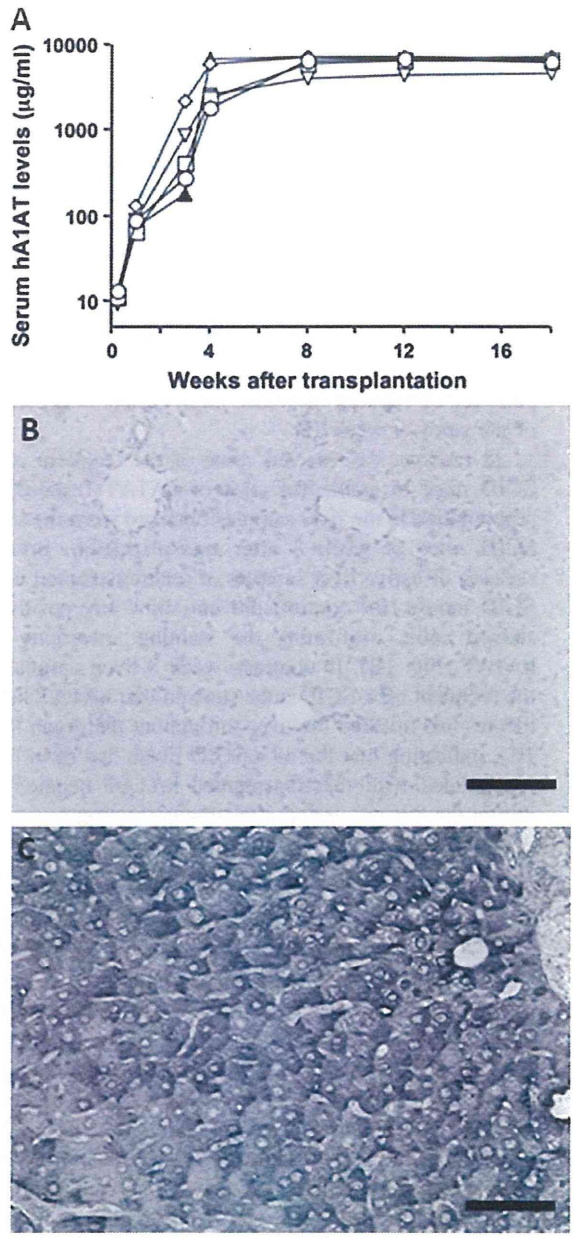

Figure 1. Proliferation and propagation status of the transplanted hA1AT hepatocytes in uPA/SCID mouse livers. (A) Proliferation status of the human α-1 antitrypsin (hA1AT) hepatocytes. At day 0, hA1AT hepatocytes were transplanted into the liver of urokinase-type plasminogen actiovator-severe combined immunodeficient (uPA/SCID) mice ($n = 5$) and recipient serum hA1AT levels were measured by ELISA to determine the level of proliferation and persistence of the transplanted cells. (B, C) hA1AT immunohistochemical staining of the naive uPA/SCID liver (B) and uPA/SCID liver harvested at 8 weeks after the transplantation of hA1AT hepatocytes (C). Scale bars: 100 μm (B, C).

RESULTS

Proliferation of hA1AT Allogenic Hepatocytes in uPA/SCID Livers

Hepatocytes isolated from 13-week-old hA1AT-TG mouse were transplanted into uPA/SCID mice ($n = 5$). Serum hA1AT was detected, which ranged between 8,000 and 14,000 ng/ml, in all of the five recipient uPA/SCID mice at day 2 after transplantation. Following measurements after day 2 showed continuous increases in the serum hA1AT levels through to week 8 (Fig. 1A). In four out of the five recipient mice, the serum hA1AT levels reached the same levels as detected in normal hA1AT-TG mice. The elevated serum hA1AT levels persisted throughout the observation period until the end of the study at week 18.

At random, we selected some of the recipient uPA/SCID mice to assess the state of hA1AT hepatocytes repopulation in the liver samples obtained from the uPA/SCID mice at week 8 after transplantation. hA1AT staining of native liver samples of nontransplanted uPA/SCID mouse (for control) did not show any positively stained cells, confirming the staining specificity for hA1AT (Fig. 1B). In contrast, week 8 liver samples of the recipient uPA/SCID mice showed that hA1AT hepatocytes had invaded the liver throughout the organ (Fig. 1C), indicating that the uPA/SCID livers had been fully repopulated with the transplanted hA1AT hepatocytes during the 8-week period after transplantation.

Isolation of Repopulated hA1AT Hepatocytes From uPA/SCID Livers and Creation of Hepatocyte Sheet

Our confirmation that there was full repopulation of uPA/SCID livers with hA1AT hepatocytes after week 8 allowed us to continue to determine whether these donor repopulated hepatocytes could be a viable option for isolation and recovery to develop them in the hepatocyte sheet protocol. To achieve this goal, we isolated and recovered the repopulated hA1AT hepatocytes form one uPA/SCID recipient at week 12. After the Percoll isodensity centrifugation step, 14.5×10^6 viable hepatocytes were obtained. These recovered hA1AT hepatocytes (recovered hepatocytes) were then plated on the PIPAAm culture dishes at a density of 0.75×10^6 per 35-mm dish as previously described (17) (Fig. 2). We also isolated hA1AT hepatocytes (fresh hepatocytes) from hA1AT mouse and plated the hepatocytes on the PIPAAm culture dishes at the same cell density. Plating efficiency (percent of attached hepatocytes per plated hepatocytes) assessed 24 h after the cell plating were $73.8 \pm 8.8\%$ and $75.5 \pm 5.3\%$ in the recovered hepatocytes and fresh hepatocytes, respectively (four different wells in each group, no statistically significant differences between groups). At day 3 of the culturing protocol, the recovered hepatocytes were found to reach

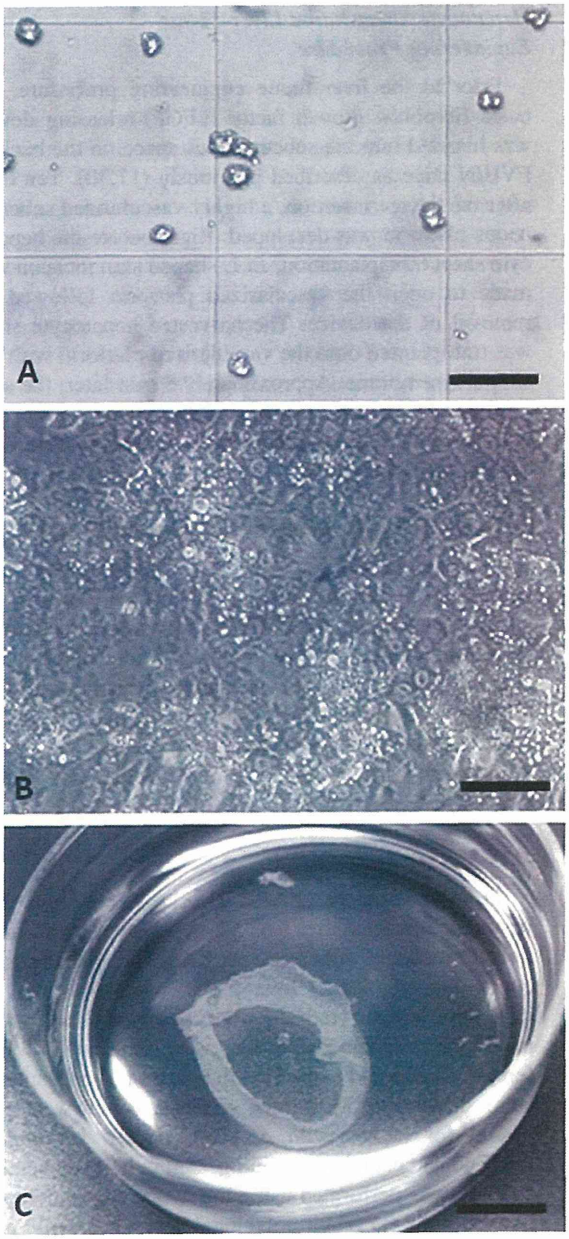

Figure 2. Morphological validation of the recovered hA1AT hepatocytes from the uPA/SCID livers that had been repopulated with the hA1AT hepatocytes. (A) Morphology of the isolated hepatocytes from uPA/SCID recipient. Photo was taken following the trypan blue exclusion test. (B) Recovered hA1AT-hepatocytes cultured on the poly(*N*-isopropylacrylamide) (PIPAAm) dish for 3 days documenting the confluent nature of the cells. (C) Observation of the cultured hA1AT-hepatocytes from the PIPAAm dish as a uniform tissue sheet. Scale bars: 100 μm (A, B), 1 cm (C).

confluence. Lowering the culture temperature from 37°C to 20°C for 15 min resulted in the spontaneous detachment of the cultured hepatocytes from the culture plates as a uniform cell sheet.

Liver Tissue Engineering Using Hepatocyte Sheet Made of Recovered Hepatocytes

The recovered hepatocyte sheet was then used for liver tissue engineering with the FVB/N mice using a similar procedure as previously described (17). Hepatocyte sheet was transplanted to the subcutaneous site that had been prevascularized following the insertion of a bFGF-releasing device. Functional activity of the engineered liver tissues was assessed by measuring serum hA1AT levels in the recipient FVB/N mice. As shown in Figure 3A, recipient mice showed stable and persistent serum hA1AT levels (ranged between 2,000 and 5,000 ng/ml), suggesting that the engineered liver tissues could be viable and stably maintained throughout the 90-day experimental period.

Subcutaneous tissue samples taken 90 days after the tissue engineering procedure were assessed for histological examination. H&E staining and hA1AT immunostaining revealed that liver tissues were engineered in the subcutaneous space with the hepatocyte-specific phenotypes (Fig. 3B, C). PAS staining revealed that the engineered liver tissues were positive for the function of glycogen synthesis and storage (Fig. 3D). Negative signals in the PAS staining on salivary amylase-pretreated samples confirmed staining specificity for cellular glycogen (Fig. 3E). It is important to note that there was no evidence of tumor formation observed in any of the engineered tissues.

DISCUSSION

The present study describes an in-mouse hepatocyte propagation system by which uPA/SCID mouse livers were found to fully reconstitute transplanted allogeneic mouse primary hepatocytes. This full reconstitution event was conducted by active and continuous hepatocyte proliferation as evidenced by the progressive increase in the hA1AT serum levels of the recipient uPA/SCID mice and massive occupation of hA1AT staining-positive hepatocytes in the recipient livers. The present study also showed that these propagated donor hepatocytes were able to be recovered, cultured, and subsequently used in the creation of hepatocyte sheets. The de novo generated hepatocytes sheets were able to be transplanted into a prevascularized subcutaneous space resulting in the successful engineering of an ectopic functional liver tissue.

We have established several innovative experimental approaches to create functional liver system in vivo (5,11–13,16,17,30). One of the key technologies in our series of developments is the creation of a uniform hepatocyte sheet using the PIPAAm culture dishes (17,27). The present study demonstrated that the hepatocytes recovered from the propagated livers of uPA/SCID mice were adhesive to the PIPAAm dish surfaces, and appeared to be at a similar efficiency of newly isolated primary hepatocytes that have not been previously isolated and propagated. The attached hepatocytes in the present study showed natural cell extension and became confluent by day 3 of culture. These cells showed the same morphological alterations associated with normal primary hepatocytes. This morphological similarity suggests that the microtubules and microfilaments were likely intact in the recovered hepatocytes.

This study as well as previous studies by our group have shown that hepatocyte sheets can be obtained by natural detachment of the primary cultured hepatocytes from the PIPAAm culture surfaces through a simple change in the ambient temperature for a brief period of time (17) without the need for any proteolytic enzyme treatment (28,29). This simple cell harvesting approach does not damage the cell-to-cell contact allowing for the retention of the intricate cell–cell intercommunication systems (i.e., bile canaliculi, desmosomes, gap junctions) (17). Although the ultrastructural examination was not performed in the present study, it is reasonable to postulate that the hepatocyte sheet made of the recovered hepatocytes possessed intercellular communications previously observed in our system using newly isolated hepatocytes considering that these functional intercellular communications are important in maintaining the structure of monolayered hepatocyte sheet. The integrity of the hepatocyte sheet using the recovered donor hepatocytes is similar to that of de novo isolated cells, and is readily transferable to a support membrane for the transplantation process.

We have previously reported that the uPA/SCID mice have the innate property to actively proliferate donor primary hepatocytes of xenogenic origin, including human hepatocytes, following transplantation into the liver (22, 23,31). The functional preservation of the human hepatocytes during their active proliferation phase in the uPA/SCID livers had been previously established as determined by the production of a hepatocyte-specific coagulation factor IX (FIX) (23). As an extension of the FIX findings, we have recently found that human hepatocytes in the uPA/SCID livers can maintain their cellular machinery to produce other hepatocyte-specific coagulation factors and anticoagulation factors (unpublished data). The availability of the propagated human hepatocytes in the uPA/SCID system for in vitro analyses was established in an earlier study by Yoshitsugu et al. (31). Since the recovered human hepatocytes can be cultured and express various drug-metabolizing enzymes,

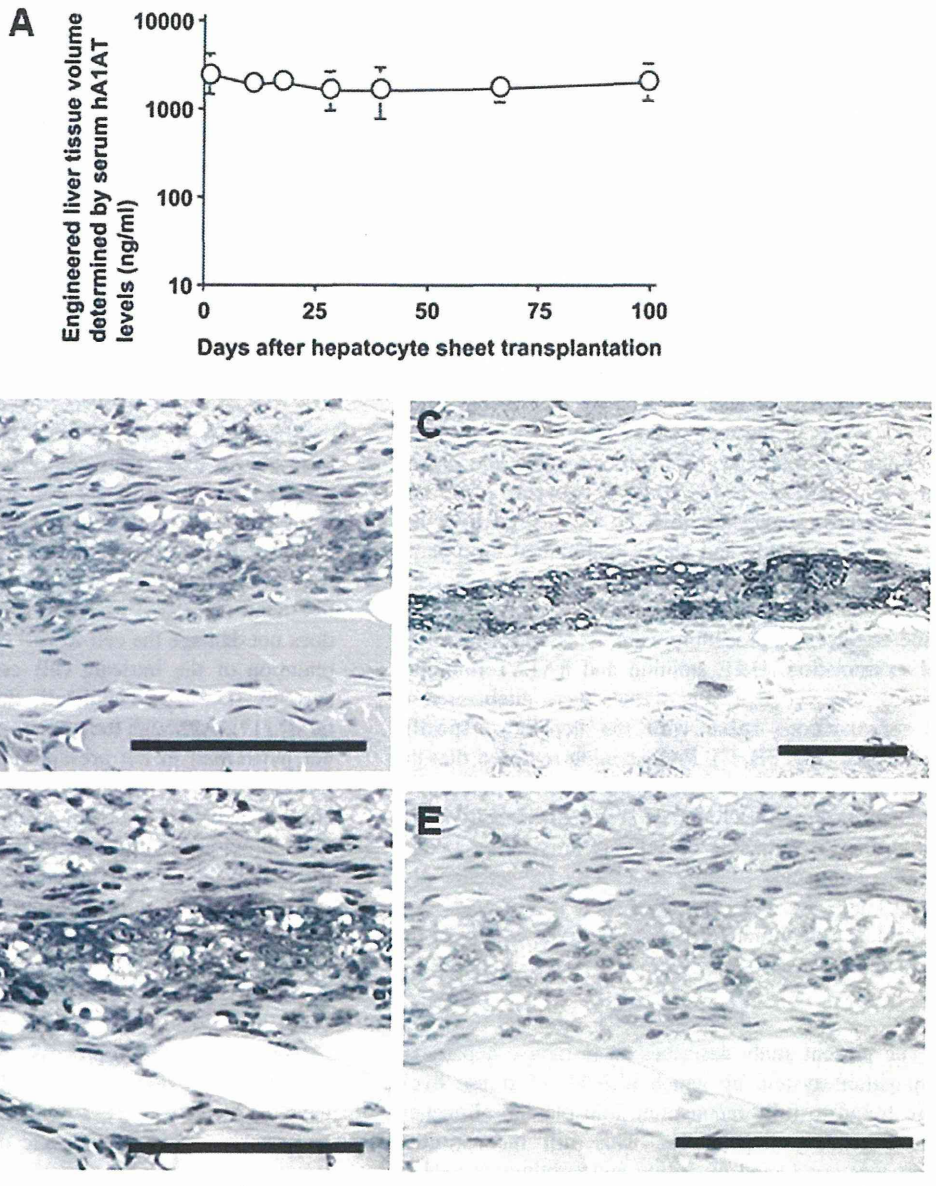

Figure 3. Functional maintenance of the engineered liver tissue within the subcutaneous space. (A) Functional volume of the liver tissues engineered in the subcutaneous space was determined by measuring recipient serum hA1AT levels by ELISA. Hepatocyte sheet composed of the hA1AT hepatocytes as shown in Figure 2 was generated and this harvested hepatocyte sheet was then transplanted into the subcutaneous space where an active vascular network had been induced using a basic fibroblast growth factor (bFGF)-releasing device. Histological findings by (B) H&E staining, (C) immunohistochemical staining for hA1AT, and (D, E) Periodic Acid Schiff (PAS) staining of the engineered liver tissue from samples obtained at day 90 after the hepatocytes sheet transplantation. Functions for cellular glycogen synthesis and storage were confirmed by PAS staining in sections that were either nontreated (D) or pretreated with salivary amylase (E). Scale bars: 100 μm (B–E).

it was demonstrated that these recovered hepatocytes could be a valuable cell source for the study of a variety of liver functions (31). From these previous studies as well as the findings in our current study, we suggest that the propagated hepatocytes in the uPA/SCID livers could be as effective as the original primary hepatocytes with respect to their cellular functionalities. Although the present study clearly demonstrated that the hepatocytes obtained by in-mouse propagation system are useful for the creation of hepatocyte sheets, their availability for other hepatocyte-based approaches have not been investigated. Future studies will need to be conducted if the recovered hepatocytes can be transplanted and/or engraftable to other sites, such as the liver parenchyma itself or other ectopic sites within the body other than the subcutaneous space.

For potential clinical application for this type of hepatocyte-based therapeutic approach, the potential risk of oncogenesis deriving from the transplanted hepatocytes needs to be addressed, particularly due to the active level of proliferation that is being undergone following the transplantation procedure (8). In the present study, the gross morphology of all six of the recipient uPA/SCID livers at the time of sacrifice and/or hepatocyte isolation was normal. Histological investigation of the liver specimens did not show any cancerous or precancerous lesions. In parallel to these findings, no cancer development was observed in our previous study in which human hepatocytes were transplanted into the same strain of mice following the active proliferative phase of the donor hepatocytes (23). The best to our knowledge and as recently reviewed by Marongiu et al. (8), cancer cell development from the transplanted normal primary hepatocytes has not been reported in the uPA/SCID system. Other types of small animal models that are able to coordinate the process of proliferation of transplanted hepatocytes toward the replacement of recipient livers have been established, including the fumaryl-acetoacetatehydroxylase-null Rag2^{-} mouse (1), the retrorsine-treated (6–8), or monocrotaline-treated rat models (25). In none of the models has there been any evidence of cancer cell development being documented. As far as we are aware from the previously documented and the present findings, it could be summarized that the risk of the cancer cell development from the normal hepatocytes during in-animal hepatocyte proliferation process appears minimal.

In all, we have shown that the use of the uPA/SCID mice as a viable recipient model to actively proliferate allogenic primary hepatocytes in their livers for subsequent use in the development of newly propagated hepatocytes or as a cell sheet system. These results taken together provide strong evidence that isolated hepatocytes can be efficiently propagated using an in-mouse liver procedure, and can be a potential valuable method to generate a new source of hepatocytes for hepatocyte-based therapies.

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