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ORIGINAL ARTICLE

A bioartificial liver device based on three-dimensional culture of genetically engineered hepatoma cells using hollow fibers

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Abstract The bioartificial liver (BAL) device is an extracorporeal liver support system incorporating living hepatocytes. A major problem in BAL device development is to obtain a high number of functional cells. In this study, we focused on a genetically engineered mouse hepatoma cell line, Hepa/8F5, in which elevated liver functions are induced via over-expression of liver-enriched transcription factors activated by doxycycline (Dox) addition. We applied a three-dimensional culture technique using hollow fibers (HFs) to Hepa/8F5 cells. Hepa/8F5 cells responded to Dox addition by reducing their proliferative activity and performing liver-specific functions of ammonia removal and albumin secretion. The functional activities of cells depended on the timing of Dox addition. We also found that Hepa/8F5 cells in the HF culture were highly functional in a low rather than high cell density environment. We further fabricated an HF-type bioreactor with immobilized Hepa/8F5 cells as a BAL device. Although ammonia removal activity of this BAL device was lower than that of the small-scale HF bundle, albumin secretion activity was slightly higher. These results indicated that the BAL device with immobilized Hepa/8F5 cells was highly functional with potential to show curative effects in liver failure treatment.

Keywords Genetically engineered hepatoma cell line · Bioartificial liver · Three-dimensional culture · Hollow fiber

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Introduction

As one of the largest organs in our body, the liver is essential for survival. Despite the high regenerative ability of the liver, drugs and viral infection cause serious damage and reduce liver-specific functions and regeneration. Severe liver dysfunction results in serious diseases with high mortality rates. To this end, liver transplantation is a successful treatment option for patients with end-stage liver disease. However, its application has been limited due to the shortage of organ donors.

Extracorporeal liver support systems, which support liver functions from outside the patient's body, are classified into two types: purely mechanical artificial liver support systems based on membrane

filtration and adsorption, and bioartificial liver (BAL) support systems containing living hepatocytes. A BAL system with immobilized hepatocytes is expected to show not only detoxification but also a synthetic ability. Various types of BAL systems have already been extensively studied, and several of them have also been evaluated in clinical trials (Millis et al. 2002; Sauer et al. 2002; Demetriou et al. 2004; van de Kerkhove et al. 2004). However, there is still room for discussion on suitable cell sources in BAL devices for clinical applications. Primary human hepatocytes are the most preferred cell source for BAL systems. However, organ shortage hampers the acquisition of human hepatocytes. Porcine hepatocytes are readily obtained in large quantities and able to perform liver-specific functions as well as human hepatocytes. Most clinical BAL studies have used primary porcine hepatocytes (Mundt et al. 2002; Demetriou et al. 2004; van de Kerkhove et al. 2004). However, there are serious problems concerning the use primary porcine hepatocytes as well, such as xenozoonosis risk (Wilson 2008) and immune reactivity. Stem cells are other alternative cell source candidates. Embryonic stem (ES) cells (Martin 1981; Evans and Kaufman 1981) and induced pluripotent stem cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007) have recently gained attention as new cell sources due to their infinite proliferation capacity and ability to differentiate into hepatocytes (Mizumoto et al. 2012, 2018; Sakiyama et al. 2017). On the other hand, before employing pluripotent stem cells in BAL devices, problems such as teratoma formation risk and low efficiency of differentiation into hepatocytes must be resolved. Human hepatoma-derived C3A cells are among other prospective cell sources. These cells were previously utilized in an extracorporeal bioartificial liver system (Millis et al. 2002). A previous study reported that C3A cells exhibited comparable liver-specific functions such as production of albumin and fetoprotein and nitrogen metabolizing capacity (Nyberg and Misra 1998). However, C3A cells lack ammonia removal and many other drug metabolism abilities (Wang et al. 1998).

A genetically engineered hepatocyte cell line, which can switch between proliferation and differentiation states, can also be an alternative cell source. Hepa/8F5 cells are genetically modified mouse hepatoma cells with enhanced liver function due to overexpression of liver-enriched transcription factors activated by doxycycline (Dox) addition (Yamamoto et al. 2012, 2018, available from RIKEN BioResource Research Center (Tsukuba, Japan) [RCB4661]). Here, we used the hollow fiber (HF) method where cells are immobilized inside plasma separation hollow fibers to culture Hepa/8F5 cells (Fig. 1). This culture system allows the formation of cellular aggregates inside HFs. In our previous study using the HF culture, primary hepatocytes formed multicellular aggregates inside HFs, achieving high cell densities and long-term maintenance of liver-specific functions (Mizumoto et al. 2008). We also found that the HF culture method is a more effective tool for inducing the differentiation of ES cells into hepatocytes (Amimoto et al. 2011). The HF culture is also easy to scale up, allowing the design of an HF-type bioreactor. We have already designed several BAL devices for primary hepatocytes (Aoki et al. 2008) and ES cells (Mizumoto et al. 2018) and have verified their clinical efficacy using a rat liver failure model. Thus, in the present study, we tried to find new possibilities through the genetic engineering of hepatocyte cell lines as a cell source for the BAL systems. We assessed the liver-specific functions of Hepa/8F5 cells in HF culture and designed an HF-type BAL module.

Materials and methods

Culture conditions of Hepa/8F5 cells

Hepa/8F5 cells were grown on tissue culture dishes coated with collagen Type I (Asahi Glass Co., Ltd., Tokyo, Japan) at 37 °C in humidified air with 5% CO_2 using high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS),

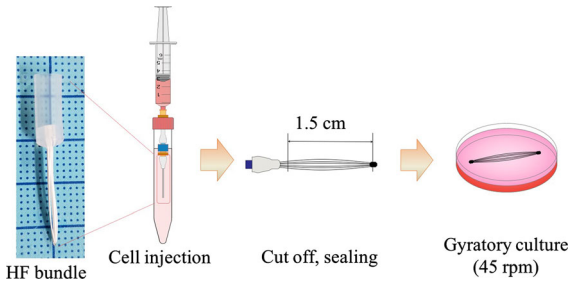


Fig. 1 A schematic illustration of the culture methods used in this study

100 U/ml penicillin (Millipore, Billerica, MA, USA), and 100 μg/ml streptomycin (Millipore).

HF culture

Figure 1 illustrates the HF culture method used in this study. In the HF culture, an HF bundle comprised of 7 HFs (inner diameter: 140–145 μm; outer diameter: 240–285 μm; kindly donated by Unitika Ltd., Osaka, Japan) was used. Subcultured Hepa/8F5 cells were resuspended in the culture medium at a density of 1.0×10^5 cells/ml. 1 ml of cell suspension was injected into the lumen of the HFs, and the bundle was cut to a length of 1.5 cm. The bundle containing the cells was placed in a cell culture dish, and the culture medium was added. The cells were cultured on a rotary shaker at 45 rpm and 37 °C in humidified air with 5% CO_2. The medium was supplemented with 0.1 μg/ml Dox (Sigma Aldrich) to induce the expression of liver-specific functions of Hepa/8F5 cells. The medium was changed every other day.

Cell number counting

HFs were cut into small pieces and the nuclei were eluted using a Polytron homogenizer (Kinematica AG, Littau/Luzern, Switzerland) in a citric acid solution. Then, a stabilizing buffer equal to the volume of citric acid solution was added to neutralize the sample solution. After that, the nuclei were counted using a NucleoCounter (ChemoMetec A/S).

Evaluation of liver-specific functions

In order to evaluate ammonia removal and albumin secretion activities, the culture medium was replaced with fresh medium supplemented with 1 mM ammonium chloride at each evaluation. The ammonia removal rate was calculated based on the decrease in the ammonia concentration during 24 h following medium replacement. The ammonia concentration was measured using the Wako ammonia test (WAKO). The concentration of albumin secreted into the medium during 24 h was detected by performing the enzyme-linked immunosorbent assay using an ELISA starter accessory package (Bethyl Laboratories, Montgomery, TX, USA) and a mouse albumin ELISA Quantitation Kit (Bethyl Laboratories).

Immunofluorescence staining

The Hepa/8F5 cells cultured in HFs were collected, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections. (100 μm) were prepared using a cryostat (CM1100, Leica, Wetzlar, Germany). Sections were fixed in acetone for 10 min at 4 °C, washed for 5 min three times in phosphate-buffered saline (PBS), and then incubated in a blocking solution (PBS containing 10% skimmed milk powder and 6% glycine) for 20 min. The sections were again washed for 5 min three times in PBS and then incubated in a blocking solution containing Hoechst 33342 (1:1000) for 1 h at room temperature. Images were captured with an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Design of an HF-based BAL module

We previously reported hepatic differentiation of mouse ES cells in an HF-type bioreactor and application of this system as a BAL device (Mizumoto et al. 2018). In this study, we slightly modified the design of this HF-type BAL device. Figure 2a shows a schematic diagram of the BAL device. The BAL consisted of polycarbonate housings and a cell culture space within a single-layer textile HF sheet. The HF sheet was comprised of 250 HFs with a length of 6 cm each. The lumen volume for cell culture of the HF sheet was 0.231 cm^3. Each HF was woven into a textile sheet, in which the gap between the HFs was fixed by warp threads. A regular arrangement of the HFs enables the medium to flow consistently through the BAL device. As the medium flows around the HFs, oxygen, nutrients, and metabolic waste products are exchanged through the HF membrane. The housing included medium inlet/outlet ports and three cell injection ports. Cell injection ports were installed to immobilize cells inside the HFs homogeneously. The volume of the BAL module was 3.0 cm^3.

Subcultured Hepa/8F5 cells were resuspended at a density of 2.5×10^6 cells/ml in the culture medium. 6 ml of the cell suspension (1.5×10^7 cells) was injected into the lumen of the HFs. Figure 2b shows a schematic diagram of the culture system. The BAL module was connected to the culture line circuit, and cells were cultured at 37 °C in humidified air with 5% CO_2. The BAL module was then continuously

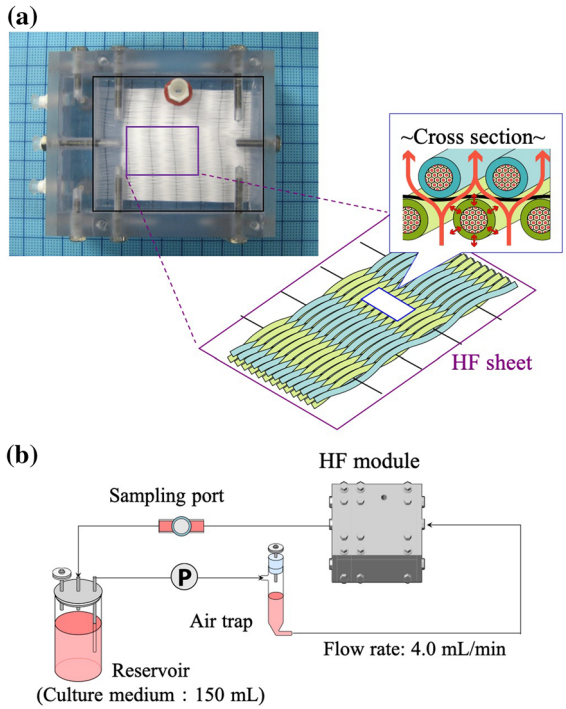


Fig. 2 Schematic diagram of the HF-type bioreactor and its culture system. **a** Illustration of the bioreactor. The bioreactor consists of polycarbonate housings and a single-layer textile HF sheet. Cells were immobilized and cultured in the lumen of the HFs. The culture medium flows through the HFs. **b** Illustration of a perfusion culture system for the bioreactor

perfused overnight with 150 ml of culture medium recirculating at a speed of 4 ml/min. 0.1 µg/ml Dox (Sigma–Aldrich) was added from day 0 to induce liver-specific functions. The medium was replaced every other day.

Statistical analysis

Results are presented as the mean ± standard deviation. Statistical analysis was performed using Welch's *t* test using R software (version 3.1.2). $P < 0.05$ was considered statistically significant.

Result

Evaluation of cell growth and liver-specific functions of Hepa/8F5 cells in HF culture

We evaluated changes in the phenotypes of Hepa/8F5 cells cultured in the HFs upon Dox addition. We

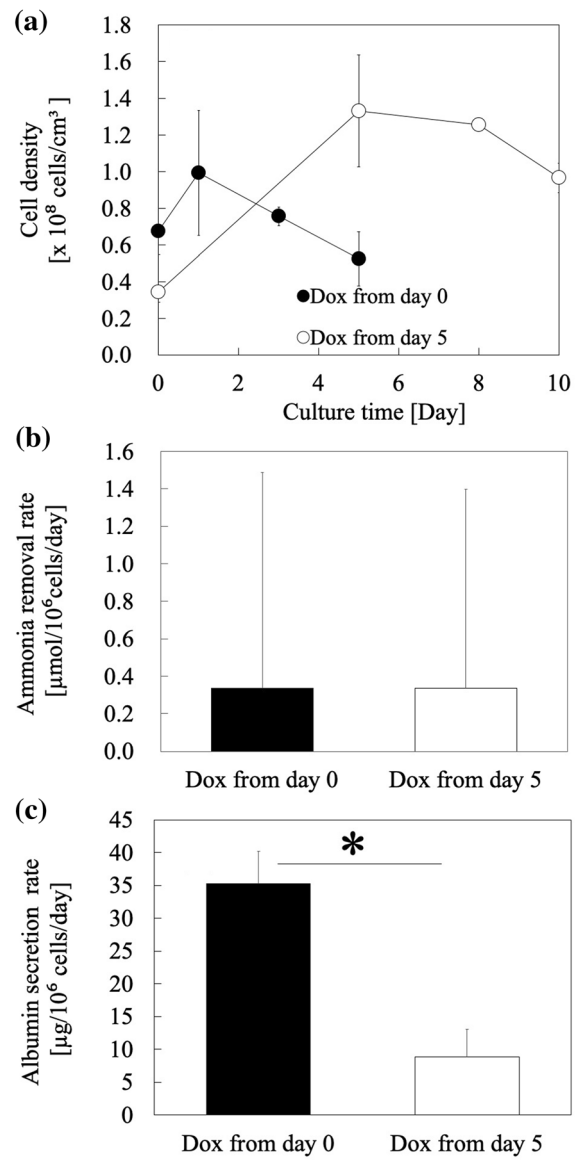


Fig. 3 HF culture of Hepa/8F5 cells with different Dox addition times. **a** Time course of cell density inside the HFs with two Dox addition times, Dox addition on day 0 (closed circle), and day 5 (open circle). **b** Comparison of ammonia removal rates at 5 days after induction by Dox addition on day 0 and 5. **c** Comparison of albumin secretion rates at 5 days after induction by Dox addition on day 0 and 5. Data are presented as the mean ± standard deviation. Asterisk (*) indicates significant differences ($P < 0.05$)

compared the effects of two Dox addition time points: day 0 and day 5. Figure 3a shows cell density changes inside HFs. Following Dox addition on day 0, number of cells decreased after day 1 whereas addition of Dox on day 5 led to a decrease in number of cells after day

5. These results showed that the Hepa/8F5 cells responded to Dox addition, albeit with a slight lag. Figure 3b and c show liver-specific ammonia removal and albumin secretion activities at 5 days after induction by Dox. There was no significant difference between the ammonia removal activities of the two Dox addition conditions. On the other hand, albumin secretion activity of the Hepa/8F5 cells upon Dox addition on day 5 was significantly higher than that on day 0.

Effect of cell inoculation conditions on cell distribution and density inside HFs

Our findings from the previous section indicate that Hepa/8F5 cell density inside HFs affect their phenotypes. In these experiments, we applied centrifugation on the HF-bundle after injection to enhance cell density and cell-to-cell interaction. Next, we compared two cell inoculation conditions: with and without centrifugation after cell injection into HFs. Figure 4a shows cell densities inside HFs for both conditions. Cell proliferation was suppressed by Dox addition on day 0 under both conditions. The cell density remained higher with than without centrifugation throughout the culture time. We also evaluated the morphologies of cell nuclei inside HFs. Figure 5 shows cell nuclei distributions. With centrifugation, cell nuclei tended to accumulate along the direction of the centrifugal force. In contrast, the nuclei were uniformly distributed throughout the HFs without centrifugation.

Liver-specific functions of Hepa/8F5 cells under different inoculum conditions

We evaluated albumin secretion and ammonia removal activities of Hepa/8F5 cells under two different inoculum conditions. Figure 4b shows the changes in ammonia removal activities. The activities were higher with than without centrifugation. Figure 4c shows albumin secretion activities of Hepa/8F5 cells under both conditions. In contrast to ammonia removal, the albumin secretion activities of cells were almost the same regardless of centrifugation.

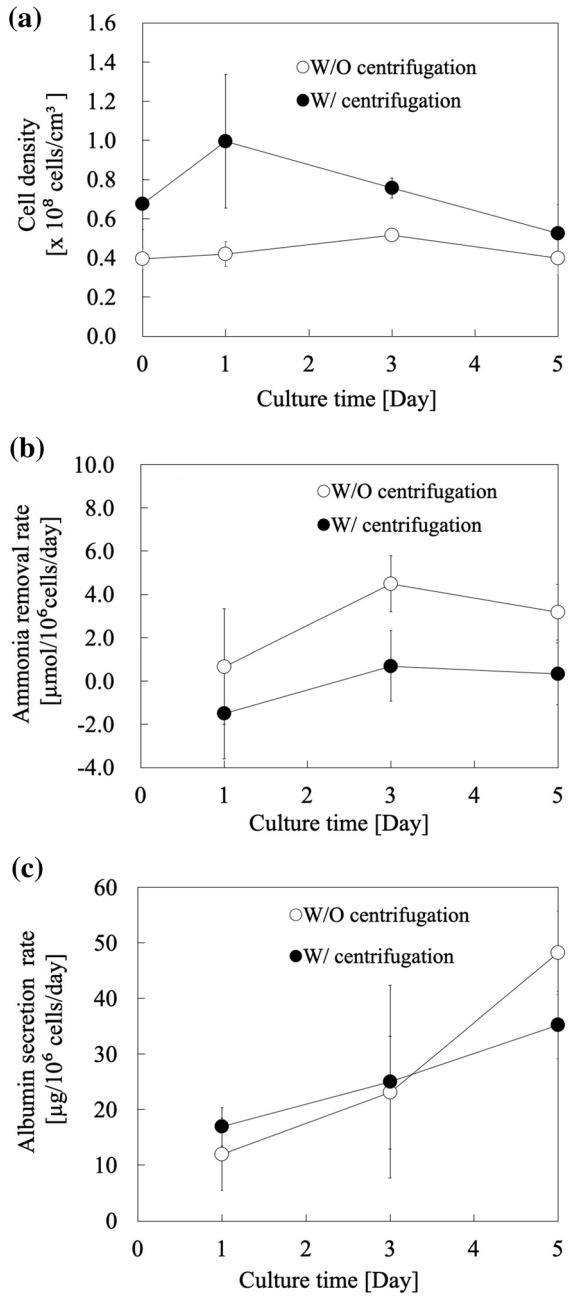


Fig. 4 HF culture of Hepa/8F5 cells with different seeding conditions. **a** Time course of cell density inside the HFs under different seeding conditions, with (closed circle) or without centrifugation (open circle) after cell injection inside HFs. **b** Comparison of ammonia removal rates between two conditions. **c** Comparison of albumin secretion rates between two conditions. Data are presented as the mean ± standard deviation

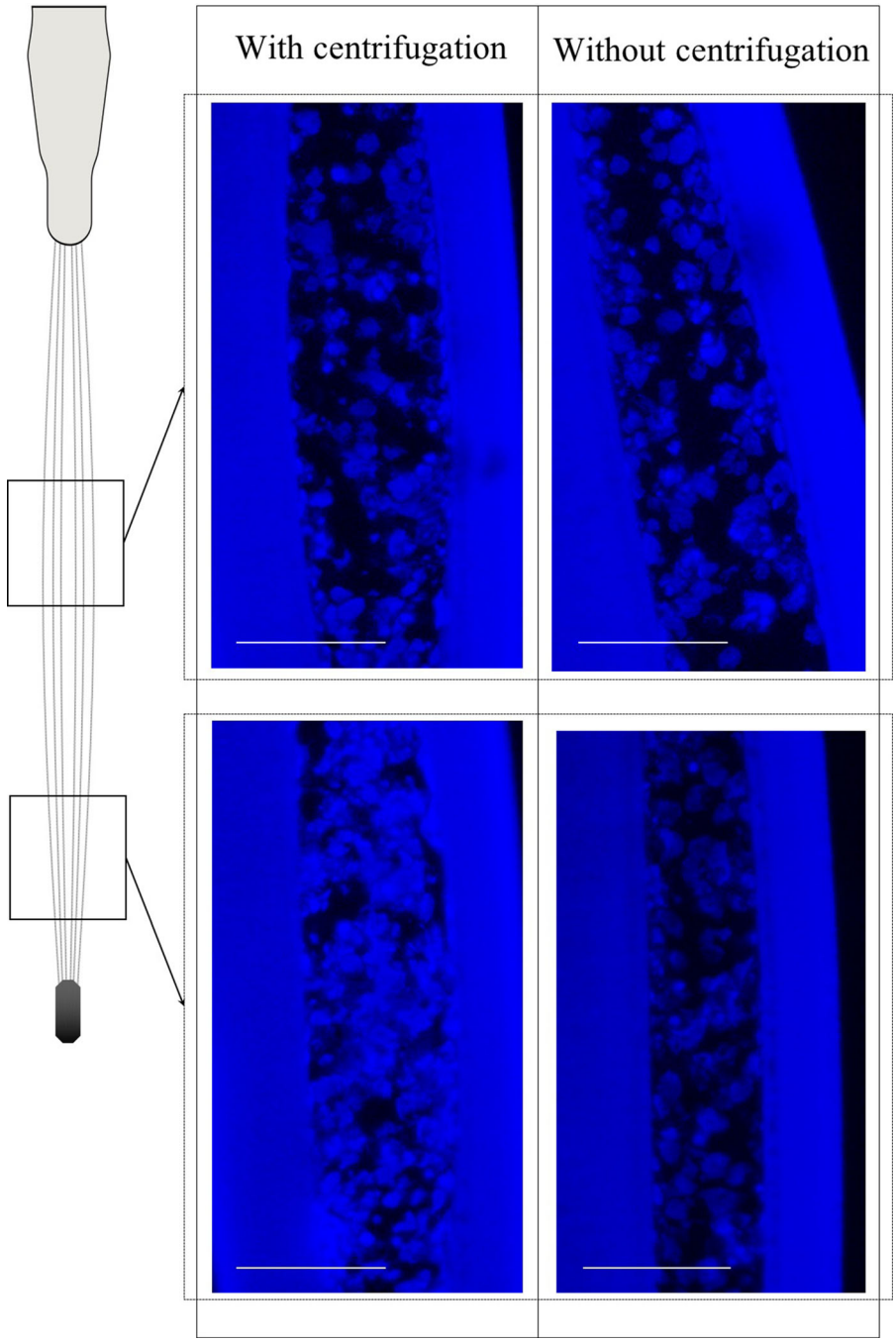


Fig. 5 Morphological observation of cell nuclei inside HFs with or without centrifugation. Scale bar: 200 μm

Evaluation of a BAL device with Hepa/8F5 cells in perfusion culture

We fabricated a HF bioreactor as a BAL device. Hepa/8F5 cells were immobilized into the bioreactor, and perfusion culture was performed. Figure 6 shows liver-specific functions of Hepa/8F5 cells in the bioreactors. Ammonia removal activity was detected after 3 days of culture (Fig. 6a). The maximum ammonia removal rate in the Hepa/8F5-BAL module

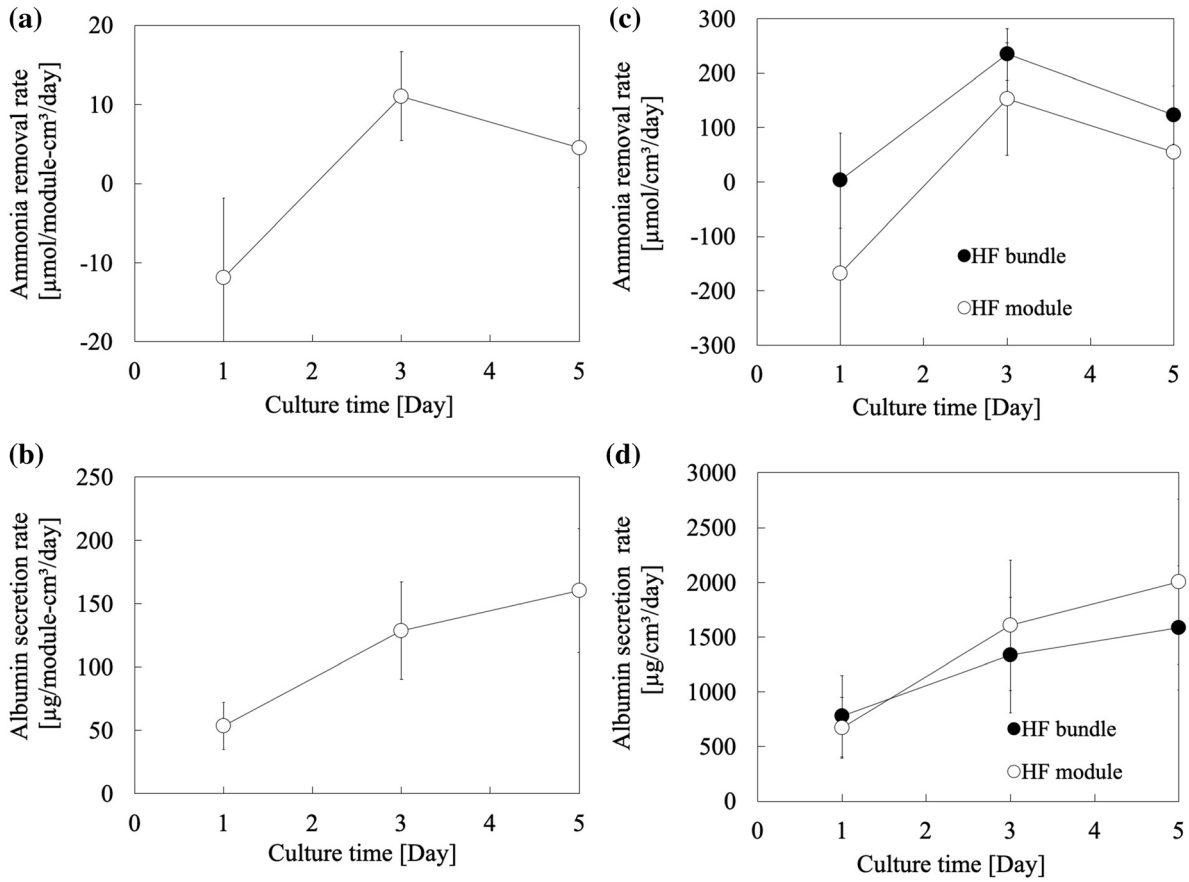
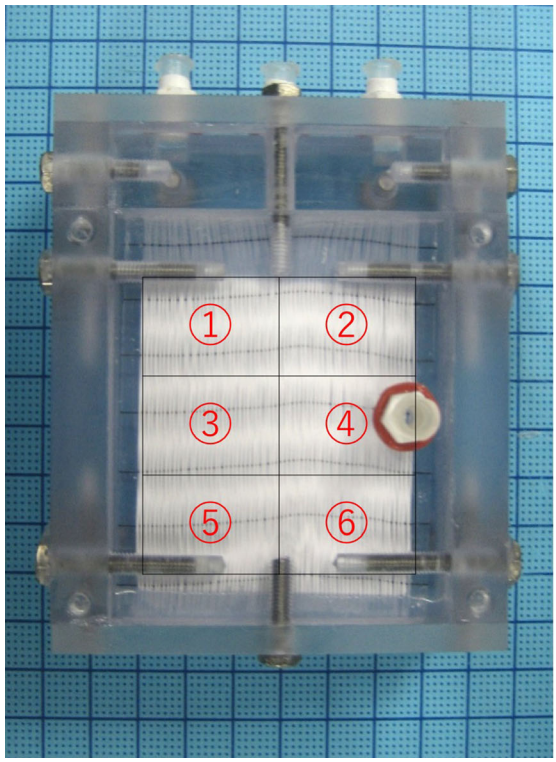


Fig. 6 Perfusion culture of Hepa/8F5 cells using a HF-type bioreactor. **a** Changes in ammonia removal rates of the bioreactor during the culture. The rates were normalized by a unit volume of the bioreactor. **b** Changes in albumin secretion rates of the bioreactor during the culture. The rates were normalized by a unit volume of the bioreactor. **c** Comparison of ammonia removal rates between the HF bundle (closed circle) and the HF module (open circle). The rates were normalized by a unit volume of HFs. **d** Comparison of albumin secretion rates between the HF bundle (closed circle) and the HF module (open circle). The rates were normalized by a unit volume of HFs. Data are presented as the mean ± standard deviation

was 11 μmol/module-cm^3/day. Albumin secretion activity was detected after day 1, and the activity increased with culture time (Fig. 6b). The maximum albumin secretion rate in the Hepa/8F5-BAL module was 160 μg/module-cm^3/day. We also compared the functional activities of Hepa/8F5 cells immobilized in the module (perfusion culture) with those immobilized in the HF bundle (gyratory culture). The activities were normalized by the unit volume of HFs to obtain reference values for designing a BAL device. Albumin secretion and ammonia removal activities in perfusion culture were slightly higher and lower than those in gyratory culture, respectively (Fig. 6c, d). Total cell number inside BAL was 8.29 × 10^6 cells at the end of perfusion culture. The average cell density inside HFs was 3.59 × 10^7 cells/cm^3 (Fig. 7).

Discussion

One of the most important factors determining the success of a BAL system is the source of cells. Although various cell types are currently being investigated as possible sources of hepatocytes for BAL devices, including primary porcine hepatocytes, primary human hepatocytes, liver cell lines, and stem cells, problems associated with each source hinder their practical use. A high capacity to proliferate is a desirable characteristic for cells used in BAL systems,



Cell number in each part	①	0.71 x 10^6 cells
	②	0.84 x 10^6 cells
	③	1.37 x 10^6 cells
	④	1.50 x 10^6 cells
	⑤	1.94 x 10^6 cells
	⑥	1.93 x 10^6 cells
Total cell number		8.29 x 10^6 cells
Cell density		3.59 x 10^7 cells/cm^3

Fig. 7 Cell numbers in different parts of the bioreactor. At the end of the perfusion culture, the HFs immobilized in the bioreactor were divided into six parts and cell number in each part was measured

due to the requirement for a large number of cells. However, characteristics such as elevated proliferation and high hepatic functional activity are often incompatible, and a mechanism by which cell fates can be switched between proliferation and differentiation may serve as an alternative solution. Based on this perspective, genetically engineered hepatocyte cell lines such as Hepa/8F5 may serve as alternative cell sources. Hepa/8F5 cells can switch their phenotype between proliferation and differentiation states upon the addition of an induction agent. We evaluated the possibility of using this cell line in artificial liver using HF culture.

In our first approach to use Hepa/8F5 cells in the BAL device, we added Dox after cell expansion to induce cell differentiation. However, this approach did not yield satisfactory results, as the functional level of Hepa/8F5 cells was very low. Thus, we took another approach to initiate differentiation immediately after inoculation of Hepa/8F5 cells. We injected Hepa/8F5 cells into the lumen of HFs and cultured them in the presence of Dox. Under these conditions, Hepa/8F5 cell growth was suppressed, and albumin secretion activities were up-regulated (Fig. 3c). However, ammonia removal activity remained low (Fig. 3b). We further focused on the cell density inside HFs. By avoiding the centrifugation inside HFs, Hepa/8F5 cells dispersed more homogeneously in the HF and achieved a higher ammonia removal activity (Fig. 4b). Average functional levels of Hepa/8F5 cells on day 3 were as follows: ammonia removal rate was 4.48 ± 1.29 μmol/10^6 cells/day and average albumin secretion rate was 23.08 ± 10.12 μg/10^6 cells/day. In our previous HF culture study, ammonia removal and albumin secretion rates of primary mouse hepatocytes were 2.08 μmol/10^6 cells/day and 16.82 μg/10^6 cells/day, respectively (Amimoto et al. 2011). These results indicate that Hepa/8F5 cells in HF culture had high functional activity comparable to that of primary hepatocytes.

We also found that in the case of HF culture, it was difficult to induce liver-specific functional activities in the Hepa/8F5 cells after proliferating to a high cell density state. It is unlikely that Dox was ineffective as

Dox addition suppressed cell proliferation. In our preliminary investigations, we found that even when the Dox concentration was increased tenfold, the magnitude of the liver-specific functional activity did not change. However, cell density inside the HFs may affect these activities in two ways: through diffusion and metabolism. A high cell density state may inhibit mass transfer, especially that of high molecular weight substances such as albumin. The high cell density state could also cause a depletion of substances such as oxygen. Oxygen depletion is likely to occur at the center of the high cell density environment in the HFs. Additionally, the resulting anaerobic respiration will likely lead to acidosis. Kashiwagura et al. reported that the rate of urea synthesis (i.e., ammonia metabolism) in rat hepatocytes was pH-dependent, and this activity declined in both acidic and alkaline conditions (Kashiwagura et al. 1984). Furthermore, Ulrich et al. reported that albumin synthesis in HepG2 cells was significantly decreased at lower pH (Ulrich et al. 1999). From these reports, we can deduce that the poor functional activities in these cells were due to a low pH as a consequence of high cell density.

Changes in cell volume may also affect functional activity levels. Upon Dox addition, Hepa/8F5 cells transform into a more epithelial-like morphology, resulting in an increase in cell volumes (Yamamoto et al. 2012). On the other hand, an increase in cell volume is rather difficult in the high cell density environment within the closed space inside the HFs. This may also affect ammonia removal ability. Further research focusing on the hypoxic region inside Hepa/8F5 cell aggregates is needed to clarify the mechanisms behind changes in functional activity levels and cell volumes in HF culture.

Maintaining a uniform low cell density environment inside the HFs is another difficulty. Here, the use of spheroids formed by single cells can be considered as an alternative method for achieving highly functional culture configurations. Forming spheroids under Dox addition does not limit cell volume changes. Moreover, spheroids can be configured to obtain ideal sizes to prevent oxygen depletion by controlling cell seeding conditions. In a previous study, we succeeded in constructing a high-performance hepatic tissue culture by using a bottom-up method in which spheroids were stacked inside the HFs (Okudaira et al. 2017). This bottom-up method can be applied to the Hepa/8F5 cells as well.

When evaluating the BAL device, it is difficult to trace the changes in the number of cells inside the device over time. Therefore, we have calculated the functional activities of these cells as per unit volume of the hollow fibers and compared them to those in the HF bundle. Consequentially, the functional activities between the two were almost the same, and thus the scale-up process from the HF bundle to the BAL device was performed with relative ease. While albumin secretion activities of Hepa/8F5 cells in perfusion culture were slightly higher than those in gyratory culture, ammonia removal activities were lower (Fig. 6c, d). Low levels of ammonia removal activity may occur due to a heterogeneous cell density distribution inside the BAL device. The results of cell number measurements in each part of the BAL device verified such heterogeneous distribution of cells (Fig. 7). If the cell density inside the HF affects the functional activity levels of cells, in particular ammonia removal, this heterogeneity might have reduced the performance of the BAL. To this end, further studies focusing on maintaining a uniform cell density in the BAL device are needed.

In a previous study, we developed the same type of HF BAL device containing mouse ES cells and applied this ES-BAL device to treat liver failure in rats (Mizumoto et al. 2018). The ES-BAL module showed some efficacy in liver regeneration. Ammonia removal and albumin secretion rates of the ES-BAL device per unit volume were 5.75 μmol/module-cm^3/day (approximately one-third of that in the module containing primary mouse hepatocytes) and 158.31 μg/cm^3-module/day (equivalent to that in the module containing primary mouse hepatocytes), respectively. Comparing these results to those we obtained in this study, we conclude that Hepa/8F5-BAL device was highly functional with potential to show curative effects in liver failure treatment.

Conclusion

We assessed liver-specific functions of Hepa/8F5 cells in HF culture. Hepa/8F5 cells were able to perform liver-specific functions of ammonia removal and albumin secretion at low cell densities. We also designed an HF-type BAL module with Hepa/8F5 cells. Although ammonia removal activity of the BAL module was lower than that of the small-scale HF

bundle, albumin secretion activity was slightly higher. These results indicate that, despite certain problems that need to be addressed in future studies, the BAL module containing immobilized Hepa/8F5 cells was highly functional with potential to show curative effects in liver failure treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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