



Title	Vascularized subcutaneous human liver tissue from engineered hepatocyte/fibroblast sheets in mice
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Citation	Biomaterials, 65, pp.66-75; 2015
Issue Date	2015-10
URL	<a href="http://hdl.handle.net/10069/36001">http://hdl.handle.net/10069/36001</a>
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1 **Vascularized subcutaneous human liver tissue from engineered hepatocyte/fibroblast**  
2 **sheets in mice**

3

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## 1 **Abstract**

2 Subcutaneous liver tissue engineering is an attractive and minimally invasive approach used  
3 for curative treat hepatic failure and inherited liver diseases. However, graft failure occurs  
4 frequently due to insufficient infiltration of blood vessels (neoangiogenesis), while the  
5 maintenance of hepatocyte phenotype and function requires *in vivo* development of the  
6 complex cellular organization of the hepatic lobule. Here we describe a subcutaneous human  
7 liver construction allowing for rapidly vascularized grafts by transplanting engineered cellular  
8 sheets consisting of human primary hepatocytes adhered onto a fibroblast layer. The  
9 engineered hepatocyte/fibroblast sheets (EHFSs) showed superior expression levels of  
10 vascularization-associated growth factors (vascular endothelial growth factor, transforming  
11 growth factor beta 1, and hepatocyte growth factor) *in vitro*. EHFSs developed into  
12 vascularized subcutaneous human liver tissues (VSLTs) contained glycogen stores,  
13 synthesized coagulation factor IX, and showed significantly higher synthesis rates of  
14 liver-specific proteins (albumin and alpha 1 anti-trypsin) *in vivo* than tissues from  
15 hepatocyte-only sheets. The present study describes a new approach for vascularized human  
16 liver organogenesis under mouse skin. This approach could prove valuable for establishing  
17 novel cell therapies for liver diseases.

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19

## 1 **1. Introduction**

2 Construction of functional organs with capillary networks *in vitro* and *in vivo* is an  
3 attractive for the ultimate goal of regenerative medicine [1–6]. Construction of the  
4 transplantable liver tissue is clearly essential to establish systems necessary to treat liver  
5 disease using regenerative medicine. However, the reconstruction of liver structures and  
6 maintenance of their functions *in vitro* and *in vivo* are extremely difficult because the liver  
7 tissue contains complex liver-specific structures and displays a wide variety of functions [7].

8 To overcome the problem that liver-specific functions of cultured hepatocytes decrease  
9 quickly, many researchers have reported that long-term maintenance of liver-specific  
10 functions was achieved using co-culture and/or 3D culture [8–12]. In addition, the  
11 reconstruction of liver sinusoids *in vitro* has been achieved by tri-culture of hepatocytes,  
12 hepatic stellate cells, and endothelial cells (ECs) on a microporous polyethylene terephthalate  
13 membrane [13]. However, the size of the reconstructed liver tissue has usually been limited  
14 because hepatocytes consume oxygen at a particularly high rate [14,15]. Several researchers  
15 have demonstrated vascular fabrication in cultured tissue using co-culturing with ECs [3],  
16 printed rigid 3D filament networks of carbohydrate glass [4], and decellularized liver matrix  
17 [16]. However, it has been difficult to construct a functional vascularized liver tissue *in vitro*.

18 The construction of the liver tissue *in vivo* also presents the need for a vascular bed and  
19 neovascularization into the transplanted tissue to ensure efficient engraftment and  
20 maintenance of proper function. Toward this goal, three-dimensional cell aggregates are  
21 usually transplanted into the omental pouch or subrenal capsule, where local blood vessels  
22 with high angiogenic potential facilitate tissue survival [2,5,17–19]. In addition, recellularized  
23 liver graft using decellularized technology were transplantable organs [16]. However, there  
24 are several issues in these transplantation sites such as risks associated with the type of

1 surgery involved in blood vessels.

2 Vascularized tissue construction under the skin is widely regarded as potentially the safest,  
3 quickest, least invasive grafting strategy, retransplantation, and easy removal of transplanted  
4 tissues in case of graft failure or tumorigenic transformation [18,20–22], but it has yet to be  
5 achieved, because graft failure occurs frequently due to insufficient infiltration of blood  
6 vessels [2,5,17,23]. To overcome the issue, previous papers have showed remarkable  
7 approaches using cell sheet engineering and neovascularized method. Repeated  
8 transplantation of mouse primary hepatocyte sheets after per-transplant vascularization under  
9 skin by implanting a device for the continuous release of angiogenic growth factors [24].  
10 Others showed reconstructed tissue with capillary networks *in vitro* connected with local  
11 blood vessels by surgical methods [3,16]. However, these methods have limitations of  
12 promptness of treatment such as acute liver disease because it takes at least several days to  
13 prepare transplantable tissue from hepatocytes and to prepare the vascular bed.

14 In this study, we demonstrate a simple and rapid method for producing vascularized  
15 subcutaneous human liver tissue (VSLT) *in vivo* by transplantation of engineered  
16 hepatocyte/fibroblast sheets (EHFSs) without addition of stem cells or ECs. We show the  
17 maintenance of liver-specific functions and reconstructed structures.

18

19

## 20 **2. Materials and methods**

### 21 ***2.1. Human primary hepatocyte isolation***

22 Ethical approval was obtained from the Human Ethics Review Committee of Nagasaki  
23 University School of Medicine for human hepatocyte isolation, storage, culture, and  
24 transplantation into mice, and informed consent was obtained from all human hepatocyte

1 donors. Resected human liver tissues (approximately 30 g) were obtained during liver surgery  
2 (Table 1). Human primary hepatocytes were isolated from human liver tissues by perfusing  
3 collagenase (130 U/mL, Wako Pure Chemical, Osaka, Japan) [25,26]. The cell suspension in  
4 25% Percoll Plus solution (GE Healthcare, Tokyo) was centrifuged at  $70 \times g$  for 7 min at  $4^{\circ}\text{C}$   
5 to further purify hepatocytes and enrich viable cells. Cell viability was determined by the  
6 trypan blue exclusion test, and suspensions with  $>80\%$  viable cells were used for culture and  
7 construction of sheets. The medium for isolation was Dulbecco's modified Eagle's medium  
8 (Wako Pure Chemical) supplemented with 10% fetal bovine serum (FBS), 10 mM  
9 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 2 mM L-glutamine, 100 U/mL penicillin,  
10 and 100  $\mu\text{g/mL}$  streptomycin (all from Invitrogen, Carlsbad, CA).

11

## 12 ***2.2. Human fibroblast culture***

13 Normal human diploid fibroblast TIG-118 cells, which derived from human skin (female,  
14 12 years old), were purchased from Health Science Research Resources (JCRB0535; Osaka,  
15 Japan) and cultured as a continuous monolayer in 90-mm tissue culture dishes (Nalgene Nunc  
16 International, Rochester, NY, USA) containing 10 mL Minimum Essential Medium  
17 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100  
18  $\mu\text{g/mL}$  streptomycin. The TIG-118 cells at 90% confluence were treated with 0.25%  
19 trypsin-EDTA (Invitrogen), and the cell suspension was obtained.

20

## 21 ***2.3. Human hepatocyte sheet construction***

22 Two types of human primary hepatocyte sheets were constructed using  
23 temperature-responsive culture dishes (TRCDs) (UpCell; CellSeed, Tokyo) in accordance  
24 with our previous report [27]. To construct EHFSs, human primary hepatocytes obtained as

1 described were plated at  $1.04 \times 10^5$  cells/cm<sup>2</sup> ( $1.0 \times 10^6$  cells/dish) onto a confluent layer of  
2 TIG-118 cells plated 3 days previously at  $2.29 \times 10^4$  cells/cm<sup>2</sup> ( $2.2 \times 10^5$  cells/dish) onto  
3 non-coated TRCDs (Fig. 1A). To construct hepatocyte-only sheets (HSs), hepatocytes were  
4 inoculated onto TRCDs coated with FBS. All the human primary hepatocytes were cultured in  
5 Supplemented ISOM's Media (BD Biosciences, San Jose, CA) supplemented with 10% FBS,  
6 100 U/mL penicillin, and 100 µg/mL streptomycin until 24 h after inoculation. The  
7 Supplemented ISOM's Media was replaced with 2 mL of Hepato-STIM Culture Medium (BD  
8 Biosciences) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100  
9 µg/mL streptomycin. This medium was changed 1 and 3 days after inoculation. All cells were  
10 cultured under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. After 4 days of  
11 hepatocyte culture or co-culture, plates were incubated at 20°C to induce the formation of  
12 detached cell sheets. Fibroblast-only sheets (FSs) were also constructed by following the  
13 same process. Samples of media were collected after 1 to 3 days of culture and stored at  
14 -20°C until assayed for human GFs involved in angiogenesis.

15

#### 16 **2.4. Hepatocyte sheet transplantation under mouse skin**

17 Ethical approval for hepatocyte sheet transplantation was obtained from the Animal Care  
18 and Use Committee and the Recombinant DNA Experiment Safety Committee of Nagasaki  
19 University and performed according to all protocols approved by the Regulations. The  
20 abdominal subcutaneous space for transplantation was created by a tear between the  
21 cutaneous and skeletal muscle layer in NOD SCID mice (NOD.CB17-*Prkdc*<sup>scid</sup>/J; Charles  
22 River Japan Inc., Kanagawa, Japan) and in NOG mice (NOD.Cg-*Prkdc*<sup>scid</sup> *Il2rg*<sup>tm1Sug</sup>/Jic;  
23 Central Institute for Experimental Animals, Kanagawa, Japan) (males, 6–17 weeks old,  
24 weighing 20–35 g) (Fig. 1B). The hepatocyte-containing sheets (EHFSs or HSs) after 4 days

1 of culture were subcutaneously-transplanted using a support membrane or a glass plate to  
2 facilitate handling, and the supporters were removed at several miniatures after contact of cell  
3 sheet into subcutaneous site. One cell sheet ( $1.0 \times 10^6$  hepatocytes) was transplanted into each  
4 mouse. To demonstrate the advantages of EHFS, a suspension consisting of hepatocytes ( $1.0$   
5  $\times 10^6$  cells) and fibroblasts ( $1.5 \times 10^6$  cells) was injected under a mouse's skin. At 1, 2, and 4  
6 weeks after transplantation, 100–200  $\mu$ L of blood was collected from the tail of NOD SCID  
7 mice, and the serum samples were stored at  $-20^\circ\text{C}$  until assayed for the concentration of  
8 liver-specific human proteins.

9

## 10 **2.5. Histology**

11 Cell sheets and VSLTs in NOD SCID mice were fixed with 4% paraformaldehyde  
12 phosphate buffer solution (Wako Pure Chemical) for 24 h to 72 h. Fixed samples were  
13 embedded in paraffin, cut into 5- $\mu$ m cross-sections, mounted on MAS-coated slides  
14 (Matsunami Glass, Osaka, Japan), and deparaffinized for standard histological staining with  
15 hematoxylin and eosin (HE), orange G, azocarmin G, and aniline blue (Azan), or periodic  
16 acid-Schiff (PAS) (all from Muto Pure Chemicals, Tokyo). For immunostaining, sections were  
17 heated in 10 mM Tris-HCl buffer (pH 9.0) containing 1 mM EDTA using a microwave or  
18 autoclave for antigen retrieval, incubated in 3% hydrogen peroxide solution for 10 min to  
19 quench endogenous peroxidase activity or Biotin-Blocking System (Dako Japan, Kyoto), and  
20 then blocked in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and  
21 0.1% Tween 20 for 1 h at room temperature. Blocked sections were incubated overnight at  
22  $4^\circ\text{C}$  in TBS + 5% BSA, 0.1% Tween 20, and with the following antibodies: rabbit anti-human  
23 albumin (hALB) (ab2406; 1:3000), mouse anti-human alpha 1-antitrypsin (hA1AT) (ab9399;  
24 1:1500), sheep anti-human blood coagulation factor IX (hF9) (ab128048; 1:100), mouse



1 anti-cytokeratin 18 (CK18) (ab668; 1:500), mouse anti-human vimentin (hVim) (ab8069;  
2 1:500), rabbit anti-cluster of differentiation 31 (CD31) (ab28364; 1:50), rabbit anti-human  
3 CD31 (hCD31) (ab76533; 1:250), rabbit anti-Ki67 (ab16667; 1:100) (all from Abcam,  
4 Cambridge, MA), or rabbit anti-E-cadherin (E-cad) (sc-7870; 1:100) (Santa Cruz  
5 Biotechnology, Santa Cruz, CA). Sections were then incubated for 1 h at room temperature in  
6 an appropriate secondary antibody: horseradish peroxidase (HRP)-conjugated goat anti-rabbit  
7 IgG (A0545; 1:300), biotin-conjugated goat anti-rabbit IgG (B8895; 1:800), fluorescein  
8 isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (F9887; 1:320), FITC-conjugated  
9 rabbit anti-mouse IgG (F9137; 1:200), tetramethylrhodamine isothiocyanate  
10 (TRITC)-conjugated rabbit anti-mouse IgG (T2402; 1:400), FITC-conjugated ExtrAvidin  
11 (E2761; 1:80), TRITC-conjugated ExtrAvidin (E3011; 1:150) (all from Sigma-Aldrich Japan,  
12 Tokyo), or biotin-conjugated donkey anti-sheep IgG (ab97123; 1:500) (Abcam).  
13 HRP-conjugated secondary binding was visualized using the Dako liquid DAB+ substrate  
14 chromogen system (Dako Japan). Nuclei stained with hematoxylin or  
15 4',6-diamidiono-2-phenylindole (DAPI; DOJINDO, Kumamoto, Japan). Slides stained with  
16 HRP-conjugated secondary antibodies were mounted with Permount (Fisher Scientific,  
17 Atlanta, GA) and slides stained with fluorophore-conjugated secondary antibodies with  
18 ProLong gold antifade mounting medium (Invitrogen). Bright-field and fluorescence images  
19 were captured using an optical microscope (BX53; Olympus, Tokyo) and a confocal laser  
20 scanning microscope (FV10i; Olympus), respectively.

21

## 22 ***2.6. Sheet size and thickness measurements***

23 The exterior photographs of cell sheets were captured to evaluate the cell sheet sizes [27].

24 The sizes of cell sheets were calculated as the ratio of the cell sheet area to the original TRCD

1 culture surface area. To evaluate the thicknesses of the cell sheets, the HE stained  
2 cross-sectional images of the cell sheets were captured. These areas and thickness were  
3 measured using the NIS-Elements software program (Nikon, Tokyo).

4

### 5 **2.7. Ultrastructural analysis (TEM)**

6 EHFSs and VSLTs in NOG mice at 2 weeks after transplantation were fixed with 2.5%  
7 glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in phosphate-buffered 1%  
8 osmium tetroxide for 2 h at 4°C, and then dehydrated through a graded ethanol series. The  
9 dehydrated samples were embedded in Epon 812 (TAAB Laboratories Equipment Ltd.,  
10 Berkshire, England). Ultrathin sections were cut with a diamond knife on an ultramicrotome  
11 (Ultracut S, Leica, Austria), double-stained with uranyl acetate and lead nitrate, then imaged  
12 under an electron microscope (JEM-1200EX, JEOL, Tokyo) at an accelerating voltage of  
13 60–80 kV.

14

### 15 **2.8. Human growth factors and liver-specific functions assay (ELISA)**

16 Human platelet-derived growth factor BB (PDGF-BB), vascular endothelial growth factor  
17 (VEGF), transforming growth factor beta 1 (TGF- $\beta$ 1), basic fibroblast growth factor (bFGF),  
18 hepatocyte growth factor (HGF), and epidermal growth factor (EGF) in culture media  
19 samples were measured by using DuoSet ELISA Development Systems (R&D Systems,  
20 Minneapolis, MN) according to the manufacturer's instructions. Human ALB and A1AT in  
21 serum samples of NOD SCID mice were measured using rabbit anti-human albumin (6  
22  $\mu$ g/mL), HRP-conjugated goat anti-human albumin (10  $\mu$ g/mL) (MP Biomedicals,  
23 LLC-Cappel products, Irvine, CA), goat anti-human A1AT (5  $\mu$ g/mL; Bethyl Laboratories,  
24 Montgomery, TX), or HRP-conjugated goat anti-human A1AT (7  $\mu$ g/mL; Fitzgerald Industries

1 International, Inc., Concord, MA).

2

### 3 ***2.9. Statistical analysis***

4 Data are presented as mean  $\pm$  standard deviation (SD) from at least 6 time points. Means  
5 of continuous numerical variables were compared by Welch's *t*-test, one-way analysis of  
6 variance (ANOVA) (Tukey's multiple comparison test), or two-way ANOVA (Sidak's multiple  
7 comparison test) (GraphPad Prism version 6.00 for Windows; GraphPad Software, San Diego,  
8 CA). Values of  $**P < 0.01$  and  $*P < 0.05$  were considered statistically significant.

9

10

## 11 **3. Results**

### 12 ***3.1. Characteristic morphologies of the EHFS***

13 Human primary adult hepatocytes attached onto the TIG-118 cell layer within 1 h, and  
14 these adherent hepatocytes eventually assumed the typical cuboidal shape of mature  
15 hepatocytes (Fig. 2A). In contrast, primary hepatocytes cultured alone on a FBS-coated  
16 TRCD surface required approximately 4 h to adhere. It normally takes at least 4 days for  
17 cultured hepatocytes alone to become fully confluent. EHFSs and HSs were harvested by  
18 reducing the culture temperature from 37°C to 20°C for approximately 1 to 2 h (for EHFSs)  
19 or 4 to 12 h (for HSs) on day 4 (Fig. 2B). The reduced temperature initiated the natural  
20 detachment of the cells from the edge of the TRCD surface, resulting in floating cell sheets in  
21 the culture medium that shrank to approximately 20% of the original culture surface area (Fig.  
22 2B, C). The area of EHFSs were slightly-larger than those of HSs.

23 Histological cross-sections of both types of hepatocyte sheet showed ubiquitous  
24 hepatocyte survival as revealed by HE staining, as well as hepatocytic expression of both

1 hALB and the hepatocyte marker CK18 (Fig. 2D). In EHFSs, almost all hVim-positive  
2 fibroblasts (particularly those displaying mesenchymal cell cytoskeleton markers) had  
3 migrated from the bottom to the top of the sheet during the 4 days in co-culture with  
4 hepatocytes. The cells present in HSs were hVim-negative. The endothelial cell marker  
5 hCD31 was expressed only at very low levels in EHFSs and HSs. These EHFSs were  
6 approximately 1.6-times thicker than HSs (Fig. 2E). Hepatocytes in EHFSs reconstructed bile  
7 canals (BCs), tight junctions (TJs), desmosomes (Ds), and gap junctions (GJs), and adhered to  
8 fibroblasts as revealed by TEM (Fig. 2F).

9

### 10 **3.2. Angiogenic growth factor syntheses in vitro**

11 To indicate the possibility of using a one-step subcutaneous hepatocyte transplantation  
12 procedure without a pre-transplant vascularization step, synthesis rates of  
13 vascularization-associated growth factors *in vitro* were assayed. EHFSs exhibited significantly  
14 higher synthesis rates of the angiogenic factors VEGF, TGF- $\beta$ 1, and HGF than the HSs from 1  
15 to 3 days in culture (Fig. 3B, C, E). The synthesis of TGF- $\beta$ 1 and HGF from fibroblasts were  
16 upregulated and down-regulated, respectively, by co-culture with hepatocytes. In contrast,  
17 PDGF-BB, bFGF, and EGF were not detected in either type of cell sheet (Fig. 3A, D, F).

18

### 19 **3.3. VSLT construction process from EHFS**

20 Time-dependent VSLTs were investigated by staining with HE, two types of CD31  
21 (non-specific species and human origin), and cell proliferation marker Ki67. The transplanted  
22 EHFSs were initially laminated and necrosis occurred at a part of hepatocytes on 1 day after  
23 transplantation (Fig. 4A). Although hepatocytes at the outer end of VSLTs were hypertrophic  
24 at 7 days, cells were rarely observed at 14 days after transplantation. ECs (CD31-positive)

1 were observed 7 days after transplantation, and many neovascular vessels (Ki67-positive ECs)  
2 grew into the liver tissue until 14 days (Fig. 4A–C). In contrast, little expression of Ki67 in  
3 hepatocytes was revealed (Fig. 4A). The transplanted EHFSs formed grossly-visible VSLT,  
4 which had numerous blood vessels at the edge by day 7 after transplantation (Fig. 4D). The  
5 VSLTs indicated an increased red color by day 14 after transplantation (Fig. 4E).

6

### 7 ***3.4. Liver-specific structures of the VSLT***

8 Indeed, VSLT of 100–300  $\mu\text{m}$  thickness at 2 weeks after transplantation contained  
9 hepatocytes surrounded by collagen fibers as confirmed by HE and Azan staining,  
10 respectively (Fig. 5A). Moreover, VSLT had liver-specific features such as glycogen stores  
11 and hALB, hA1AT, and hF9 syntheses as revealed by PAS staining and immunostaining,  
12 respectively (Fig. 5A, B). The aggregated hepatocytes formed several linear structures  
13 surrounded by human fibroblasts (hVim-positive) and abundant blood vessels  
14 (hCD31-positive) and adherens junctions (E-cad) between hepatocytes (CK18-positive). TEM  
15 images of VSLTs showed that hepatocytes are adjacent to microvessels supported by  
16 fibroblasts through the microvilli (MVs) (Fig. 5C). The VSLTs constructed the ultrastructural  
17 features of mature liver, such as BCs and several types of cell adhesions (TJs and Ds). In  
18 contrast, transplanted HSs formed simple hepatocyte monolayers that covered only a small  
19 area of the transplant site (Fig. 5A).

20

### 21 ***3.5. Liver-specific functions of the VSLT in vivo***

22 The *in vivo* liver-specific functionality of transplanted EHFSs is underscored by the  
23 concentrations of human proteins detected in the sera of recipient mice. The hALB synthesis  
24 rate was approximately 23-times higher than that in liver tissue from transplanted HSs one

1 week after transplantation (Fig. 6A). Although there was a minor decrease after  
2 transplantation, the VSLTs maintained higher hALB and hA1AT expression levels for 4 weeks  
3 (Fig. 6A, B). The transplantation of EHFS was more effective than the transplantation of  
4 co-suspension of hepatocytes and fibroblasts (Fig. 6C).

5

6

#### 7 **4. Discussion**

8 Both types of hepatocyte sheets could be harvested at 4 days after the seeding of  
9 hepatocytes (Fig. 2B). Interestingly, EHFSs were more easily detached due to the forceful  
10 contraction of fibroblasts and formed thicker morphology (Fig. 2D, E). In addition, EHFSs  
11 could be harvested from TRCDs only one day after the seeding of hepatocytes onto fibroblast  
12 confluent (Supplementary Fig. 1A). The most rapid construction of EHFSs was achieved  
13 about 4 h after the inoculation of TIG-118 cells (Supplementary Fig. 1B). The TIG-118 cell  
14 layer could form on FBS-coated TRCD within 1 h when seeded at high cell density ( $1.56 \times$   
15  $10^5$  cells/cm<sup>2</sup>), and EHFSs could be harvested soon after the adhesion of hepatocytes. These  
16 advantages of EHFS could aide in easy manipulation during transplantation and could be used  
17 for emergency such as acute liver failure. Similar advantages for rapid construction and sheet  
18 characteristics were described for our previously engineered HepaRG cell/fibroblast sheets  
19 compared to HepaRG cell sheets from monoculture [27].

20 Subcutaneous transplantation of the EHFSs would allow for minimally invasive  
21 hepatocyte-based therapy as well as possible retransplantation and removal of grafts if  
22 necessary [18,20–22]. However, graft failure occurs frequently [2,5,17,23]. The major reason  
23 for engraftment failure has been the insufficient number of blood vessels at the site of  
24 transplantation and the lack of vascular infiltration into the new tissue. To overcome this

1 limitation, previous studies have induced subcutaneous vascularization by implanting a device  
2 for controlled release of acidic fibroblast growth factor, bFGF, or VEGF, but it took at least 10  
3 days for vascular bed formation [24,28,29]. Quite amazingly, grossly-visible VSLT, which  
4 had numerous blood vessels, were observed by day 7 after transplantation (Fig. 4D) without  
5 pre-transplant vascularization. This result will be caused by the continuous releasing of the  
6 angiogenic factors from the EHFSs (Fig. 3B, C, E) instead of implanting a device for  
7 subcutaneous vascularization. Furthermore, the mRNA expressions (VEGF, TGF- $\beta$ 1, and  
8 HGF) of VSLTs from EHFSs maintained at least 2 weeks after transplantation, and the  
9 expression levels compared favorably with those of EHFSs (Supporting information and  
10 Supplementary Fig. 2).

11 We show the model of VSLT construction in Fig. 7. The VSLTs were thick tissue  
12 comprising hepatocytes despite the low expression of Ki67 (Fig. 4A), which means that VSLT  
13 thickness resulted from the survival and self-aggregation of transplanted hepatocytes,  
14 followed by neoangiogenesis (Fig. 4B, C). The regulation of hepatocyte growth can be  
15 blamed on high TGF- $\beta$ 1 synthesis from fibroblasts (Fig. 3C) [30]. To improve the effect of  
16 subcutaneously transplanted hepatocytes, we should try to modify our system to allow the  
17 regulation of TGF- $\beta$ 1 synthesis. The aggregated hepatocytes in the outer end of VSLTs were  
18 hypertrophy at 7 days after transplantation because a temporary increasing of blood pressure  
19 will occur in the areas due to the discontinuity of blood vessels (Fig. 4A, D). The  
20 phenomenon was usually observed under high portal blood pressure condition after the partial  
21 hepatectomy [31]. Vascular network could perfuse blood when these discontinuous blood  
22 vessels connected with themselves; thus, the VSLTs indicated an increased red color (Fig. 4E).  
23 Hypertrophied hepatocytes were rarely observed at 14 days after transplantation (Fig. 4A)  
24 because the local blood pressure had decreased from vascular network formation. To reveal

1 the origin of ECs, the immunochemical staining (non-specific species CD31 and human  
2 CD31) of VSLTs at 7 and 14 days after EHFS transplantation were performed. As a result,  
3 almost all ECs at 7 days were from the host mice (non-specific species CD31-positive,  
4 hCD31-negative) (Fig. 4A). The number of CD31-positive cells at 14 days was higher than  
5 that at 7 days; thus, these vascular vessels grew into VSLTs from mice. However,  
6 hCD31-positive ECs could also be detected at 14 days. Very few human ECs in the cell  
7 suspension may proliferate during later period.

8 VSLTs showed high synthesis of liver-specific functions and reconstruction of structures  
9 (Fig. 5). At 2 weeks after EHFS transplantation, VSLTs consisted of several linearly located  
10 hepatocytes and abundant blood vessels, which were similar to hepatic lobule structures (Fig.  
11 5A, B) [7]. In detail, hepatocytes are adjacent to microvessels through MVs, which is  
12 remarkably similar to the space of Disse (Fig. 5C). These structures permit VSLTs of 300- $\mu$ m  
13 thickness to remain alive despite exceeding the limit of cell sheet thickness for effective  
14 oxygen diffusion/supply (approximately 40  $\mu$ m) [32–34]. Liver-specific ultrastructures, such  
15 as BCs, between hepatocytes were also reconstructed *in vivo*. These human hepatocytes  
16 expressed liver-specific functions *in vivo*, including hALB, hA1AT, and hF9 (Fig, 5B). In the  
17 future, VSLT could also assist in the development of several useful assays, such as novel  
18 systems to assess drug transport, and the treatment of hemophilia.

19 The human ALB and A1AT synthesis activities of VSLTs were exceedingly higher than  
20 those in the liver tissue derived from transplanted HSs (Fig, 6A, B). Takebe *et al* [2] reported  
21 that the kidney subcapsule was a superior transplantation site compared with various ectopic  
22 sites, such as cranium and mesentery. However, they also showed that human adult  
23 hepatocytes ( $4.0 \times 10^6$  cells) transplanted under the kidney subcapsule of mouse could not  
24 keep producing high levels of hALB and showed a peak at day 15 (approximately 1200



1 ng/mL). Chen *et al* [5] reported that their system for creating human ectopic artificial liver  
2 was ineffective for engraftment to the subcutaneous space. In contrast, VSLTs showed high  
3 levels of function despite the fact that VSLTs consisted of no more than  $1.0 \times 10^6$   
4 subcutaneously implanted hepatocytes. At 4 weeks after transplantation, VSLTs maintained  
5 these functions (hALB,  $1132 \pm 553$  ng/mL; hA1AT,  $193 \pm 62$  ng/mL), although there were no  
6 significant differences. HE stained images of VSLT showed hepatocyte survival and support  
7 the high liver-specific protein synthesizes (Supplementary Fig. 3A). On the other hand, the  
8 hepatocytes from HS were not readily apparent (Supplementary Fig. 3B). EHFS was more  
9 effective than co-suspension, as evidenced by results from well engraftment of the sheet  
10 format cell organoid (Fig, 6C). Almost all hepatocyte at 1 week after co-suspension  
11 transplantation were hypertrophic and formed thin tissue (Supplementary Fig. 3C). This fact  
12 highlighted the benefit of hepatocyte sheet such as enough extracellular matrix components  
13 and well-graft on site without wide spreading. However, we were concerned that  
14 exceedingly-high TGF- $\beta$ 1 synthesis of EHFS may trigger a part of hepatocytes to be apoptosis  
15 [31] and possibly-caused this minor decrease. Thus, this cell therapy has the possibility of  
16 improvement in additional cell sources and/or medications for long-term cell therapy of  
17 chronic liver failure.

18 One of the most important differences from previous techniques is the safety of the cell  
19 sources. The use of mature hepatocytes and fibroblasts eliminates the risks of unanticipated  
20 differentiation fate, tumorigenic transformation [35–37], and tumor growth by mesenchymal  
21 stem cell accumulation at the tumor site [38,39]. Next, the VSLTs were constructed from  
22 easily cultured cells using simple procedures. This system employed normal human dermal  
23 fibroblasts, which are easily cultured and proliferate rapidly, and requires no separate *in vitro*  
24 or *in vivo* vascularization methods before transplantation, such as mixing human umbilical

1 vein endothelial cells with the cultured tissue and/or pre-implanting a bFGF-releasing device  
2 at the transplantation site. In addition, this system could allow for rapid large-scale production  
3 of transplantable tissue because it takes as little as 4 h for construction of EHFSs after seeding  
4 of fibroblasts. Such a system could be used in emergent circumstances associated with liver  
5 surgery, such as autologous hepatocyte transplantation, because EHFSs can be produced  
6 during the operation. This simple and rapid procedure is a major advance toward clinical  
7 application of subcutaneous liver tissue transplantation. Finally, more improvement of  
8 transplantation efficiency could require optimization of other cell sources, sheet forming  
9 process, combination of growth factors, and/or pre-transplant vascularization; thus, we will  
10 investigate these subjects as the next step for treatment liver diseases. Moreover, identical  
11 expression patterns of angiogenic factor synthesise *in vitro* and liver-specific functions *in vivo*  
12 were observed when HepaRG cells were employed instead of human primary hepatocytes to  
13 create EHFSs (Supplementary Fig. 4, 5). Thus, this versatile fibroblast system could be used  
14 for the construction of subcutaneous organs by using several functional cells such as islet of  
15 the pancreas.

16

17

## 18 **5. Conclusion**

19 We demonstrate viable VSLT derived from simple one-time subcutaneous transplantation  
20 of EHFSs. This system, which consists of cell sheet technology and co-culturing fibroblasts  
21 without pre-transplant procedures, was able to overcome the low transplantation efficiency of  
22 previous subcutaneous transplantation procedures. In addition, the transplant site and cell  
23 types employed could realize goals for the procedure to be minimally invasive and drastically  
24 safe. The resulting VSLTs could be used for the treatment of acute hepatic failure and

1 inherited liver diseases. Furthermore, this system can be applied not only to hepatocytes but  
2 also to other cell types. Thus, our novel fibroblast system could be used to rapidly construct  
3 subcutaneous vascularized organs.

4

5

#### 6 **Source of funding**

7 This work was supported in part by Grants-in-Aid for Young Scientists to Y. Sakai (no.  
8 25861161) and Scientific Research to S. Eguchi (no. 23591868 and no. 26461916), Takeda  
9 Science Foundation to Y. Sakai, the Regenerative Medicine Support Project in Nagasaki  
10 Prefecture, and Creation of Innovation Centers for Advanced Interdisciplinary Research Areas  
11 Program in Project for Developing Innovation Systems, from the Ministry of Education,  
12 Culture, Sports, Science, and Technology (MEXT) of Japan to K. Ohashi.

13

14

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- 10  
11



1 **Figure Legends**

2 **Fig. 1.** Construction and transplantation of EHFSs. (A) Schematic of EHFS construction. (B)  
3 The processes of hepatocyte sheet transplantation under mouse skin.

4

5 **Fig. 2.** Characterization of EHFSs. (A) Phase-contrast micrographs of hepatocytes cultured on  
6 TRCDs. Above, human primary hepatocytes and fibroblasts; below, hepatocytes alone. Scale  
7 bar, 100  $\mu\text{m}$ . (B) Macroscopic and phase-contrast images of EHFSs and HSs. Scale bar, 100  
8  $\mu\text{m}$ . (C) Areas ( $n \geq 11$  from 2 independent preparations) of EHFSs (open columns) and HSs  
9 (closed columns). Mean  $\pm$  SD. (D) HE and immunofluorescence staining of sheet  
10 cross-sections. Scale bar, 50  $\mu\text{m}$ . (E) Thicknesses ( $n \geq 9$  from 4 independent preparations) of  
11 EHFSs (open columns) and HSs (closed columns). Mean  $\pm$  SD,  $^{***}P < 0.01$  (Welch's *t*-test).  
12 (F) TEM images of EHFS cross-sections. Scale bar, 1  $\mu\text{m}$ . Hep, hepatocyte; Fib, fibroblast; N,  
13 nucleus; MV, microvillus; BC, bile canal; TJ, tight junction; D, desmosome; GJ, gap junction;  
14 N.S., not significant.

15

16 **Fig. 3.** *In vitro* synthesis rates of growth factors involved in angiogenesis. (A) Human  
17 PDGF-BB, (B) VEGF, (C) TGF- $\beta$ 1, (D) bFGF, (E) HGF, and (F) EGF ( $n \geq 17$  from 2  
18 independent cell preparations). Open, closed, and shaded columns represent EHFSs, HSs, and  
19 FSs, respectively. Mean  $\pm$  SD,  $^{***}P < 0.01$  and  $^{*}P < 0.05$  (one-way ANOVA). N.D., not  
20 detected.

21

22 **Fig. 4.** Development of VSLTs *in vivo*. (A) Temporal HE and immunohistochemical staining  
23 of VSLT cross-sections at 1, 7, and 14 days after EHFS transplantation. Scale bar, 100  $\mu\text{m}$ .  
24 (B) Vessel area and (C) number of Ki67-positive ECs in VSLTs at 7 and 14 days after EHFS

1 transplantation (n = 6 from more than 4 independent cell preparations). Mean  $\pm$  SD, \**P* < 0.05  
 2 (Welch's *t*-test). (D) Macroscopic image and phase-contrast micrograph of blood vessels in  
 3 the VSLTs at 7 days after EHFS transplantation. Scale bar, 1 mm. (E) Macroscopic images of  
 4 skin at 7 and 14 days after EHFS transplantation. Scale bar, 5 mm. The dashed lines indicate  
 5 the VSLTs.

6  
 7 **Fig. 5.** Functional VSLTs under mouse skin at 2 weeks after transplantation. (A) HE, Azan,  
 8 and PAS staining of VSLT cross-sections. Scale bar, 100  $\mu$ m. (B) Immunofluorescence  
 9 staining of VSLT cross-sections. Scale bar, 50  $\mu$ m. (C) TEM images of VSLT cross-sections.  
 10 Scale bar, 2  $\mu$ m. Hep, hepatocyte; Fib, fibroblast; EC, endothelial cell; RBC, red blood cell,  
 11 MV, microvillus; BC, bile canal; TJ, tight junction; D, desmosome.

12  
 13 **Fig. 6.** *In vivo* liver-specific functions in mice. (A) hALB and (B) hA1AT concentrations in  
 14 NOD SCID mouse serum (n  $\geq$  10 from 2 independent cell preparations). (C) hALB  
 15 concentrations in NOD SCID mouse serum at 1 week after transplantation (n  $\geq$  6 from 2  
 16 independent preparations). Open, closed, and shaded columns represent EHFSs, HSs, and  
 17 co-suspension of hepatocytes and fibroblasts, respectively. Mean  $\pm$  SD, \*\**P* < 0.01 and \**P* <  
 18 0.05 (two-way or one-way ANOVA). N.S., not significant; N.D., not detected.

19  
 20 **Fig. 7.** Model of VSLT construction.

21  
 22 **Table 1.** Human liver tissue resource for primary hepatocyte isolation.

23  
 24 **Supplementary Fig. 1.** Rapid construction of EHFSs. (A) One-day construction of EHFSs.

1 (B) Rapid construction of EHFSs using a high density of fibroblasts. Scale bar, 100  $\mu$ m.

2

3 **Supplementary Fig. 2.** mRNA expression levels of growth factors involved in angiogenesis  
4 of VSLTs at 7 and 14 days after EHFS transplantation. (A) Human VEGF, (B) TGF- $\beta$ 1, (C)  
5 HGF (n = 3 from one cell preparation). Mean  $\pm$  SD. The expression levels of EHFS were set  
6 as 1.0. N.D., not detected.

7

8 **Supplementary Fig. 3.** HE stained images of hepatocyte tissues under mouse skin. (A)  
9 Hepatocyte tissues from EHFS and (B) from HS at 4 weeks after transplantation. (C)  
10 Hepatocyte tissue from co-suspension of hepatocytes and fibroblasts at 1 week after  
11 transplantation. Scale bar, 100  $\mu$ m.

12

13 **Supplementary Fig. 4.** *In vitro* synthesis rates of growth factors involved in angiogenesis  
14 using HepaRG cells. (A) Human PDGF-BB, (B) VEGF, (C) TGF- $\beta$ 1, (D) bFGF, (E) HGF, and  
15 (F) EGF (n = 12 from 2 independent cell preparations). Open, closed, and shaded columns  
16 represent EHFSs, HSs, and FSs, respectively. Mean  $\pm$  SD, \*\* $P$  < 0.01 and \* $P$  < 0.05 (one-way  
17 ANOVA).

18

19 **Supplementary Fig. 5.** *In vitro* liver-specific functions using HepaRG cells. (A) hALB and  
20 (B) hA1AT (n  $\geq$  5 from one cell preparation). Open and closed columns represent EHFSs and  
21 HSs, respectively. Mean  $\pm$  SD, \*\* $P$  < 0.01 and \* $P$  < 0.05 (two-way ANOVA).

22

23 **Supplementary Table 1.** TaqMan gene expression assay numbers and GenBank accession  
24 numbers for real-time PCR analysis.

Figure 1

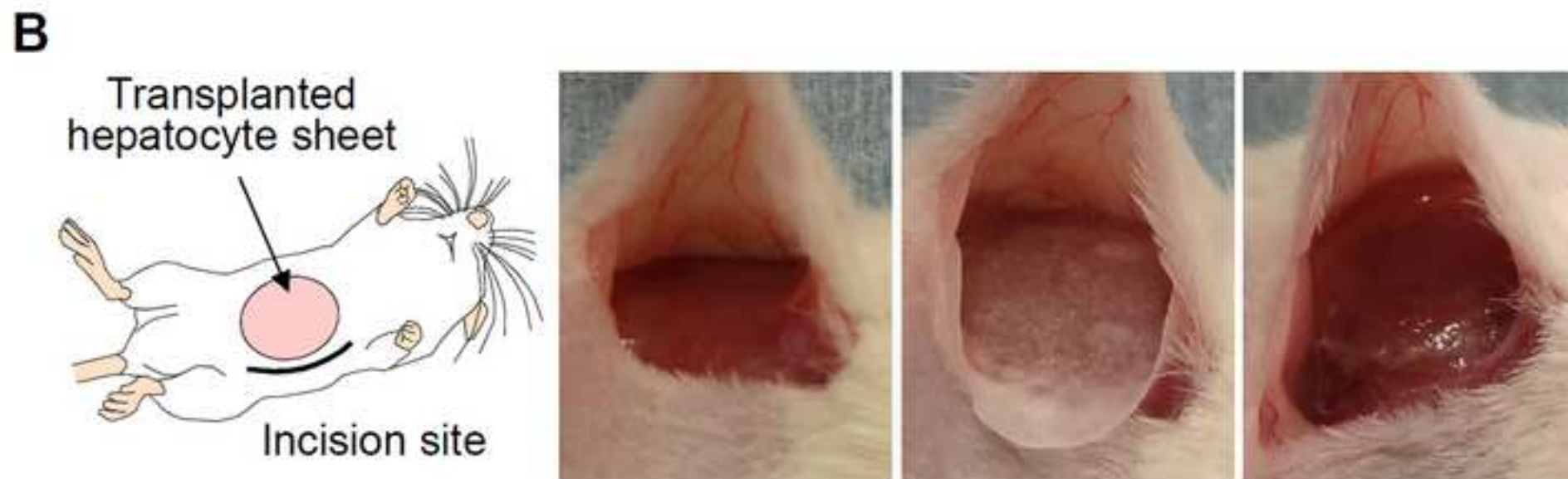
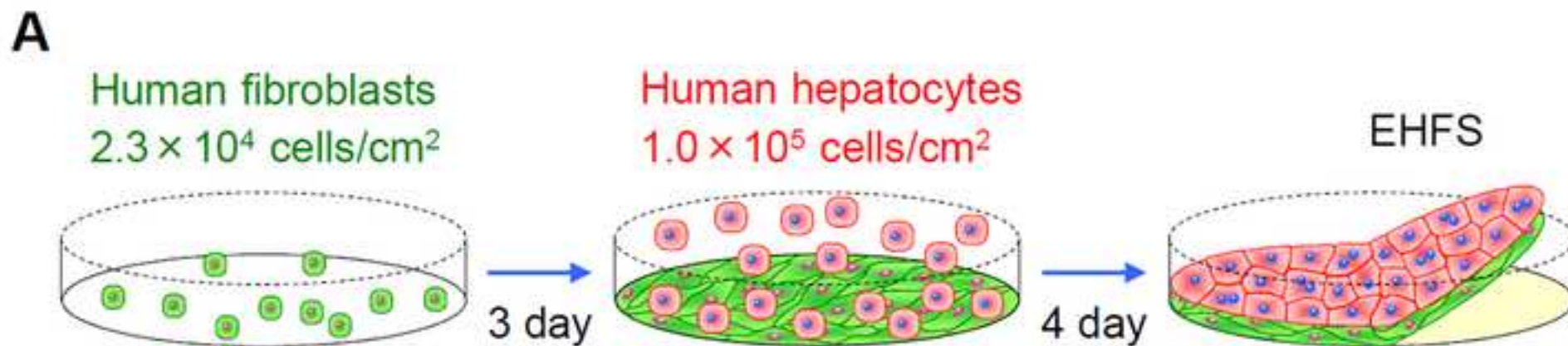


Figure 2

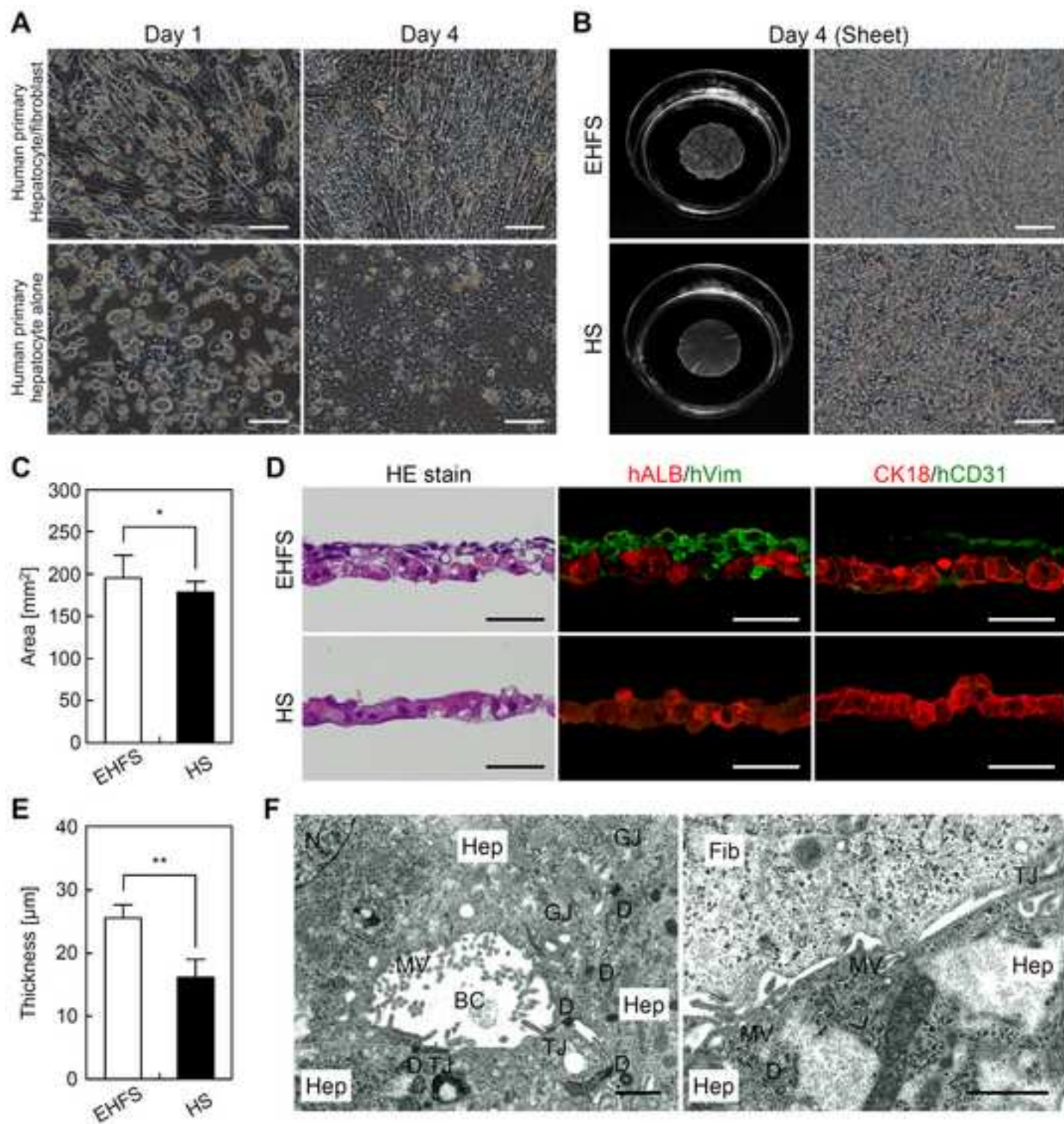


Figure 3

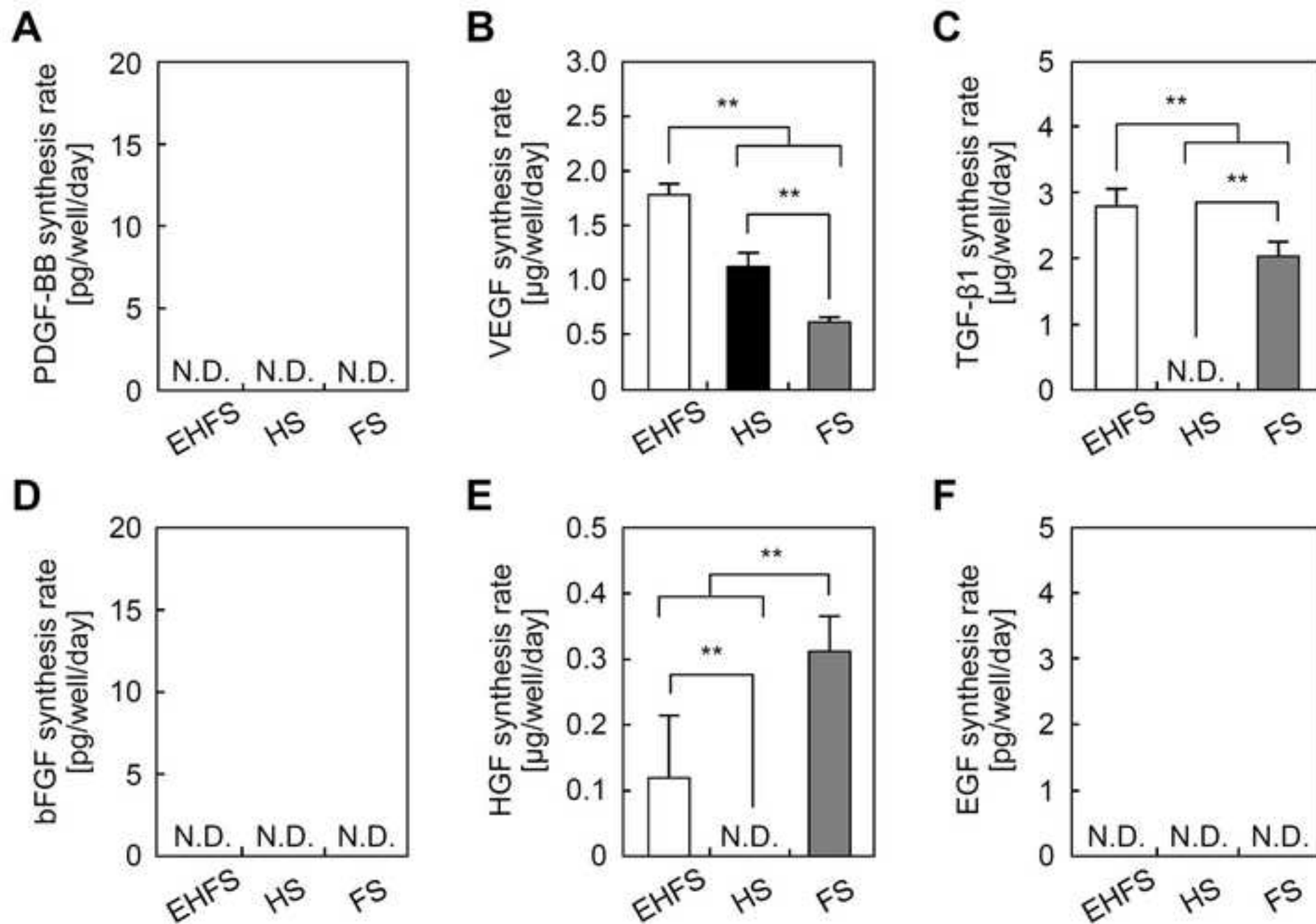




Figure 4

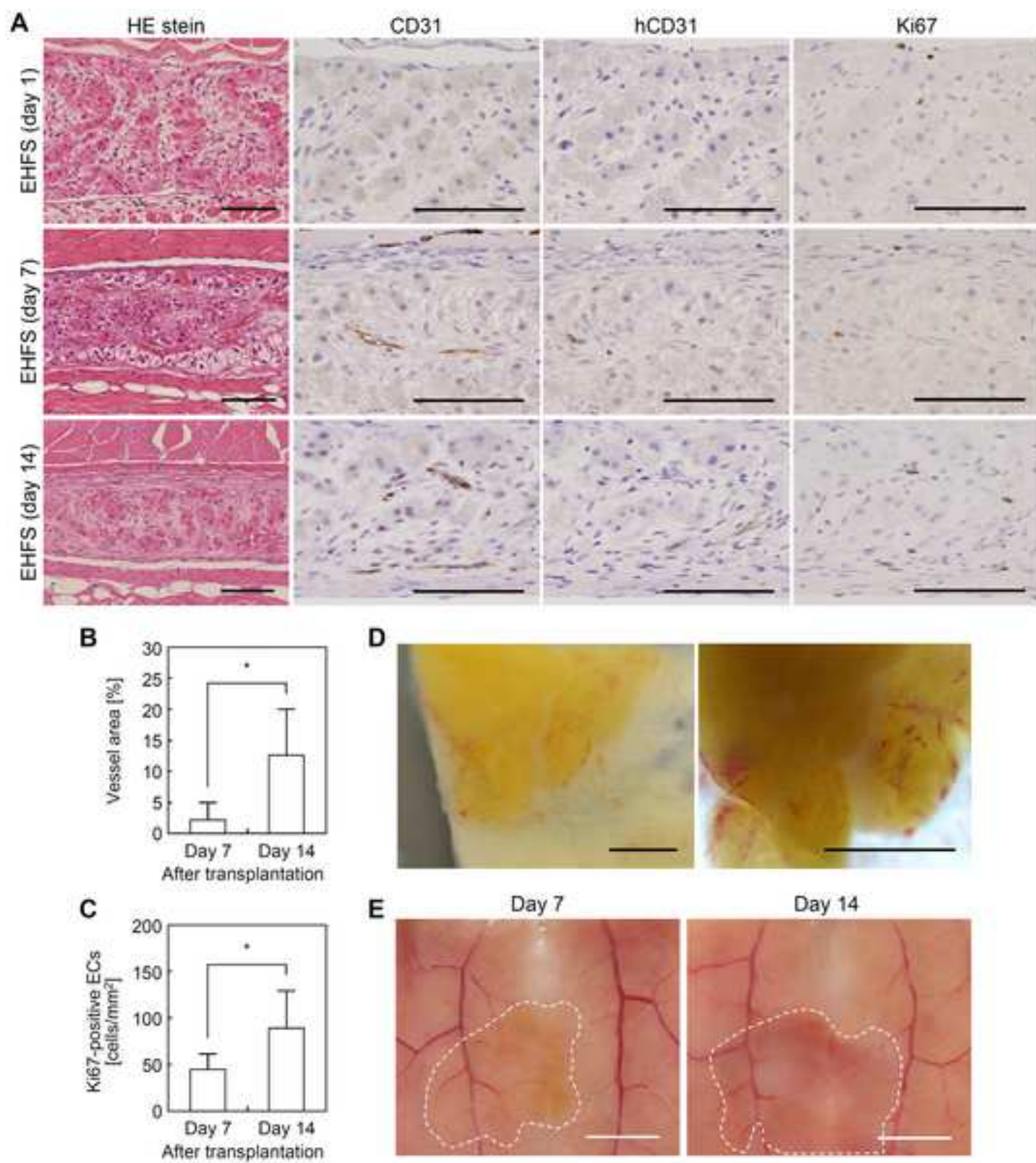


Figure 5

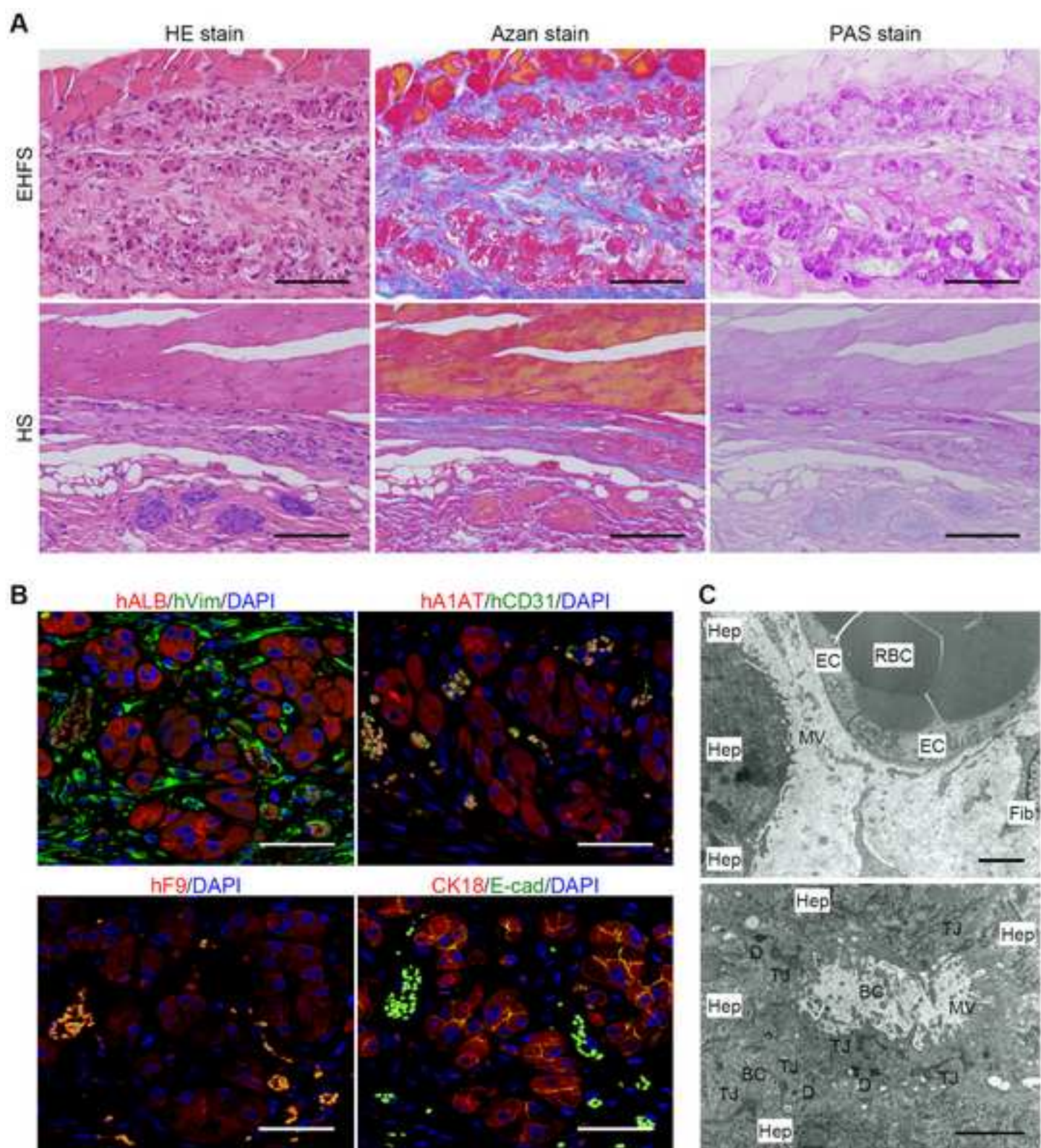




Figure 6

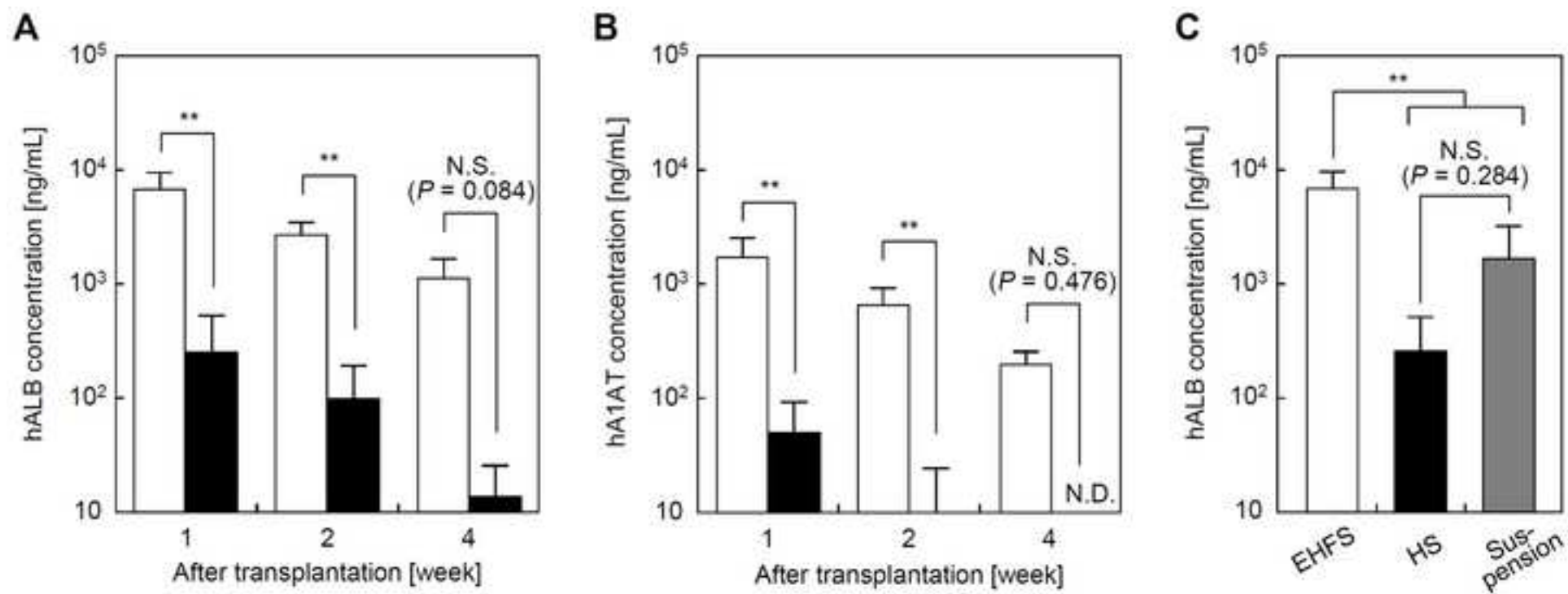
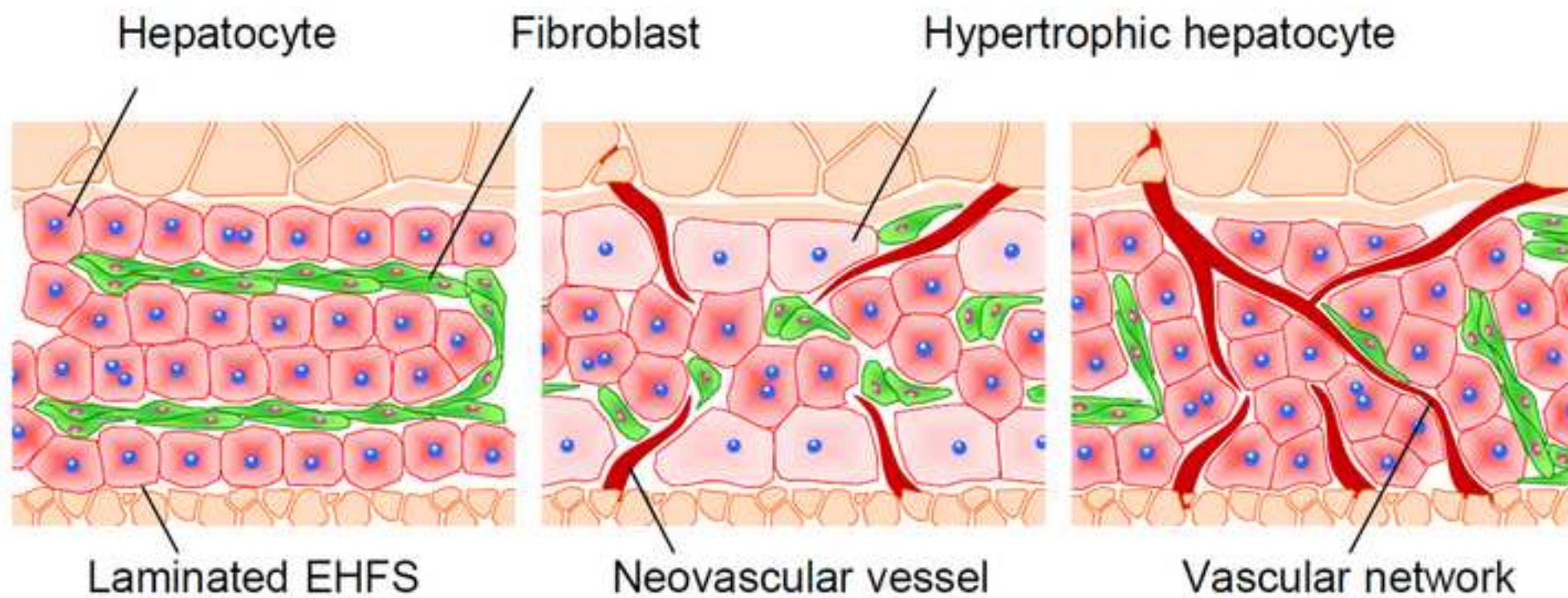
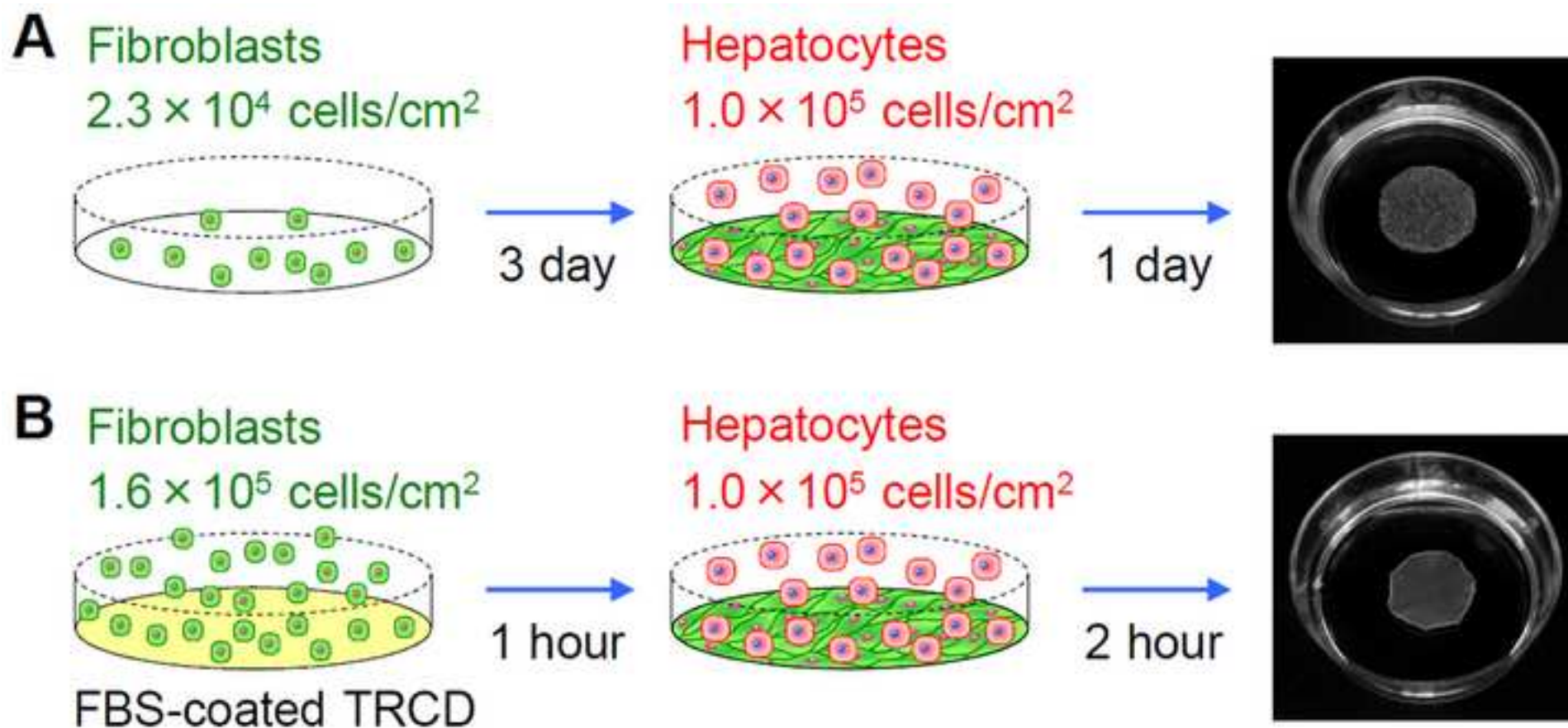


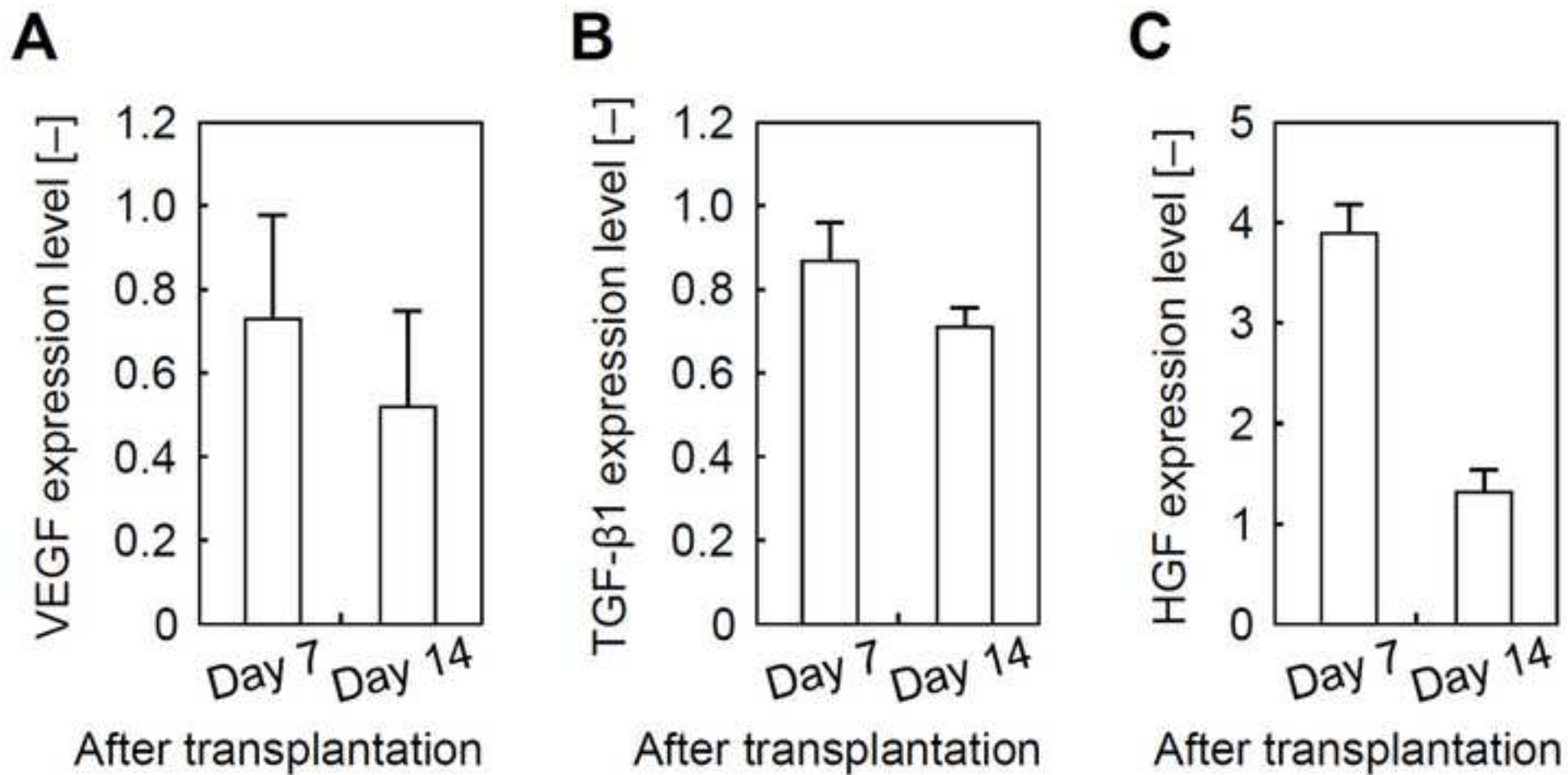
Figure 7



**Table 1.** Human liver tissue resource for primary hepatocyte isolation.

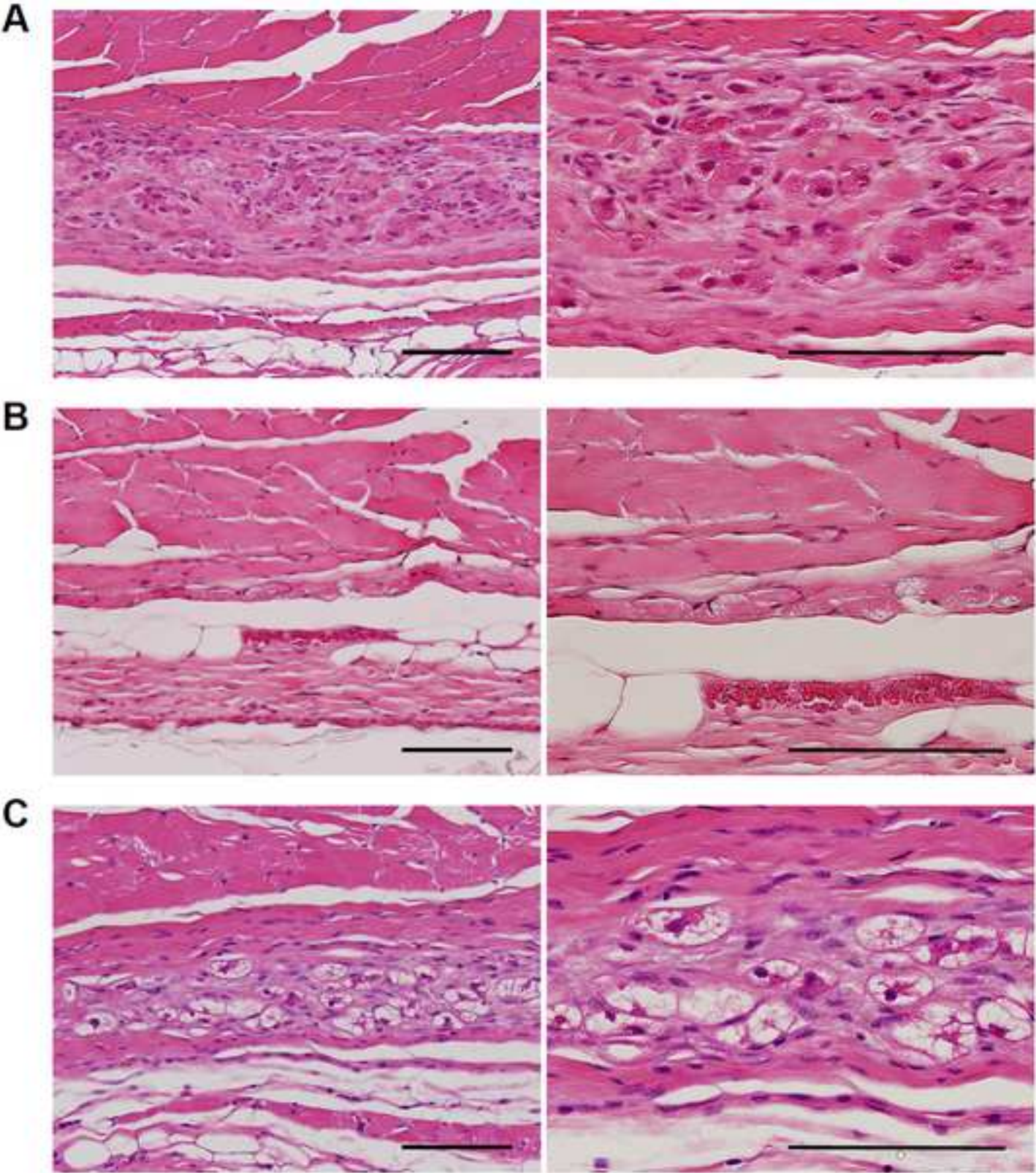
	Age	Sex	Disease	Viability [%]
1	70's	M	Hepatocellular carcinoma	93.1
2	70's	M	Intrahepatic cholangiocarcinoma	95.1
3	70's	M	Hepatocellular carcinoma	93.2
4	60's	M	Hepatocellular carcinoma	91.8
5	60's	F	Hepatocellular carcinoma	83.3
6	80's	M	Intrahepatic cholangiocarcinoma	89.7
7	70's	M	Intrahepatic cholangiocarcinoma	93.1
8	60's	F	Cholangiocarcinoma	85.8
9	70's	M	Hepatocellular carcinoma	92.4
10	60's	M	Metastatic liver tumor	95.4
11	70's	F	Intrahepatic cholangiocarcinoma	85.1
12	70's	F	Intrahepatic bile duct cystadenocarcinoma	93.1
13	60's	M	Hilar cholangiocarcinoma	89.8



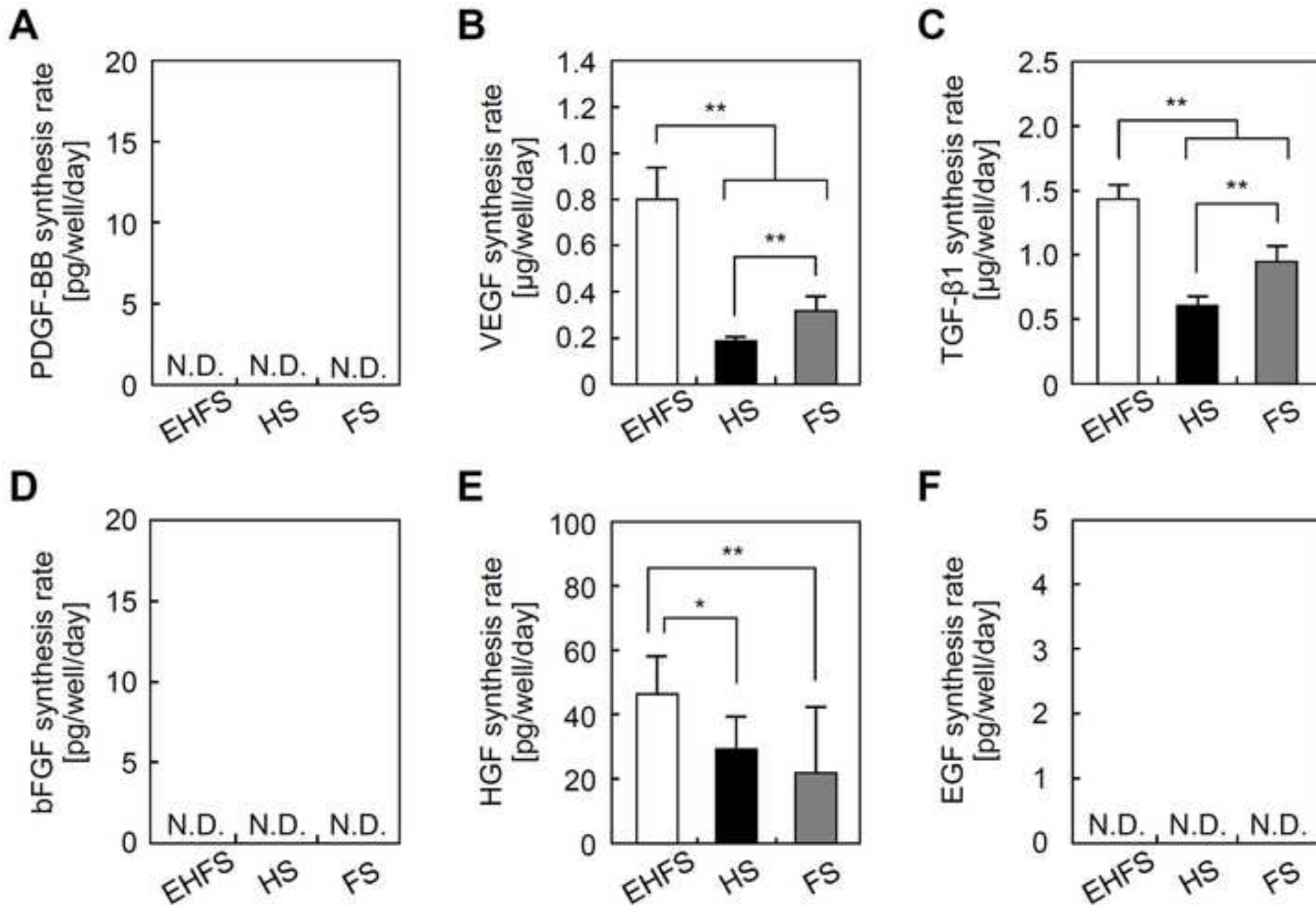


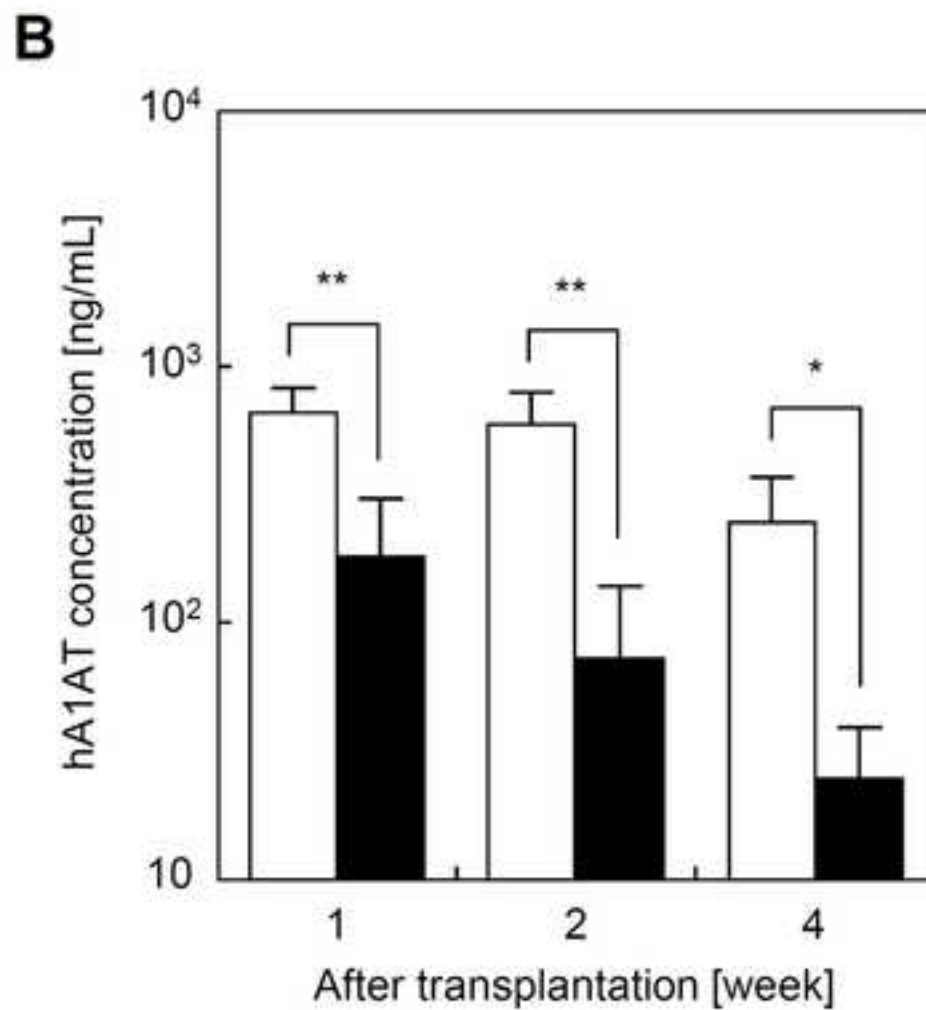
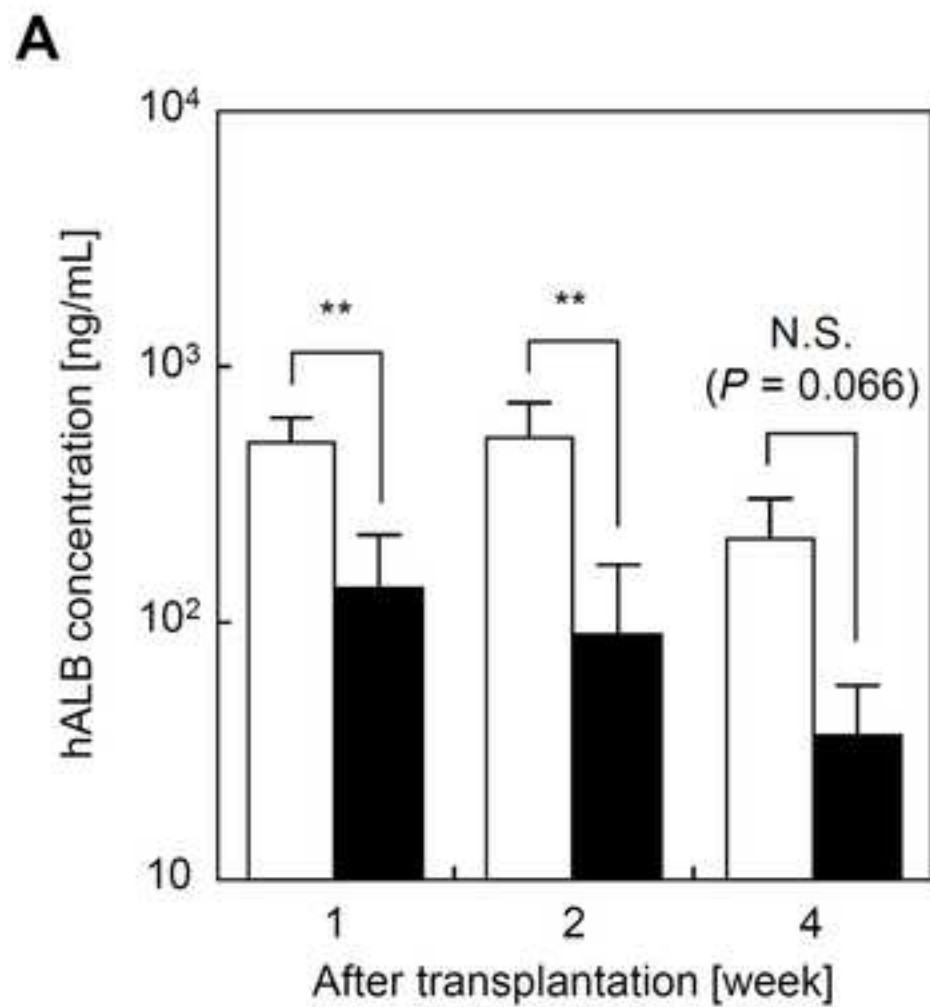


Supplementary Figure 3



Supplementary Figure 4







**Supplementary Table 1.** TaqMan gene expression assay numbers and GenBank accession numbers for real-time PCR analysis.

Gene symbol	Gene name	TaqMan Assay no.	GenBank no.
VEGF	Vascular endothelial growth factor A	Hs00900055_m1	NM_003376.5
TGF- $\beta$ 1	Transforming growth factor, beta 1	Hs00998133_m1	NM_000660.4
HGF	Hepatocyte growth factor	Hs00300159_m1	NM_000601.4
ACTB	Actin, beta	Hs99999903_m1	NM_001101.3

## Supporting Information for

# Vascularized subcutaneous human liver tissue from engineered hepatocyte/fibroblast sheets in mice

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## Supporting Information

### S1. Evaluation of mRNA expression

#### S1.1. Total RNA extraction

Vascularized subcutaneous human liver tissues (VSLTs) in NOG mice at 7 and 14 days after EHFS transplantation, human liver tissues, and engineered hepatocyte/fibroblast sheets at 4 days after hepatocyte culture (EHFSs) were used for mRNA extraction using a spin column (NucleoSpin RNA II; Nippon Genetics Co. Ltd., Tokyo) according to the manufacturer's instructions.

#### S1.2. cDNA synthesis

cDNA was synthesized from 0.2 µg total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo). The samples were then stored at -20 °C until they were processed for polymerase chain reaction (PCR) analysis.

#### S1.3. Real-time PCR

PCR was performed on an Applied Biosystems StepOnePlus Real-time PCR system using the TaqMan Gene Expression Assay Kit (Applied Biosystems; Supplementary Table 1). In brief, PCR amplification was performed using a reaction mixture containing 1 µL cDNA sample, 0.5 µL TaqMan Gene Expression Assay probe, 5 µL TaqMan Fast Universal PCR Master Mix solution (Applied Biosystems), and 13.5 µL nuclease-free water. Each amplification cycle consisted of 1 s at 95 °C and 20 s at 60 °C. The number of amplification cycles was 40 cycles.

#### S1.4. Data analysis of PCR

The comparative cycle time ( $\Delta\Delta CT$ ) method was used to quantify the gene expression levels according to the manufacturer's protocol. The expression levels of human VEGF, TGF- $\beta$ 1, and HGF were normalized to that of ACTB (EHFSs was set as 1.0) to establish the expression of angiogenic growth factors *in vivo*. Data are presented as mean  $\pm$  standard deviation and corresponded to 3 time points from one cell preparation.