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Fabrication of a fiber-type hepatic tissue by bottom-up method using multilayer spheroids

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Liver regenerative medicine, a therapy using cultured hepatocytes or hepatic tissues, has the potential to replace liver transplantation. However, this therapeutic strategy has challenges to overcome, including in construction of the hepatic tissues. As an approach to fabricating functional 3D hepatic tissues, we focused on hepatocyte spheroids, which have high cell density and maintain high liver-specific functions. We employed a bottom-up method using spheroids, arranging hepatocytes and endothelial cells regularly at the time of tissue construction. This enabled a vascular network to be formed within the three-dimensional hepatic tissue. We included NIH/3T3 cells, known to promote vasculature formation by endothelial cells. We fabricated hepatocyte spheroids covered with human umbilical vein endothelial cells (HUVECs) and NIH/3T3 cells (EC-3T3-covered hepatocyte spheroids) and constructed the hepatic tissues by stacking these cell types in hollow fibers. We then performed histological and functional analyses of the resulting hepatic tissues. The hepatic tissues constructed by stacking EC-3T3-covered hepatocyte spheroids showed high liver-specific functions; that is, ammonia removal and albumin secretion. The HUVECs formed endothelial networks. In addition, hypoxia-inducible factor-1α (HIF-1α) expression was suppressed in the hepatic tissue throughout the culture period and the hepatic tissue was sufficiently strong for use in certain analyses and applications. In summary, we fabricated a functional 3D hepatic tissue by the bottom-up method using hepatocyte spheroids covered with HUVECs and NIH/3T3 cells.

[**Key words:** Tissue engineering; Hollow fiber; Bottom-up method; Hepatocyte; Endothelial cell; NIH/3T3 cell; Spheroid]

The liver has a complex architecture and performs a variety of functions in the body. Even though the liver is highly regenerative, drugs, alcohol or viral infections can cause serious damage and reduce its function and regeneration. Extensive liver damage can lead to liver failure, for example in cirrhosis, hepatitis or cancer. A definitive treatment for patients with end-stage liver failure is liver transplantation, but the shortage of donors and risks of immune rejection exclude this option for many patients. Therefore, liver regenerative medicine, a therapy using cultured hepatocytes or hepatic tissues, has attracted attention as an alternative to transplantation. However, liver regenerative medicine has many challenges, including how to maintain the many hepatocyte functions in the regenerated tissues over long periods of time. Hepatocytes are widely known to lose their liver-specific functions rapidly in suspension or monolayer culture. Another challenge is construction of the hepatic tissue. Because the liver has a complex structure, including hepatic lobules and sinusoids, it is difficult to fabricate hepatic tissue with a structure similar to that of the living organ.

There are several methods for maintaining hepatocyte function. One is to construct a three-dimensional (3D) tissue. It is widely known that liver-specific functions and survival and specific characteristics of hepatocytes can be maintained by using a 3D, rather than monolayer, culture method (1). Spherical organization, that is, a spheroid structure, is commonly used because it is easy to construct many spheroids and to control their sizes. Another promising technique is to co-culture hepatocytes with heterologous cells. Many investigators reported improved liver-specific functions in hepatocytes co-cultured with stromal cells, including fibroblasts and endothelial cells (2–5). In addition, the level of improvement of liver-specific functions changed according to the co-culture cell types and their distributions, and even whether the hepatocytes were in contact with the heterologous type cells (5).

To construct an implantable hepatic tissue, it is necessary to construct a tissue of high cell density from a sufficient mass of hepatocytes, because there is limited space for implantation in the body. In addition, it is also important to supply sufficient oxygen and nutrients to all cells. For hepatocytes, which have a high oxygen requirement, a sufficient oxygen and nutrient supply is essential for survival and to maintain their high levels of function (6). To address these challenges, there have been many reports of various construction methods for 3D hepatic tissue in vitro. Some studies used de-cellularized liver matrices, formed by removing cells from the living tissue (7). In this manner, the extracellular matrix can serve as a template for the structure of the living tissue, including liver lobules and sinusoids (8). Other studies used 3D printing technology, employing an ink-jet method to build 3D structures mimicking vital organs (9). This technique can be used to generate structures of various shapes, such as cubes, sheets, rods and tubes (10). Other reports described construction of liver tissues using gels. This

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method can produce tissue constructs of specific shapes, such as strings, by suspending the cells during the sol-to-gel transition (11,12). Holes can be drilled in these structures to ensure the oxygen supply channels (13). Recently, some investigators fabricated micro liver tissues by co-culturing hepatocytes, vascular endothelial cells and mesenchymal cells (14). Although a number of liver tissue construction methods have been reported, no method has yet been described for construction of high-performance liver tissue of a useful size. In addition, previously reported methods required expensive devices or specialized materials. Thus, a number of challenges still exist.

To construct a 3D hepatic tissue model, we focused on using hepatocyte spheroids, which have high cell densities and maintain high levels of liver-specific functions. In this study, we employed a bottom-up method using spheroids (Fig. 1A). In a previous study, we fabricated hepatocyte spheroids covered with human umbilical vein endothelial cells (HUVECs) and constructed 3D hepatic tissues by stacking cells at a high density (15,16). In this manner, we arranged hepatocytes and HUVECs regularly at the time of tissue construction and a vascular network was formed within the 3D hepatic tissue (16). Among the merits of this method, it does not require specialized or costly materials and equipment and, in addition, can be easily scaled up. In the previous study, we evaluated cell viability and liver-specific functions of hepatic tissues constructed by stacking HUVEC-covered hepatocyte spheroids. We found that co-culturing HUVEC-covered hepatocyte spheroids inside hollow fibers improved liver-specific functions and cell survival. In the current study, we added NIH/3T3 cells, which are known to promote vascularization of endothelial cells in spheroids. Thus, we fabricated hepatocyte spheroids covered with HUVECs and NIH/3T3 cells (EC-3T3-covered hepatocyte spheroids) and constructed hepatic tissues by stacking these spheroids. We demonstrated the utility of this bottom-up method using EC-3T3-covered hepatocyte spheroids by evaluating liver-specific functions and oxygen supply to the cells in the hepatic tissue. In addition, we showed the suitability of this construct for transplantation by collecting the hepatic tissue from hollow fibers.

MATERIALS AND METHODS

Cell preparation HUVECs (Lonza, Basel, Switzerland) were cultured in endothelial growth medium-2 (EGM-2, Lonza) at 37°C in a humidified atmosphere equilibrated with 95% air, 5% CO_2.

NIH/3T3 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum and penicillin/streptomycin solution (Merck Millipore, Darmstadt, Germany) at 37°C in a humidified atmosphere equilibrated with 95% air, 5% CO_2.

Primary rat hepatocytes were isolated from a male Wistar rat (7–8 wk old, Kyudo, Saga, Japan) by the collagenase perfusion method (17). Experimental protocols were approved by the Ethics Committee on Animal Experiments at Kyushu University. Viable cells (85–95% yield) were used for spheroid cultures. Isolated hepatocytes were suspended in hepatocyte culture medium consisting of high-glucose Dulbecco's Modified Eagle Medium with 50 μg/L epidermal growth factor (EGF; BT-201, Biomedical Technologies, Stoughton, MA, USA), 10 mg/L insulin (Sigma–Aldrich, St. Louis, MO, USA), 60 mg/L proline (Sigma–Aldrich), 7.5 mg/L hydrocortisone (Wako Pure Chemical Industries, Osaka, Japan), 3.7 g/L $NaHCO_3$, 5.985 g/L HEPES, 50 mg/L linoleic acid, 0.1 μM copper ($CuSO_4 \cdot 5H_2O$), 3 nM selenium (H_2SeO_3), 50 pM zinc ($ZnSO_4 \cdot 7H_2O$), antibiotics (58.5 mg/L penicillin and 100 mg/L streptomycin) and EGM-2 Single Quots (Lonza, containing hydrocortisone, human vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (bFGF), R3-IGF-1α, ascorbic acid, heparin, 2% fetal bovine serum, human EGF and GA-1000).

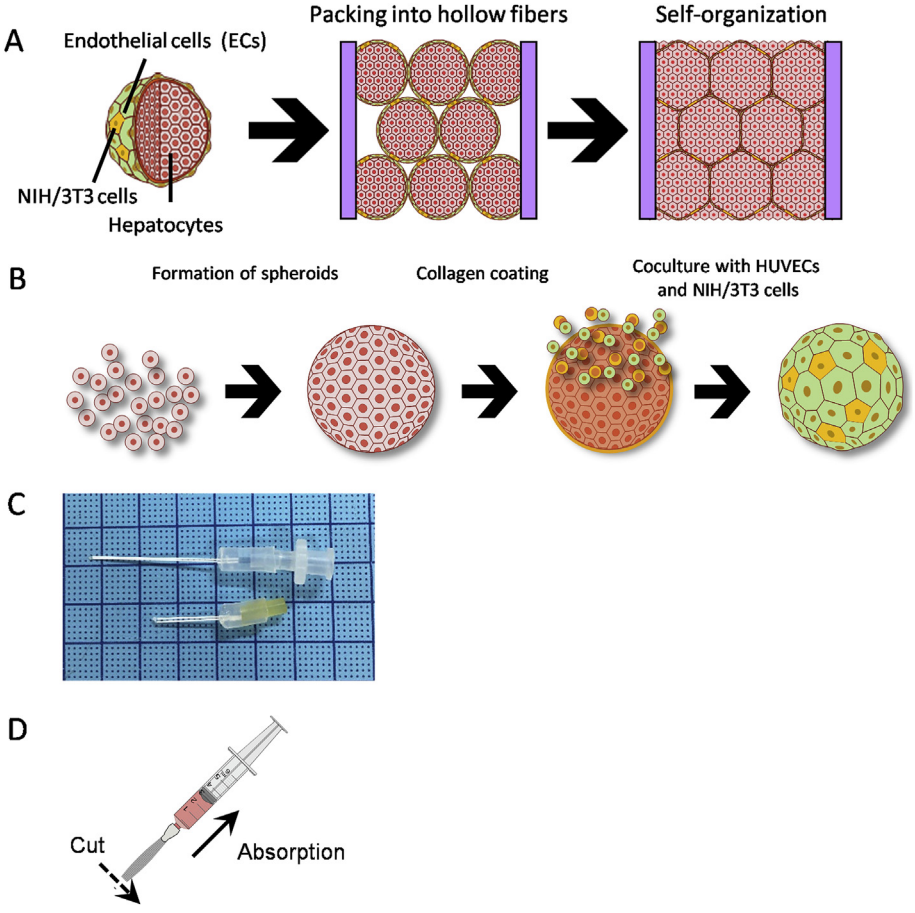

FIG. 1. Illustrations of culture methods used in this study. (A) 3D hepatic tissue construction by the bottom-up method using spheroids. (B) Culture of a HUVEC-3T3-covered hepatocyte spheroid. (C) Photograph of hollow fiber bundles. Upper bundle is for functional evaluation and lower bundle is for collection. (D) Method of collecting hepatic tissues.

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