Reconstitution of hepatic tissue architectures from fetal liver cells obtained from a three-dimensional culture with a rotating wall vessel bioreactor

Reconstitution of hepatic tissue architectures

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1 **Abstract**

2 Reconstitution of tissue architecture *in vitro* is important because it enables
3 researchers to investigate the interactions and mutual relationships between cells and cellular
4 signals involved in the three-dimensional (3D) construction of tissues. To date, *in vitro*
5 methods for producing tissues with highly ordered structure and high levels of function have
6 met with limited success although a variety of 3D culture systems have been investigated.
7 In this study, we reconstituted functional hepatic tissue including mature hepatocyte and
8 blood vessel-like structures accompanied with bile duct-like structures from E15.5 fetal liver
9 cells, which contained more hepatic stem/progenitor cells comparing with neonatal liver cells.
10 The culture was performed in a simulated microgravity environment produced by a rotating
11 wall vessel (RWV) bioreactor. The hepatocytes in the reconstituted 3D tissue were found to
12 be capable of producing albumin and storing glycogen. Additionally, bile canaliculi between
13 hepatocytes, characteristics of adult hepatocyte *in vivo* were also formed. Apart from this,
14 bile duct structure secreting mucin was shown to form complicated tubular branches.
15 Furthermore, gene expression analysis by semi-quantitative RT-PCR revealed the elevated
16 levels of mature hepatocyte markers as well as genes with the hepatic function. With RWV
17 culture system, we could produce functionally reconstituted liver tissue and this might be
18 useful in pharmaceutical industry including drug screening and testing and other applications
19 such as an alternative approach to experimental animals.

20

21

- 1 **Keywords: Tissue reconstitution, Liver development, Bile canaliculi, Three-dimensional**
- 2 **(3D), Rotating wall vessel (RWV), Stem cell**
- 3

1 **Introduction**

2 The liver is essential for systemic metabolism, particularly detoxification, lipid
3 metabolism, glucose metabolism, and secretion of serum factors that are required for
4 maintenance of homeostasis in mammals. The liver has a complex structure comprising
5 hepatocytes lined with sinusoidal endothelial microvasculature, bile ducts, Kupffer cells, and
6 stellate cells (1). The formation of a 3D structure is thought to be essential for the proper
7 function of liver tissues and maturation of liver cells.

8 The first morphological sign of the developing embryonic liver is the formation of
9 the hepatic diverticulum, an outpocketing of thickened ventral foregut epithelium adjacent to
10 the developing heart, observed at embryonic day (E) 9.0 (2). The anterior portion of the
11 hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, while the posterior
12 portion forms the gall bladder and extrahepatic bile ducts. At E9.5, the hepatic endodermal
13 cells delaminate from the epithelium and invade the adjacent septum transversum
14 mesenchyme to form the liver bud (3-5). Between E10-15, the liver bud undergoes a period
15 of accelerated growth as it gets vascularized and colonized by hematopoietic cells to become
16 the major fetal hematopoietic organ.

17 The hepatic endodermal cells consist of not only hepatocytes but also hepatic stem
18 cell and hepatic progenitor cells. These cells contribute to the expansion of volume of liver
19 tissue during embryonic development. Our group previously succeeded in isolating and
20 identifying hepatic stem/progenitor cells in the mouse fetus (6-8). Hepatic stem/progenitor
21 cells possess both proliferative and bipotent properties with respect to hepatocytes and

1 cholangiocytes. Both vascular endothelial and hematopoietic cells constitute a highly ordered
2 network to produce sufficient functions *in vivo* in a 3D arrangement, which, to our current
3 knowledge, cannot be reproduced in 2D culture. Due to the limitations of 2D culture,
4 knowledge regarding tissue construction from cells is limited. Production of tissues with
5 highly ordered structures and high levels of function *in vitro* has not been successful although
6 several 3D culture systems have been tested for liver tissue production.

7 Understanding the mechanisms of construction of tissue from the cells will provide
8 new insights in the field of developmental biology and in regenerative medicine. Thus, we
9 sought to construct 3D hepatic tissue from liver cells using a rotating wall vessel bioreactor
10 (RWV), which utilizes a circular vessel with a gas-exchange membrane and that rotates
11 vertically to provide culture medium flow in a simulated microgravity environment (9-12)
12 Previously, we reported on the formation of hepatic-like tissue from mouse neonatal liver
13 cells (13). However, it has not been achieved that construction of hepatic tissue composed of
14 matured, adult-type hepatocytes, bile ducts and blood vessels. We assumed that the limited
15 reconstitution of liver tissue was due to the lower content of highly proliferative and bipotent
16 stem/progenitor cells in the neonatal liver.

17 In this study, we demonstrate the reconstruction of liver-like tissue from E15.5 fetal
18 liver cells having highly ordered structure and several metabolic functions, using RWV-based
19 3D culture systems.

1 **Materials and Methods**

2 Mouse fetal liver cell preparation

3 Fetal liver cell suspensions were prepared from fetal ICR mice (CLEA, Tokyo,
4 Japan) as previously described (14). Briefly, E15.5 fetal livers were dissected in Hanks'
5 balanced salt solution (Sigma) containing 0.05% collagenase and 5 mmol/L CaCl₂ (pH 7.4)
6 and incubated for 10 min at 37°C with gentle shaking. A single cell suspension was washed 3
7 times in our standard medium, a 1:1 mixture of Dulbecco's modified Eagle's medium and
8 F-12 (Sigma, St. Louis, MO), supplemented with 10% fetal calf serum and several other
9 factors (6). Human recombinant HGF (50 ng/mL) (Sigma) and epidermal growth factor
10 (EGF) (20 ng/mL) (Sigma) were added to the medium just before use. Cell viability after
11 each treatment exceeded 90% as assessed by trypan blue dye exclusion.

12

13 Hanging drop and Spinner flask culture of fetal mouse liver cells

14 Fetal liver cells were suspended in 20 µL medium containing 1×10^6 cells per drop
15 to obtain a Hanging drop. Culture was carried out for 10 days. Subsequently, cell aggregates
16 were transferred to a suspension culture dish for an additional 10 days of culture. The fetal
17 liver cells at a concentration of 5×10^6 cells/mL were cultured in a 100-mL Spinner flask
18 with gentle shaking using a Spinner.

19

20 RWV culture of fetal mouse liver cells

21 RWV culture was performed as previously described with a slight modification (13).

1 A Rotating Wall Vessel (RWV) (Synthecon, Houston, TX) with a 10 ml capacity was used for
2 these experiments. 10 ml of medium containing cells at a density of 1×10^6 cells/ml was
3 poured into a 10 ml capacity of RWV. The medium in the RWV was changed every 2 days.
4 Since the culture medium rotates with the vessel, the speed of rotation was adjusted to
5 counteract the sedimentation rate of the tissue so that the tissue remained suspended in the
6 fluid. The rotation speed used in these experiments was 15.0-18.5 rpm depending on the size
7 of the tissues. Briefly, liver cells were cultured in our standard medium (see above). As a
8 control, 10 ml of medium including fetal liver cells were cultured on a $\phi 10$ cm cell-culture
9 dish (Becton Dickinson) coated with type IV collagen at 5×10^6 cells/mL, which was same
10 density as that used for the RWV culture.

11

12 Histology and immunohistochemistry

13 The 3D tissue samples obtained from the RWV culture were washed with PBS, fixed
14 with 4% PFA for 12 h, and embedded into paraffin after dehydration. Sections (3 μ m) were
15 prepared and stained with hematoxylin-eosin (HE), Hoechst, Alcian blue, PAS, and Masson
16 trichrome stain according to standard procedures. The percentage of Hoechst positive cells
17 were quantified after staining and plotted against the total number of counted cells by Bright
18 field. Immunostaining was performed using rat anti-mouse Ki67 monoclonal antibody (Dako),
19 rabbit anti-laminin polyclonal antibody (Sigma), rabbit anti-type IV collagen polyclonal
20 antibody (LSL, Japan), mouse anti-cytokeratin 7 monoclonal antibody (Dako), rabbit
21 anti-albumin polyclonal antibody (Biogenesis, Poole, UK), and goat anti-PECAM-1

1 polyclonal antibody (Santa Cruz Biotechnology, Inc.).

2

3 TEM

4 The 3D tissue samples obtained from the RWV culture were fixed with 2.5%
5 glutaraldehyde/PBS for 2 h at 4°C, and embedded in epoxy resin. Half-thin sections were
6 stained with toluidine blue to detect endogenous structure. Ultrathin sections were stained
7 with uranyl acetate and lead citrate and observed using a transmission electron microscope
8 (Hitachi, Tokyo, Japan).

9

10 Measurement of albumin secretion

11 Six 3D tissue samples cultured for 10 days were placed in the wells of a nonadherent
12 24-well plate. 1 mL of cultural supernatant was collected and incubated for 0, 48, and 120 h
13 to detect the amount of albumin protein. Concentrations of were measured using the Mouse
14 Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., TX, USA) according to the
15 manufacturer's instructions. The amount of albumin secretion per 1×10^6 hepatic cells in 2D
16 samples was obtained by the same method as 3D. The cultural cell numbers in 2D as well as
17 3D were determined by digested into single cells with trypsin.

18

19 Ammonium metabolism measurement

20 Six 3D tissues samples cultured for 5 days and 10 days were placed into wells of a
21 nonadherent 24-well plate. A volume of 1 mL of culture medium containing 2 mM of

1 ammonium chloride was added to the well, and incubated for 0, 3, and 6 hours. The
2 concentration of ammonium in the culture medium was measured using AMICHECK and the
3 AMICHECK METER system (ARKRAY, Kyoto, Japan). The amount of ammonium
4 consumption per 1×10^6 cells was determined at day 5 and day 10 of culture by the
5 AMICHECK and the AMICHECK METER system. The cultural cell numbers in 2D as well
6 as 3D were assessed by digested into single cells with trypsin after the measurement of
7 ammonium consumption.

8

9 Statistical analysis

10 All data are expressed as means $\pm$ standard deviation (SD). Statistical comparisons
11 were evaluated using a one-way analysis of variance (ANOVA) and values of $p < 0.05$ were
12 considered to be significant.

13

1 **Results**

2 **Formation of 3D tissue from mouse fetal and neonate liver cells and hepatic structure** 3 **reconstitution**

4 We first compared the RWV culture with other 3D culture methods, the Hanging
5 drop method and the Spinner flask method, using fetal liver cells obtained at E15.5 (15). Cell
6 aggregates (major axis; 1600 μ m, minor axis; 600 μ m) appeared in the tissue cultured by the
7 Hanging drop method. The tissue was produced by 10 days of culture but the tissues did not
8 possess normal smooth round shape structure and only a few blood vessel-like structures
9 could be observed in this tissue (Fig. 1 A, B). Cell aggregates (major axis; 2000 μ m, minor
10 axis; 1200 μ m) also appeared in the tissue cultured by the Spinner flask. However, only a
11 few bile ducts like structures were observed in the tissue and mostly tissue aggregates were
12 necrotized (Fig. 1 C, D). Tissue structure consisting of both bile duct-like structures and
13 blood vessel-like structures did not appear when these 2 methods were used. When the RWV
14 bioreactor was used, 3D tissue (major axis; 3000 μ m, minor axis; 2500 μ m) with both bile
15 duct-like structures and blood vessel-like structures appeared after 10 days of culture from
16 mouse fetal liver cells (Fig1 E, F). The number of bile duct-like structures increased in the
17 tissue cultured by RWV bioreactor for 10 days relative to the tissue cultured for 5 days (Data
18 not shown). Although reconstitution of 3D tissues (major axis; 4000 μ m, minor axis; 2500
19 μ m) from neonatal liver cells were established (Fig1 G, H) as indicated in the previous study,
20 blood vessel-like structures were not reproduced. Qualification analysis were performed to
21 compare the number of bile duct-like structures and blood vessel-like structures within each

1 tissue formed by RWV, Spinner flask and Hanging drop methods (Fig. 1 A-H, I). A tissue
2 reconstructed from fetal liver cells using a RWV method markedly revealed to be found the
3 highest number of bile duct-like structures and blood vessel-like structures.

4 We further investigated the cell viability and the percentage of albumin positive cells
5 in tissues formed from fetal and neonatal liver cells to ascertain similarity with mature adult
6 hepatocytes. Both cell viability (fetus; $95.17 \pm 3.91\%$, neonate; $88.98 \pm 2.16\%$) (Data not
7 shown) and the percentage of albumin positive cells (fetus; $95.86 \pm 1.38\%$, neonate; $75.71 \pm$
8 5.90%) (Fig. 1 J-N) in a tissue from fetal liver cells observed to be higher than that of
9 neonatal liver cells.

10

11 Functional analysis of hepatic-like 3D tissue cultured in the RWV bioreactor.

12 The number of binuclear hepatocytes found in normal mature liver increased over
13 the culture period (Fig. 2 A, B, E). Transmission electron microscopy (TEM) revealed the
14 presence of bile canaliculi and tight junctions between hepatocytes (Fig. 3 N, O). Formation
15 of bile canaliculi between hepatocytes indicated that hepatocytes differentiated into mature,
16 adult-type hepatocytes and the 3D tissue sample has the ability to excrete drugs and toxic
17 metabolites. Ki67 positive cells, which indicate the growth state, were observed to decrease
18 among the hepatocytes in the tissue after 10 days of culture relative to the tissue that was
19 cultured for 5 days (Fig. 2 C, D, F).

20 Most of the cells within the 3D tissue were albumin-positive, confirming that the 3D
21 tissue is predominately composed of hepatocytes (Fig. 2 G, H). Periodic acid Schiff (PAS)

1 staining for glycogen revealed that many cells in tissue at the margins were PAS positive. The
2 observation of relatively high levels of stored glycogen is an indication of mature and
3 functional hepatocytes (Fig. 2 I, J). The 3D tissue samples after 10 days of culture were
4 strongly stained with PAS relative to the 3D tissue obtained after 5 days of culture (Fig. 2 I, J).
5 Masson trichrome staining indicated that collagen fibers were present in the tissue at the
6 margins (Fig. 2 K, L).

7

8 **Formation of bile duct structures**

9 A luminal, bile duct-like structure surrounded by cuboidal and columnar epithelium
10 is formed in the 3D tissue after 5 and 10 days of culture (Fig. 3 A, B). Some of the large bile
11 ducts were observed to have a branching structure and were organized in a 3D structure (Fig.
12 3 A, B). The mucus within the bile duct was stained by Alcian blue, indicating that mucin was
13 produced by cells around the bile duct (Fig. 3 D). The epithelial cells comprising the bile
14 duct-like structure after 10 days of culture were positive for Ki67. This suggests that bile duct
15 epithelium proliferates in the late phase of culture relative to hepatocytes (Fig. 3 E, F, G).
16 Immunostaining revealed that the cells forming the lumen were positive for cytokeratin 7 and
17 specific for bile duct epithelial cells (Fig. 3 C).

18 The margins of the bile duct-like structures were immunostained for laminin (Fig. 3
19 H, I) and collagen IV (Fig. 3 J, K). Both of these proteins exist in extracellular matrix, which
20 forms the basement membrane. This suggests that basal lamina is being constructed. The
21 basal lamina in the tissues after 10 days of culture increased in thickness. This was not

1 observed after 5 days of culture, indicating that the structure of basal lamina only becomes
2 well organized with additional time (Fig. 3 H–K).

3 Microstructures in this bile duct were observed using a transmission electron
4 microscope, which provides visualization of the architecture of the microvillus on the cells
5 inside the bile duct. Tight junctions and gap junctions between cells are also visible (Fig. 3 L,
6 M).

8 **Formation of blood vessels**

9 In the 3D tissue obtained after 10 days of culture, serial sections displayed 3D blood
10 vessel-like lumen structures with thin squamous endothelial cells (Fig. 4 A–E). The cells
11 constituting the luminal structure were positive for platelet endothelial cell adhesion
12 molecule-1 (PECAM-1), which is specific to vascular endothelial cells. This indicates that the
13 blood vessel-like structure is indeed formed from endothelial cells (Fig. 4 F). The formation
14 of the basal membrane in the blood vessel-like structure was also apparent from the laminin
15 staining (Fig. 4 G). Moreover, the bile duct-like structure lining the blood vessel-like
16 structure was observed. Thus it appears that the tissue structure obtained after 10 days of
17 culture closely resembles the tissue of an adult liver (Fig. 4 H, I).

19 **Expression of liver markers and analysis of liver function**

20 Gene expression analysis by semi-quantitative RT-PCR showed elevated levels of
21 mature hepatocyte markers such as α -1-antitrypsin (a protease inhibitor that is considered to

1 prevents from protease-mediated tissue destruction), glucose-6-phosphatase (a key regulator
2 of glucose metabolism), tryptophan-2,3-dioxygenase (the rate limiting enzyme for tryptophan
3 metabolism), asialoglycoprotein receptor (an important receptor for the turnover of plasma
4 glycoproteins), ornithine transcarbamylase (an enzyme involved in regulating ammonia
5 metabolism), and CYP3A (an enzyme involved in drug metabolism). The expression levels of
6 each marker increased over time (Fig. 5 A). The expression of these genes associated with
7 liver function rarely appeared in 2D culture carried out over the same time period as RWV
8 culture.

9 The ability to secrete albumin, which is one of the hallmarks of liver function, was
10 measured by ELISA. The concentration of albumin in the 3D cultured cells increased over
11 time relative to the 2D cultured cells (Fig. 5 B). To investigate ability of the 3D tissue to
12 metabolize ammonia, we added ammonium chloride to the culture medium and measured the
13 change in the concentration of ammonia. The concentration of ammonium declined during
14 the culture period (Fig. 5 C). Accordingly, these results indicate that the hepatocytes that form
15 the 3D tissue in the RWV culture might tend to express some functions in gene level similar
16 to the function of adult hepatocytes.

17

1 **Discussion**

2 Development and reconstitution of liver tissue requires not only hepatic parenchymal
3 cells but also sustentacular non-parenchymal cells to organize the complicated 3D structure,
4 which includes bile ducts formed from cholangiocytes, and blood vessels formed from
5 endothelial cells. To understand the maturation of liver cells during development and tissue
6 reconstitution, it is necessary to understand the relationships between cellular maturation and
7 3D construction of tissue architecture.

8 In this study, we reconstructed a hepatic tissue from fetal liver cells utilizing the
9 RWV bioreactor system. The RWV culture was able to reconstitute the 3D tissue from fetal
10 liver cells more efficiently than the cultures obtained using the Hanging drop or Spinner flask
11 3D culture methods. With Hanging drop method, most of reconstructed tissues were
12 unshaped or crumbled. Although the production of regular or round shape tissue was
13 identified in Spinner flask, mainly necrotized tissues were formed due to inadequate oxygen
14 or nutrients supply. The RWV bioreactor system allows gentle shear stress within the
15 chamber thus cell damages caused by mechanical agitation are reduced comparing to Spinner
16 flask [16]. Furthermore, RWV culture system showed to reduce equilibrium modulus
17 effective, dynamic stiffness effective and increase tissue permeability effective [17, 18].
18 Accordingly, in RWV culture system sufficient nutrients and oxygen are supplied to the cells
19 through cellular responses by specific biological activities in microgravity environment,
20 resulting in closely similar circumstances produced by tissues in vivo.

21 The reconstructed 3D tissue by RWV culture system was mainly reconstituted with

1 hepatocytes, which subsequently differentiated into mature, adult type hepatocytes. As
2 previous study indicated the reconstruction of bile duct-like structures within a tissue from
3 neonatal liver cells, however, we showed high numbers of reconstituted bile duct-like
4 structures and blood vessel-like structures as well as hepatocyte producing albumin in the
5 tissue formed from fetal liver cells. Moreover, bile canaliculi were also reconstituted. In the
6 fetal liver tissue used in this study, hepatic stem/progenitor cells were found to be abundant
7 relative to neonatal or adult tissue. This suggests that the formation of highly functional tissue
8 structure was promoted through proliferation and differentiation of hepatic stem/progenitor
9 cells that possess bipotency towards hepatocytes and cholangiocytes. Furthermore, fetal liver
10 tissue also includes endothelial progenitor cells. The differentiation of hepatic parenchymal
11 cells into more mature, adult type hepatocytes would be attributed to an abundance of
12 sustentacular cells including cholangiocytes and vascular endothelial cells. Therefore, fetal
13 liver cells are suitable for reconstitution of tissues with bile duct-like structure and blood
14 vessel-like structure when compared to neonatal liver cells that have lower stem/progenitor
15 cell content. Bile canaliculi with microvilli and tight junctions between hepatocytes were
16 reconstituted. This was not observed in the tissue sample formed from neonatal liver cells.
17 Construction of bile canalicular structure and tight junction between hepatocytes require
18 proper apical and lateral domain construction. This indicates that the transport proteins were
19 properly sorted to the apical membranes of bile canaliculi and the hepatocytes possess
20 metabolic functions, and drug metabolites are excreted into bile.

21 The conformation of the bile canalicular structure may be attributed to the highly

1 ordered tissue structure, which supports the maturation of hepatocytes and conformation of
2 canalicular structure. Alternatively, the conformation of endothelial cells may indirectly
3 support the formation of bile canaliculi. It has been recently reported that endothelial cell
4 produce angiocrine factor, Wnt2 and hepatocyte growth factor (HGF) via the vascular
5 endothelial growth factor(VEGF)-VEGFR2-Id1 signal cascade, which induces hepatocyte
6 proliferation. Furthermore, a defective VEGF-VEGFR2-Id1 signal causes elevated plasma
7 bilirubin levels during regeneration of the liver. This may indicate that construction of the
8 canalicular structure is indirectly supported by the sinusoidal endothelium (19).

9 The hepatocyte conformation also affects the blood vessel structure, including the
10 sinusoid structure. It has been found that signals between parenchymal cells and
11 non-parenchymal cells are important for reconstitution of highly ordered, highly organized
12 normal liver tissue (20-22). In the regeneration of liver, it has been reported that VEGF
13 generated from hepatic parenchymal cells stimulates sinusoidal endothelial cells to produce
14 HGF, which promotes hepatocyte growth (19, 23). Thus, the proper epithelial-mesenchymal
15 interaction and the complicated blood vessel networks observed in the tissue *in vivo* are
16 required to reconstruct highly ordered and highly matured tissue (24, 25).

17 Recent progress in ES cells and iPS cell research has opened up the possibility of
18 using these cells in drug development and regenerative medicine. Matured hepatocytes, in
19 particular, are essential for drug screening. By using RWV culture, it will be possible to
20 induce these immature liver cells derived from pluripotent stem cells to form mature,
21 adult-type hepatocytes that possess metabolic function and are capable of excreting drug

1 metabolites into bile. This will enable drug development taking into consideration the
2 difference at individual and population levels by using matured adult-type liver tissue derived
3 from pluripotent stem cells. This will also apply to regenerative medicine based on liver
4 tissue derived from patient-specific iPS cells.

5 In summary, we succeeded in reconstituting a hepatic-like tissue formation with
6 bile duct-like structures and blood vessel-like structures from mouse fetal liver cells using the
7 RWV bioreactor system. The RWV culture with fetal liver cells provided liver-like tissues *in*
8 *vitro*, which have conformations and functions similar to liver tissue formed *in vivo*. In our
9 study, it was found that the 3D tissue has the ability to perform metabolism and ammonia
10 detoxification functions. The conformation of the bile canaliculus with microvilli indicates
11 that the RWV culture from fetal liver cells will be useful to evaluate the pharmacokinetics of
12 drug candidates.

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14

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1 **Figure Legends**

2 Figure 1: Comparative analysis of hepatic-like tissue from neonatal liver cells and fetal liver
3 cells cultivated in the 3D culture systems.

4 (A-H) Comparison of 3D culture methods. H&E staining for tissue sections obtained from
5 Hanging drop (A, B), Spinner flask (C, D) and RWV (E-H) culture methods. Bile duct-like
6 structures (arrow) and blood vessel-like structures (arrowhead) are indicated in a 3D tissue
7 cultured for 10 days.

8 (I) Comparing the number of bile duct-like structures and blood vessel-like structures formed
9 within a tissue from neonatal and fetal liver cells, (I) (n = 3) (*: $p < 0.05$).

10 (J-M) Immunostaining for albumin in a tissue formed from neonatal and fetal liver cells by
11 RWV culture.

12 (N) Comparison of the amount of albumin positive cells in the tissue cultivated from neonatal
13 liver cells (J) and cultivated from neonatal liver cells (L) (n = 3) (*: $p < 0.05$).

14 (K) Higher-power magnification view of (J).

15 (M) Higher-power magnification view of (L).

16

17 Figure 2: Formation of liver-like 3D tissue from fetal liver cells cultivated in the RWV
18 bioreactor.

19 (A, B, E) (E) Comparison of the number of binuclear hepatocytes (white arrowhead) in the
20 3D tissue at 5 (A) and 10 (B) days of culture. (n = 3) (*: $p < 0.05$).

21 (C, D, F) (F) Comparison of the amount of Ki67 positive cells in the 3D tissue after 5 days

1 (C) and 10 (D) days of culture. (n = 3) (*: p < 0.05).

2 (G, H) Immunohistochemistry of albumin in the 3D tissue section after 5 (G) and 10 (H) days

3 of culture.

4 (I, J) PAS staining of the tissue section obtained after 5 (I) and 10 (J) days of culture.

5 (K, L) Masson trichrome staining revealed collagen-producing mature hepatocytes in the 3D

6 tissue after 5 (K) and 10 (L) days of culture.

7

8 Figure 3: Bile duct-like structures in the reconstituted 3D tissue.

9 (A, B) H&E staining for a bile duct-like structures within a 3D tissue cultured in the RWV for

10 5 (A) and 10 (B) days.

11 (C) Immunostaining of CK7 in the bile duct-like structure.

12 (D) Alcian blue staining showed that cells secrete mucin into the ductal lumen

13 (counterstaining with nuclear fast red).

14 (E, F) Immunostaining for Ki67 in a 3D tissue.

15 (G) Comparison of the amount of Ki67 positive cells in the 3D tissue after 5 (E) and 10 (F)

16 days of culture. (n = 3) (*: p < 0.05).

17 (H-K) Immunostaining for laminin (H, I) and type IV collagen (J, K). The basement

18 membrane formed around the bile duct-like structure after 5 (H, J) and 10 (I, K) days of

19 culture.

20 (L) Transmission electron microscope (TEM) image of the bile duct-like structure within a

21 3D tissue obtained after 10 days of culture. Note: the microvillus (black arrowheads) on the

1 bile duct epithelial cells, tight junction (arrow) and gap junction (white arrowheads) could be
2 recognized.

3 (M) Higher-power magnification view of (L).

4 (N, O) The bile canaliculus with microvillus between hepatocytes (black arrowheads) and the
5 tight junction (black arrows) were seen by TEM.

6 (O) Higher-power magnification view of (N).

7

8 Figure 4: The formation of blood vessel-like structures in the reconstituted 3D tissue.

9 (A-E) H&E staining for serial sections in the 3D tissue cultured by the RWV for 10 days. The
10 formation of blood vessel-like structures is indicated.

11 (F) Immunostaining for PECAM-1 showed the formation of the lumen by vascular
12 endothelial cells.

13 (G) Immunostaining for laminin indicates the basement membrane structure (red).

14 (H, I) The 3D tissue cultured for 10 days. Bile ducts (arrow) accompanied by blood vessels
15 (arrowhead) is indicated.

16

17 Figure 5: Liver function of the reconstituted 3D tissue.

18 (A) The expressions of genes levels responsible for liver function were analyzed by
19 semi-quantitative RT-PCR.

20 (B) The amount of albumin secretion measured by ELISA in the 3D tissue. (n = 3) (*: p <
21 0.05).

1 (C) The amount of ammonium consumption in the 3D tissue. Ammonium chloride added to
2 the medium was measured after 0, 3, and 6 h in 3D tissues cultured for 5 days and 10 days. (n
3 = 3) (*: $p < 0.05$).

FIG.1

FIG.2

FIG.3

FIG.4

FIG.5