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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Human primary hepatocyte isolation

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

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

2.2. Human fibroblast culture

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

2.3. Human hepatocyte sheet construction

Two types of human primary hepatocyte sheets were constructed using temperature-responsive culture dishes (TRCDs) (UpCell; CellSeed, Tokyo) in accordance with our previous report [27]. To construct EHFSs, human primary hepatocytes obtained as described were plated at 1.04×10^5 cells/cm^2 (1.0×10^6 cells/dish) onto a confluent layer of TIG-118 cells plated 3 days previously at 2.29×10^4 cells/cm^2 (2.2×10^5 cells/dish) onto non-coated TRCDs (Fig. 1A). To construct hepatocyte-only sheets (HSs), hepatocytes were inoculated onto TRCDs coated with FBS. All the human primary hepatocytes were cultured in Supplemented ISOM's Media (BD Biosciences, San Jose, CA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin until 24 h after inoculation. The Supplemented ISOM's Media was replaced with 2 mL of Hepato-STIM Culture Medium (BD Biosciences) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was changed 1 and 3 days after inoculation. All cells were cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After 4 days of hepatocyte culture or co-culture, plates were incubated at 20 °C to induce the formation of detached cell sheets. Fibroblast-only sheets (FSs) were also constructed by following the same process. Samples of media were collected after 1–3 days of culture and stored at −20 °C until assayed for human growth factors involved in angiogenesis.

2.4. Hepatocyte sheet transplantation under mouse skin

Ethical approval for hepatocyte sheet transplantation was obtained from the Animal Care and Use Committee and the Recombinant DNA Experiment Safety Committee of Nagasaki University and performed according to all protocols approved by the Regulations. The abdominal subcutaneous space for transplantation was created by a tear between the cutaneous and skeletal muscle layer in NOD SCID mice (NOD.CB17-*Prkdc*^{scid}/J; Charles River Japan Inc., Kanagawa, Japan) and in NOG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/Jic; Central Institute for Experimental Animals, Kanagawa, Japan) (males, 6–17 weeks old, weighing 20–35 g) (Fig. 1B). The hepatocyte-containing sheets (EHFSs or HSs) after 4 days of culture were subcutaneously-transplanted using a support membrane or a glass plate to facilitate handling, and the supporters were removed at several miniatures after contact of cell sheet into subcutaneous