Journal of Bioscience and Bioengineering
VOL. 128 No. 6, 766–772, 2019

www.elsevier.com/locate/jbiosc

In vitro and *in vivo* fabrication of stable human hepatocyte tissue in combination with normal fibroblasts derived from donors of various ages

Yusuke Sakai,* Makiko Koike, Tomomi Murai, Masaaki Hidaka, Akihiko Soyama, Mitsuhisa Takatsuki, and Susumu Eguchi

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Received 4 December 2018; accepted 17 May 2019
Available online 13 June 2019

In order to establish a minimally invasive and safe liver regenerative technology, a technique for fabricating liver tissue possessing a vascular network was developed by subcutaneously transplanting a cell sheet composed of primary human hepatocytes and normal fibroblasts. However, differences in fibroblast characteristics owing to donor age may threaten the stability of liver tissue regenerated via this technology. Herein we describe the influence of fibroblasts from multiple donors on the fabrication of engineered human hepatocyte tissues *in vitro* and *in vivo*. Primary human hepatocytes were cultured with seven strains of fibroblasts derived from the skins of donors of various ages, ranging from a fetus (12 weeks) to the elderly (69 years). Engineered hepatocyte sheets were successfully harvested for all strains. At 2 weeks after the subcutaneous transplantation of the hepatocyte sheets into mice, the highest human albumin (hALB) serum concentration was noted in the mouse containing fibroblasts from a 12 year old (TIG-118). Since the platelet-derived growth factor subunit B (PDGFB) gene expression of TIG-118 cells was significantly higher than that in the other cells, PDGFB may be considered to play an important role in the initial subcutaneous engraftment of primary human hepatocytes. Even though hALB concentration exhibited a parabolic tendency with age, there was no statistically significant difference noted within 6–8 weeks after transplantation. The present study demonstrates that this technology can produce consistent and stable hepatocyte sheets that exhibit long-term survival and liver-specific functionality *in vivo* regardless of the fibroblast donor age.

[**Key words:** Hepatocyte; Fibroblast; Donor age; Albumin; Cell sheet; Tissue engineering]

Liver tissue engineering is one of the therapeutic approaches to the treatment of lethal acute liver failure and inherited liver disease; it is also an important tool for basic research and drug metabolism assays. Numerous approaches have been taken for establishing liver regenerative technology; for example, three-dimensional hepatocyte culture (1), liver-mimetic structure formation utilizing cell arrangement and co-culture systems (2), liver bud technology based on embryology (3), and the production of artificial organs using decellularized scaffolds (4,5). Research on the origin of hepatocytes, such as primary- or stem cell-derived hepatocytes, that play a central role in the liver, and the types of cells to be complex are being actively carried out (2,3,6). However, it is still insufficient for determining whether an established technology can be stably implemented. For example, even if the same cell type is used each time, differences in cell characteristics due to differences in donors' age may result in clinical failures.

We aimed to establish a safe liver regenerative technology consisting of only cells and the proteins they produce, such as an extracellular matrix and growth factors. A cell organoid in sheet format composed of primary human and rat hepatocytes (or HepaRG cells) and normal skin-derived fibroblasts can rapidly develop into subcutaneous human liver tissue with a vascular network upon implatation (7,8). This technique allows rapid subcutaneous engraftment of hepatocytes with high probability and without pre-transplant angiogenesis treatment and/or addition of endothelial cells. Skin-derived fibroblasts are one of the stable and safe cell sources that are used as feeder cells because they are easy to harvest and culture and do not show multipotency as stem cells do. Some relationships between the donor age and fibroblast characteristics have been reported, including differences in telomere length (9), proliferative potential (10), and collagen/collagenase production (11,12). However, the effects of fibroblast donor age on the fabrication of hepatocyte sheets, the ability to produce growth factors, and the liver-specific functions *in vivo* are yet to be elucidated.

In this study, we describe the influence of fibroblasts from multiple donors on the fabrication and subcutaneous functionality of engineered primary human hepatocyte sheets. Seven fibroblast strains from individuals of diverse ages, from a fetus (12 weeks) to the elderly (69 years) were utilized as feeder cell layers. The findings of this study represent an important step toward clarifying whether stable and safe subcutaneous human liver tissue can be fabricated.

* Corresponding author. Tel.: +81 95 819 7316; fax: +81 95 819 7319.
E-mail address: y.sakai.bioeng@gmail.com (Y. Sakai).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.05.009

MATERIALS AND METHODS

Research ethics and patient consent Ethical approval was obtained from the human ethics review committee of Nagasaki University School of Medicine for human hepatocyte isolation, storage, culture, and transplantation into laboratory animals (09022449-5 and 11072745-3). Ethical approval for hepatocyte transplantation was obtained from the Animal Care and Use Committee (1304111054-3) and the Recombinant DNA Experiment Safety Committee of Nagasaki University (1304031224-3). This study was carried out in accordance with the approved guidelines. Informed consent was obtained from all human hepatocyte donors.

Primary human hepatocyte isolation Primary human hepatocytes (HH) were isolated from resected human liver tissue with the exception of tumor, which was obtained from two donors (70's and 50's, male, metastatic liver tumor) during liver surgery, by perfusing with 130 U/mL collagenase (Wako Pure Chemical, Osaka, Japan) and centrifuging at 70 $\times g$ for 7 min at 4°C in 25% Percoll Plus solution (GE Healthcare, Tokyo) (8). Hepatocytes were established to have 92.8% and 96.4% viability using a trypan blue exclusion test and were used for the subsequent experiments, respectively.

Human fibroblast culture Normal human diploid fibroblast cells, which were derived from the skin of seven donors of different ages, were purchased from Health Science Research Resources (Osaka, Japan) (Table 1). The cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen, Carlsbad, CA, USA).

Fabrication of engineered hepatocyte sheets Fresh primary human hepatocytes were plated at 1.04×10^5 cells/cm^2 onto a confluent layer of fibroblasts which were plated 3 days previously onto temperature-responsive culture dishes (TRCDs) (UpCell; CellSeed, Tokyo, Japan). The details of each fibroblast strain are provided in Table 1. The human hepatocytes with fibroblasts (TIG/HH) were cultured in Hepato-STIM culture medium (Corning Glass Works, Corning, NY, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was changed at days 1 and 3 after hepatocyte inoculation. After 4 days of hepatocyte inoculation, TRCDs were incubated at 20°C for several hours for producing engineered hepatocyte tissues in a sheet format. Samples of media at 1–3 days of culture were collected and stored at −20°C until assayed.

Subcutaneous transplantation The engineered hepatocyte sheets (1.0×10^6 inoculated hepatocytes) were subcutaneously transplanted into NOG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/ShiJic; Central Institute for Experimental Animals, Kanagawa, Japan) or NSG mice (NOD.Cg-Prkdc^{scid} I/2rg^{tm1Wjl}/SzJ; Charles River Japan Inc., Kanagawa, Japan) using a glass plate (8). All mice were males, aged 14–25 weeks old and weighed 25.5–30.4 g (average, 27.8 g) (NOG mice) or aged 10–37 weeks old and weighed 22.0–29.3 g (average, 26.1 g) (NSG mice). The mice were randomly used for the transplantation. At 2, 4, 6, and 8 weeks after transplantation, 100–200 μL of blood was collected from the tails of the NOG mice. At 1 day after transplantation, 100 μL of blood was collected from the tails of NSG mice.

Histology The engineered hepatocyte sheets and subcutaneous hepatocyte tissues at 1 day after transplantation into NSG mice were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (Wako Pure Chemical). Fixed samples were embedded in paraffin, cut into 5-μm cross-sections, and mounted on Matsunami adhesive silane-coated slides (Matsunami Glass, Osaka, Japan). The sections were deparaffinized and stained with hematoxylin and eosin (HE; Muto Pure Chemicals, Tokyo). For immunochemical fluorescent staining, deparaffinized sections were heated for 10 min with 1 mM EDTA and 0.1% Triton X-100 in 1 mM Tris–HCl buffer (pH 9.0) using a microwave. The sections were incubated in Biotin-Blocking System (Dako Japan, Kyoto, Japan) for 15 min and then blocked using 5% bovine serum albumin (BSA) and 0.1% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature. Blocked sections were incubated in TBS containing 5% BSA, 0.1% Tween 20, and the following antibodies (both from Abcam, Cambridge, MA, USA) overnight at 4°C: rabbit anti-human albumin (hALB; ab2406; 1:3000) or mouse anti-human vimentin (hVIM; ab8069; 1:500). The following sections were then incubated with an appropriate secondary antibody (all from Sigma–Aldrich Japan, Tokyo, Japan) for 1 h at room temperature: biotin-conjugated goat anti-rabbit IgG (B8895; 1:800); tetramethylrhodamine isothiocyanate (TRITC)-conjugated ExtrAvidin (E3011; 1:150); or fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (F9137; 1:200). Slides were mounted using ProLong gold antifade mounting medium (Invitrogen). Fluorescence images were captured using a confocal laser scanning microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany).

mRNA expression analysis of fibroblasts (real-time PCR) The fibroblasts were seeded at 2.08×10^4 cells/cm^2 onto TRCDs and the cells were cultured in supplemented MEM for 3 days followed by culture in supplemented Hepato-STIM culture medium. The medium was changed at days 1, 3, 4, and 6 after fibroblast seeding.

Total RNA of fibroblasts after 7 days of culture was extracted by using a spin column (NucleoSpin RNA II; Macherey–Nagel, Düren, Germany). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). PCR was performed in an Applied Biosystems StepOnePlus Real-time PCR system using the TaqMan Gene Expression Assay Kit (Applied Biosystems; Table 2). The comparative cycle time method was used for quantifying the gene expression levels, which were normalized to that of human ACTB (TIG-3S was set as 1.0). These steps were performed in accordance with the manufacturer's instructions.

Liver-specific function assay (sandwich ELISA) The concentrations of hALB in the medium and the mice serum samples were measured using enzyme-linked immunosorbent assay (ELISA) using rabbit anti-hALB (6 μg/mL) and HRP-conjugated goat anti-hALB (10 μg/mL) (MP Biomedicals, LLC-Cappel products, Irvine, CA, USA). Concentrations of human alpha 1-antitrypsin (hA1AT) were measured using goat anti-hA1AT (5 μg/mL; Bethyl Laboratories, Montgomery, TX, USA) and HRP-conjugated goat anti-hA1AT (7 μg/mL; Fitzgerald Industries International, Inc., Concord, MA, USA).

Statistical analysis Data are presented as mean ± standard deviation (SD) from at least three different samples. Means of continuous numerical variables were compared using one-way or two-way analyses of variance (ANOVA) and *t*-test on GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

TABLE 1. Human skin tissue sources for fibroblasts.

Cell name	Age	Sex	PN	PDL
TIG-3S	Fetus (12 W)	M	JCRB0544	40.9
TIG-121	8 M	F	JCRB0536	25.2
TIG-120	6 Y	F	JCRB0542	29.1
TIG-118	12 Y	F	JCRB0535	26.2
TIG-113	21 Y	F	JCRB0539	30.3
TIG-112	40 Y	F	JCRB0533	30.9
TIG-103	69 Y	F	JCRB0528	26.7

PN, product number; PDL, population doubling level; JCRB, Japanese Collection of Research Bioresources.

RESULTS

Fabrication of stable engineered hepatocyte sheets Primary human hepatocytes adhered well onto all fibroblast layers irrespective of the fibroblast donor age (Fig. 1A). In each case, engineered hepatocyte tissues were fabricated and demonstrated a complete cell sheet format after 4 days of hepatocyte culture (Fig. 1B). Immunostaining of cross-sections indicated that all fibroblasts had migrated from the bottom to the upper layer. The sheets remained viable, continued to synthesize hALB *in vitro*, and were composed of two or more layers.

Expressions of mRNAs involved in collagen synthesis and matrix metallopeptidase The expressions of mRNAs involved in collagen synthesis and matrix metallopeptidase were analyzed in cultured fibroblasts. COL1A1 expressions were no apparent relationship with donor age (Fig. 2A). MMP1 in TIG-112 cells derived from the older donor was significantly higher than that in the other cells (Fig. 2B).

Expressions of mRNAs involved in angiogenesis in fibroblasts *in vitro* The expressions of five mRNAs involved in angiogenesis were analyzed in cultured fibroblasts. PDGFB expressions in TIG-118 and TIG-113 cells were significantly higher than that in other cells (Fig. 3A). Gene expressions of VEGFA, FGF2, and EGF tended to be higher in fibroblasts derived from younger donors (Fig. 3B, D, E). TGFB1 expression in TIG-103 cells

TABLE 2. TaqMan gene expression assay numbers for real-time PCR analysis.

Gene symbol	Gene name	TaqMan assay no.
COL1A1	Collagen type I alpha 1	Hs00164004_m1
MMP1	Matrix metallopeptidase 1	Hs00899658_m1
PDGFB	Platelet-derived growth factor subunit B	Hs00966522_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
TGFB1	Transforming growth factor, beta 1	Hs00998133_m1
FGF2	Fibroblast growth factor 2 (basic)	Hs00266645_m1
EGF	Epidermal growth factor	Hs01099999_m1
ACTB	Actin, beta	Hs99999903_m1

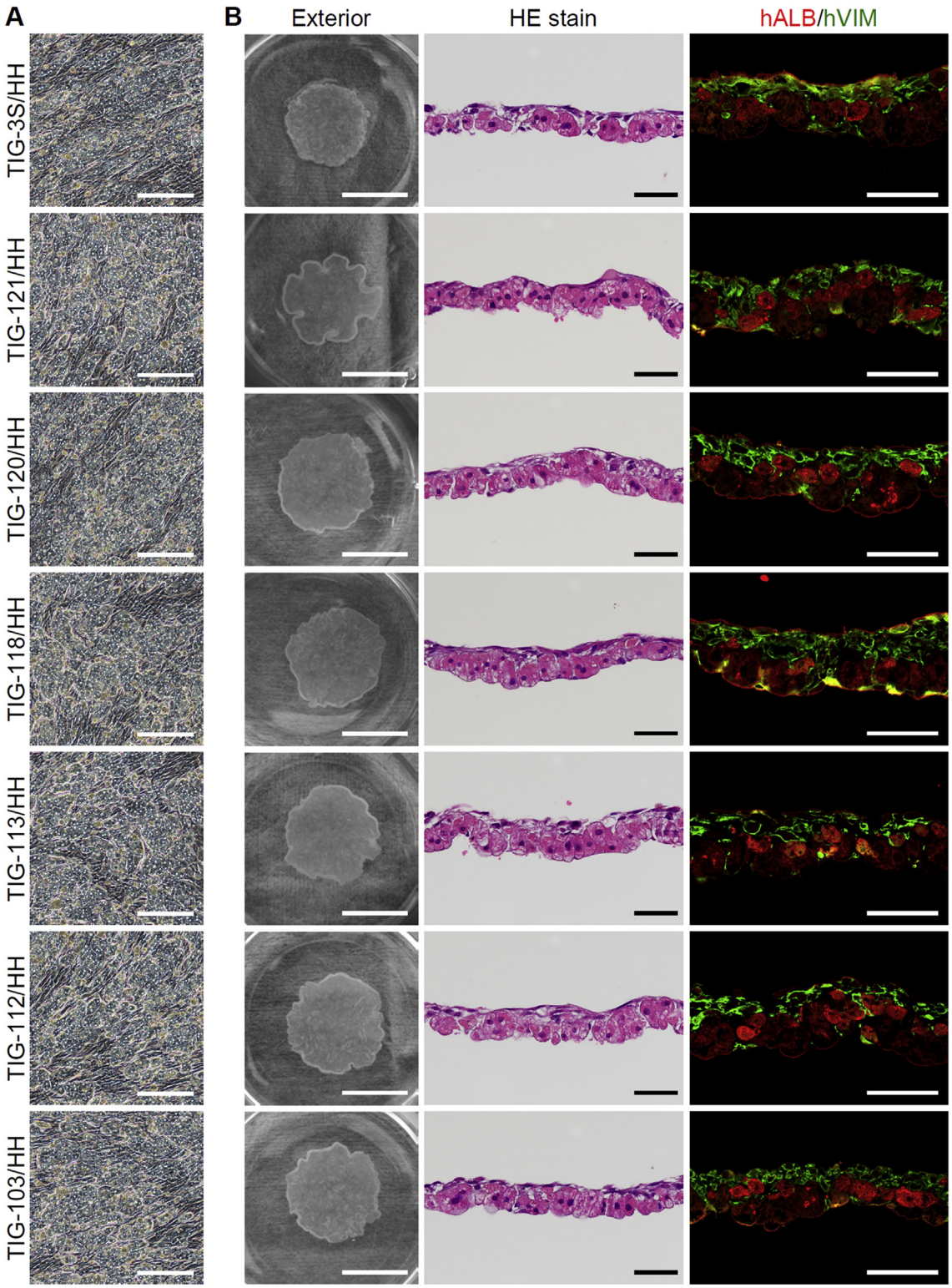

FIG. 1. Engineered human hepatocyte sheets at 4 days of hepatocyte culture. (A) Phase-contrast micrographs of adhered-cultured hepatocytes and fibroblasts. (B) Engineered human hepatocytes combining normal fibroblasts. Exterior images (left), HE stained images (middle), and immunofluorescent stained images (right). Red (TRITC), hAlb (hepatocytes); green (FITC), hVIM (fibroblasts). The bars represent 200 μm (A), 10 mm (B, left), and 50 μm (B, middle and right).

derived from the oldest donor was significantly lower than that in some of the other cells, but overall there was no apparent relationship with donor age (Fig. 3C).

Albumin synthesis activity of co-cultured hepatocytes *in vitro* Fig. 4 shows hALB synthesis rate from hepatocytes at day 1–3 of co-culture. hALB synthesis from TIG-113/HH was higher than other culture conditions. TIG-3S/HH showed the lowest synthesis rate of hALB *in vitro*.

Liver-specific functions of subcutaneous transplanted hepatocyte sheets The concentrations of hALB in the mouse

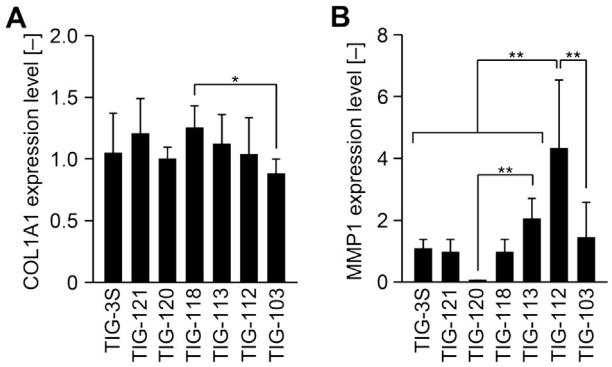

FIG. 2. *In vitro* mRNA expression of collagen synthesis and matrix metallopeptidase in fibroblasts after 7 days of culture. (A) Human COL1A1 and (B) MMP1 ($n \geq 9$ from two independent cell preparations). Data presented as the mean ± SD, $^{**}P < 0.01$ and $^{*}P < 0.05$ (one-way ANOVA).

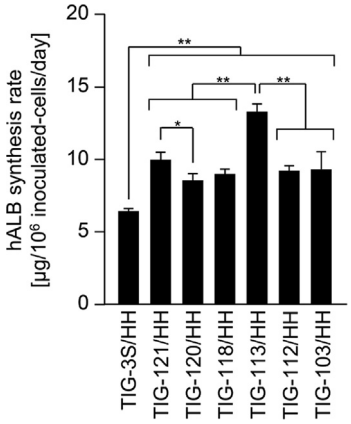

FIG. 4. *In vitro* hALB synthesis at 1–3 days of co-culture ($n = 5$). Data presented as the mean ± SD, $^{**}P < 0.01$ and $^{*}P < 0.05$ (one-way ANOVA).

sera demonstrated the survival and functionality of subcutaneously transplanted human hepatocytes. Concentrations of hALB were detectable in mouse serum in all cases for at least 8 weeks after transplantation (Fig. 5A). The hALB concentration at 2 weeks after transplantation of the sheet containing the TIG-3S cell line (TIG-3S/HH) was significantly lower (approximately 50%) than that in the other cases. The concentration of hALB tended to be slightly lower in hepatocyte sheets that were produced using fibroblasts derived from older donors (TIG-112/HH and TIG-103/HH). TIG-118/HH exhibited the highest hALB serum concentration. Four weeks after transplantation, a significant difference was observed only between TIG-118/HH and TIG-3S/HH. Even though the hALB serum concentration pattern exhibited a parabolic tendency, peaking with TIG-118/HH, there were no significant differences noted between any of the mice at 6 and 8 weeks after transplantation. Trends similar to hALB concentration patterns were also observed for hA1AT (Fig. 5B).

Morphology and function of hepatocyte tissue immediately after transplantation HE staining image of subcutaneous hepatocyte tissue from TIG-118/HH on day 1 after transplantation showed that red blood cell infiltration was partially observed at the edge of the tissue although there was no clear angiogenesis network (Fig. 6A). In contrast, it was very difficult to identify red blood cells in the tissue formed from TIG-103/HH (Fig. 6B). The hALB concentration in serum at this time was significantly higher in the tissue formed from TIG-118/HH than the tissue formed from TIG-103/HH although there is no significant (Fig. 6C).

DISCUSSION

Considerable research has focused on the reconstruction of vascular networks, which are key to oxygen supply, for improving engraftment and three-dimensional tissue fabrication involving

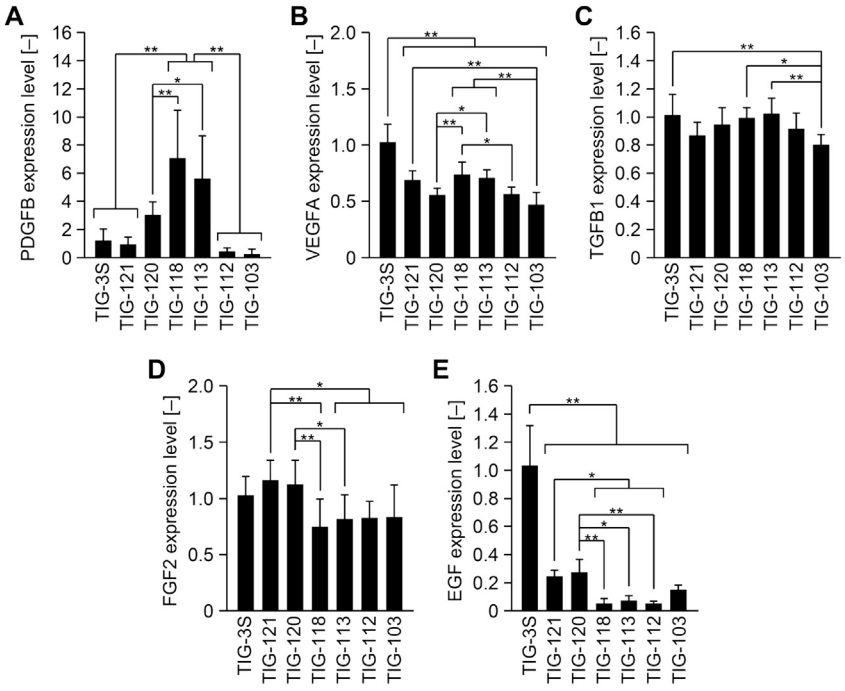

FIG. 3. *In vitro* mRNA expression of angiogenic cytokines in fibroblasts after 7 days of culture. (A) Human PDGFB, (B) VEGFA, (C) TGFB1, (D) FGF2, and (E) EGF ($n \geq 9$ from two independent cell preparations). Data presented as the mean ± SD, $^{**}P < 0.01$ and $^{*}P < 0.05$ (one-way ANOVA).

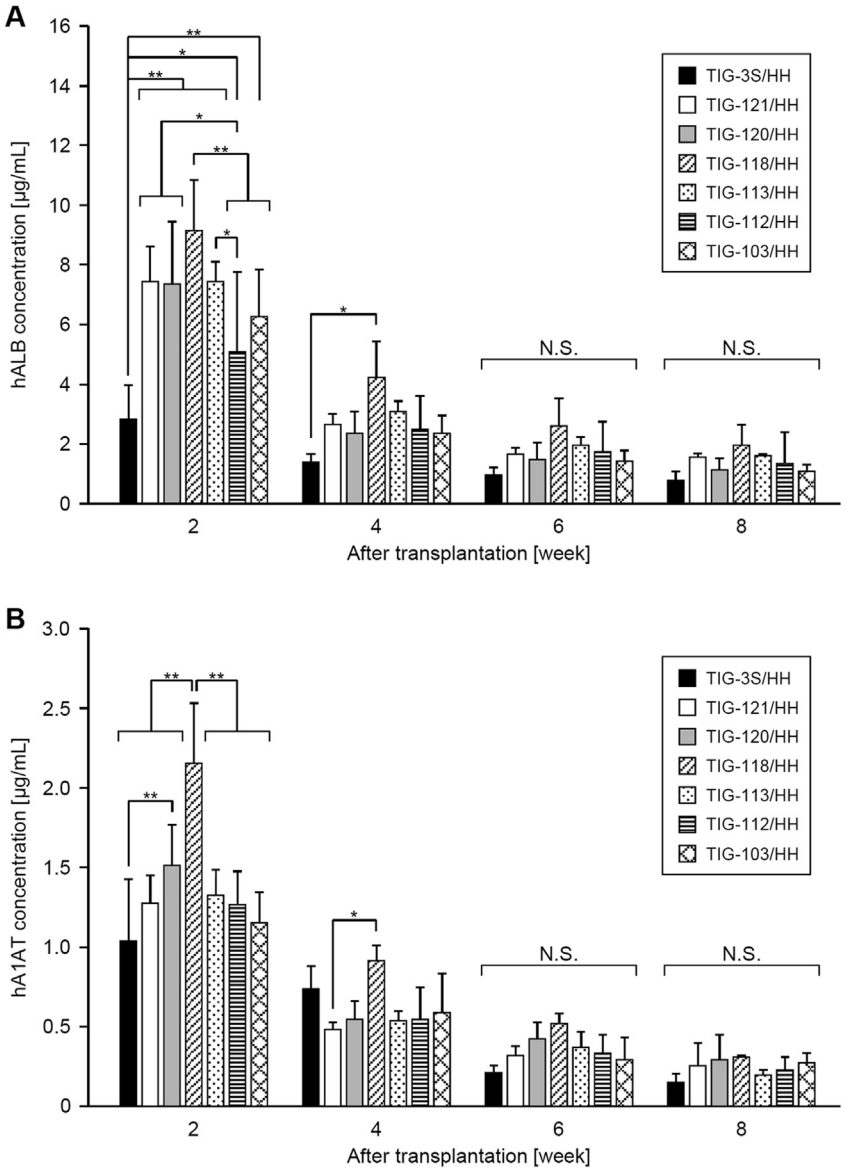

FIG. 5. *In vivo* liver-specific functions in NOG mice after subcutaneous transplantation of engineered human hepatocyte sheets. (A) hALB and (B) hA1AT concentrations in mouse serum ($n \geq 3$). Data presented as the mean ± SD, $^{**}P < 0.01$ and $^{*}P < 0.05$ (two-way ANOVA). N.S., not significant.

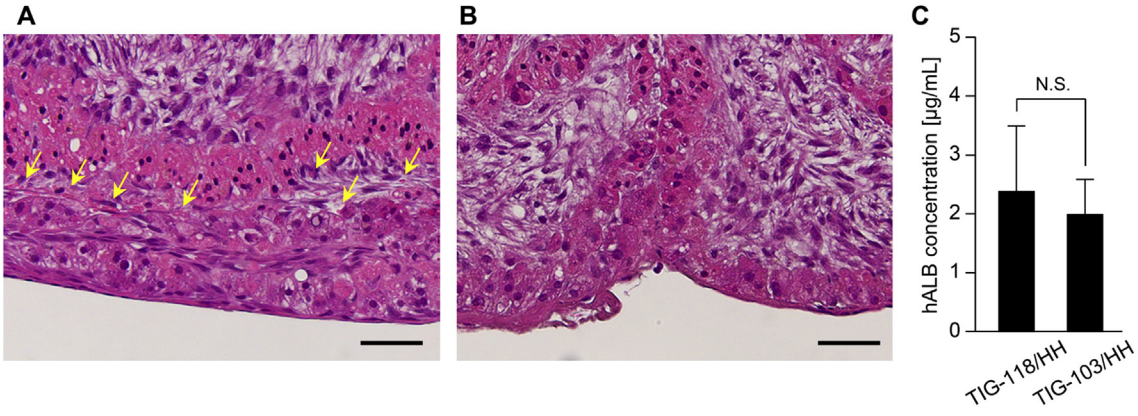

FIG. 6. *In vivo* HE stained images and liver-specific function in NSG mice after 1 day of subcutaneous transplantation. Subcutaneous hepatocyte tissues from TIG-118/HH (A) and TIG-103/HH (B). The bars represent 50 μm. The yellow arrows indicate the red blood cells. (C) hALB concentration in mouse serum at 1 day after transplantation ($n = 5$). Data presented as the mean ± SD. N.S., not significant (*t*-test).

hepatocytes, which have a high oxygen demand (13). Even though pre-transplant angiogenic treatment for preparing suitable subcutaneous sites and/or addition of endothelial cells into cultured hepatocyte organoids contribute to improvements in the subcutaneous engraftment rate of hepatocytes, these approaches involve complicated operations and long preparation periods for subcutaneous liver tissue (2,3,6,14,15). We have reported an innovative system using fibroblasts, which are easy to harvest and culture and are highly stable (16,17). Human and rat primary hepatocytes (or HepaRG cells) combined with skin-derived fibroblasts have been established to be capable of developing subcutaneous vascularized liver tissue and prolonging life in a rodent model with fatal liver damage (7,8). However, it is not clear whether consistent results can be obtained using fibroblasts from donors of different ages.

To address this issue, normal human fibroblasts established from seven healthy donors of different ages were used. The feasibility of fabricating engineered hepatocyte sheets composed of primary human hepatocytes and fibroblasts was examined. Hepatocyte sheets were successfully fabricated (Fig. 1) with all fibroblasts ranging from the fetus (12 weeks) to the elderly donor (69 years). Fibroblasts reportedly decrease collagen production and increase collagenase (matrix metalloproteinase; MMP) production with age (11,12). Some strains of the fibroblasts used in this study actually decreased COL1A1 and increased MMP1 gene expression with age (Fig. 2). These facts could limit the rapid adhesion of hepatocytes to fibroblasts and additionally inhibit stable engineered hepatocyte sheet formation and its structural maintenance. Despite this concern, hepatocytes adhered to all the fibroblasts 1 h after seeding. In addition, confluence was maintained for at least 4 days of culture. The engineered hepatocyte tissues in all cases exhibited a complete cell sheet format with a multilayered structure of sufficient thickness for handling during transplantation (8,16). The hepatocyte sheets were of uniform thickness and maintained their viability and ability to synthesize hALB *in vitro*, which is one of the hepatic functions.

As described, one important issue is the construction of a vascular network for hepatocyte engraftment and liver tissue construction (18). We evaluated, for the first time to our knowledge, the differences in growth factors involving angiogenesis between fibroblasts from donors of different ages. Most of the factors, such as VEGFA, FGF2, and EGF, were more highly expressed in fibroblasts from younger donors, and there was little difference in TGFB1 (Fig. 3B–E). Only PDGFB gene expression exhibited a tendency different from other factors (Fig. 3A), with significantly higher expressions in TIG-118 and TIG-113 cells. Since the expression of these growth factors might affect human hepatocyte functions *in vitro*, hALB concentrations from the medium samples were analyzed. Comparing all culture conditions, hALB synthesis activity of human hepatocytes co-cultured with TIG-113 cells was higher, and co-culture with TIG-3S cells was lower (Fig. 4). A very high density of fibroblasts may down-regulate hepatocyte functions (19), and the proliferative ability of the fetal fibroblasts may have been the reason for this observation because TIG-3S cell density at hepatocyte seeding was approximately 1.5 times higher than that for the other fibroblasts (Fig. S1). The high proliferative capacity of the fetus-derived fibroblast TIG-3S cells is partially consistent with reports stating that younger donors also exhibit better proliferative capacity (10). This could be one of the factors that explain the significantly lower concentrations of hALB produced by TIG-3S/HH *in vitro* and *in vivo* (Figs. 4 and 5).

There was nearly no difference in the proliferation of fibroblasts obtained from donors between 8 months and 69 years (Fig. S1). Nevertheless, there is a difference of hALB serum concentration in the early engraftment of hepatocytes (Fig. 5). This observation suggested that there are other factors that control hepatocyte survival and liver-specific functionality *in vivo* aside from fibroblast density resulting from enhanced cell proliferation ability. Interestingly, concentrations of hALB in serum exhibited tendencies similar to the PDGFB gene expression pattern (Fig. 3A). PDGFB promotes endothelial cell proliferation and lumen formation and contributes to lumen stabilization (20,21). Therefore, PDGFB gene expression is an important factor in the construction of subcutaneous human liver tissue using our fibroblast system, because it rapidly produces stable vascular networks and promotes excellent initial engraftment. This fact will greatly contribute to the selection of fibroblasts for transplanted hepatocyte sheets for use in adjuvant treatment for acute liver failure, which requires maximum effect to be achieved immediately after transplantation. On the other hand, since there was no significant difference in hALB concentrations from a long-term perspective of 6–8 weeks, the differences in fibroblast features owing to donor age may not have much effect when our technique is used for long-term treatment, such as in hereditary liver disease.

In order to evaluate the initial engraftment subcutaneously, human hepatocytes co-cultured with TIG-118 cells and TIG-103 cells, which obtained relatively huge difference in PDGFB expression, were further investigated. HE staining images of subcutaneous hepatocyte tissues on day 1 after transplantation showed that red blood cell infiltration was partially observed although there was no clear angiogenesis in co-cultured tissue with TIG-118 cells (Fig. 6A). Furthermore, the hALB concentration of the serum from co-cultured tissue with TIG-118 cells on day 1 after transplantation that tend to be high also suggests that the initial engraftment was performed with high efficiency (Fig. 6C). These results may provide evidence for supporting early angiogenesis and hepatocyte engraftment by high expression of PDGFB.

In the present study, we used fibroblasts established from multiple donors of different ages for investigating their effects on engineered hepatocyte sheet fabrication and subcutaneous transplantation. This technology provides the fabrication of stable hepatocyte tissues *in vitro* and *in vivo* regardless of the fibroblast donor age. TIG-118 cells established from the 12-year-old donor exhibited high PDGFB gene expression levels and were advantageous for early engraftment. On the contrary, fibroblast donor age had no significant effect on long-term hepatocyte survival and liver-specific functionality *in vivo*. In contrast to other similar studies that ectopically transplanted hepatocytes, this technique results in approximately 10 times higher levels of albumin synthesis and is hardly affected by fibroblast donor age (2,3,22). Therefore, we expect that this technology will be developed and become prevalent as a safe and stable liver regenerative therapy in future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.009.

ACKNOWLEDGMENTS

The authors thank Hideko Hasegawa for technical assistance in preparing slides for immune-staining. This work was supported in part by a grant from the Takeda Science Foundation to Y. Sakai and JSPS KAKENHI Grant Number 16H05908 to Y. Sakai. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. **Lin, R. Z. and Chang, H. Y.:** Recent advances in three-dimensional multicellular spheroid culture for biomedical research, Biotechnol. J., **3**, 1172–1184 (2008).
2. **Baranski, J. D., Chaturvedi, R. R., Stevens, K. R., Eyckmans, J., Carvalho, B., Solorzano, R. D., Yang, M. T., Miller, J. S., Bhatia, S. N., and Chen, C. S.:**

Geometric control of vascular networks to enhance engineered tissue integration and function, Proc. Natl. Acad. Sci. USA, **110**, 7586–7591 (2013).
3. **Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T., Zhang, R. R., Ueno, Y., Zheng, Y. W., Koike, N., and other 3 authors:** Vascularized and functional human liver from an iPSC-derived organ bud transplant, Nature, **499**, 481–484 (2013).
4. **Baptista, P. M., Siddiqui, M. M., Lozier, G., Rodriguez, S. R., Atala, A., and Soker, S.:** The use of whole organ decellularization for the generation of a vascularized liver organoid, Hepatology, **53**, 604–617 (2011).
5. **Uygun, B. E., Soto-Gutierrez, A., Yagi, H., Izamis, M. L., Guzzardi, M. A., Shulman, C., Milwid, J., Kobayashi, N., Tilles, A., Berthiaume, F., and other 4 authors:** Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix, Nat. Med., **16**, 814–820 (2010).
6. **Stevens, K. R., Scull, M. A., Ramanan, V., Fortin, C. L., Chaturvedi, R. R., Knouse, K. A., Xiao, J. W., Fung, C., Mirabella, T., Chen, A. X., and other 8 authors:** In situ expansion of engineered human liver tissue in a mouse model of chronic liver disease, Sci. Transl. Med., **9**, eaah5505 (2017).
7. **Baimakhanov, Z., Yamanouchi, K., Sakai, Y., Koike, M., Soyama, A., Hidaka, M., Takatsuki, M., Fujita, F., Kanetaka, K., Kuroki, T., and Eguchi, S.:** Efficacy of multilayered hepatocyte sheet transplantation for radiation-induced liver damage and partial hepatectomy in a rat model, Cell Transplant., **25**, 549–558 (2016).
8. **Sakai, Y., Yamanouchi, K., Ohashi, K., Koike, M., Utoh, R., Hasegawa, H., Muraoka, I., Suematsu, T., Soyama, A., Hidaka, M., and other 3 authors:** Vascularized subcutaneous human liver tissue from engineered hepatocyte/fibroblast sheets in mice, Biomaterials, **65**, 66–75 (2015).
9. **Harley, C. B., Futcher, A. B., and Greider, C. W.:** Telomeres shorten during ageing of human fibroblasts, Nature, **345**, 458–460 (1990).
10. **Schneider, E. L. and Mitsui, Y.:** The relationship between in vitro cellular aging and in vivo human age, Proc. Natl. Acad. Sci. USA, **73**, 3584–3588 (1976).
11. **Ricciarelli, R., Maroni, P., Ozer, N., Zingg, J. M., and Azzi, A.:** Age-dependent increase of collagenase expression can be reduced by alpha-tocopherol via protein kinase C inhibition, Free Radic. Biol. Med., **27**, 729–737 (1999).
12. **Varani, J., Dame, M. K., Rittie, L., Fligiel, S. E., Kang, S., Fisher, G. J., and Voorhees, J. J.:** Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation, Am. J. Pathol., **168**, 1861–1868 (2006).
13. **Guarino, R. D., Dike, L. E., Haq, T. A., Rowley, J. A., Pitner, J. B., and Timmins, M. R.:** Method for determining oxygen consumption rates of static cultures from microplate measurements of pericellular dissolved oxygen concentration, Biotechnol. Bioeng., **86**, 775–787 (2004).
14. **Ohashi, K., Yokoyama, T., Yamato, M., Kuge, H., Kanehiro, H., Tsutsumi, M., Amanuma, T., Iwata, H., Yang, J., Okano, T., and Nakajima, Y.:** Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets, Nat. Med., **13**, 880–885 (2007).
15. **Yokoyama, T., Ohashi, K., Kuge, H., Kanehiro, H., Iwata, H., Yamato, M., and Nakajima, Y.:** In vivo engineering of metabolically active hepatic tissues in a neovascularized subcutaneous cavity, Am. J. Transplant., **6**, 50–59 (2006).
16. **Sakai, Y., Koike, M., Hasegawa, H., Yamanouchi, K., Soyama, A., Takatsuki, M., Kuroki, T., Ohashi, K., Okano, T., and Eguchi, S.:** Rapid fabricating technique for multi-layered human hepatic cell sheets by forceful contraction of the fibroblast monolayer, PLoS One, **8**, e70970 (2013).
17. **Sakai, Y., Koike, M., Soyama, A., Hidaka, M., Kuroki, T., and Eguchi, S.:** Rapid production of engineered human primary hepatocyte/fibroblast sheets, Data Brief, **5**, 498–501 (2015).
18. **Sakai, Y., Koike, M., Yamanouchi, K., Soyama, A., Hidaka, M., Kuroki, T., and Eguchi, S.:** Time-dependent structural and functional characterization of subcutaneous human liver tissue, J. Tissue Eng. Regenerat. Med., **12**, 2287–2298 (2018).
19. **Sakai, Y., Koike, M., Kawahara, D., Hasegawa, H., Murai, T., Yamanouchi, K., Soyama, A., Hidaka, M., Takatsuki, M., Fujita, F., Kuroki, T., and Eguchi, S.:** Controlled cell morphology and liver-specific function of engineered primary hepatocytes by fibroblast layer cell densities, J. Biosci. Bioeng., **126**, 249–257 (2018).
20. **Battegay, E. J., Rupp, J., Iruela-Arispe, L., Sage, E. H., and Pech, M.:** PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors, J. Cell Biol., **125**, 917–928 (1994).
21. **Thommen, R., Humar, R., Misevic, G., Pepper, M. S., Hahn, A. W., John, M., and Battegay, E. J.:** PDGF-BB increases endothelial migration on cord movements during angiogenesis in vitro, J. Cell. Biochem., **64**, 403–413 (1997).
22. **Chen, A. A., Thomas, D. K., Ong, L. L., Schwartz, R. E., Golub, T. R., and Bhatia, S. N.:** Humanized mice with ectopic artificial liver tissues, Proc. Natl. Acad. Sci. USA, **108**, 11842–11847 (2011).