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3D Co-Culture of Hepatocyte, a Hepatic Stellate Cell Line, and Stem Cells for Developing a Bioartificial Liver Prototype

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Abstract. A liver organoid is an in vitro reconstruction of the liver that mimics the in vivo liver microstructure and performs liver functions. Liver organoids can be used for drug testing, as a model of liver disease pathogenesis, and as a bioartificial liver prototype material to develop promising alternative therapies for liver failure. In this study, we reconstructed liver organoids using primary rat hepatocytes, a hepatic stellate cell line (LX2), human umbilical cord-mesenchymal stem cells (UC-MSCs), and human umbilical cord blood (UCB)-CD34+ hematopoietic stem/progenitor cells. Suspensions of primary rat hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34+ cells were co-cultured using 11 ratio sets, and spheroid formation was evaluated for 2 days. Ratio sets with a positive liver organoid appearance were cultured in four different culture media, and after they were harvested, their viability was compared with that of a hepatocyte monoculture. Liver organoids were further analyzed for 14 days to assess albumin and urea production as well as relative gene expression. We found that a 5:1:2:2 cellular density ratio of hepatocytes:LX2 cells:UC-MSCs:UCB-CD34+ cells, respectively, and Williams E medium supplemented with platelet lysate, ITS, and dexamethasone were the most suitable conditions for liver organoid reconstruction. Expression of the albumin and GPT1 genes and CD31 in the liver organoid increased until day 14, while urea secretion increased until day 5. Liver organoids reconstructed through the 3D co-culture of primary rat hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34+ cells at a specific cellular ratio using an economical medium with a simple composition maintained their functions until day 14. As a future direction, these organoids can be used to develop a bioartificial liver.

Keywords: 3D co-culture; Hepatocyte; Liver function; Liver organoid; Stem cells

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1. Introduction

The liver is an important organ that performs many functions, such as protein synthesis, drug biotransformation, and detoxification (Li et al., 2017; Uygun et al., 2017). While it has a strong regenerative ability, massive liver damage can lead to liver failure (Miyaoka and Miyajima, 2013; Mazza et al., 2017). Liver transplantation, the primary available therapy for liver failure, has many shortcomings, such as difficulty in finding suitable donors, a high cost, and the need for the long-term use of immunosuppression drugs, leading to large numbers of patients awaiting liver transplantation (Li et al., 2017).

Many alternative therapies have been developed to overcome these issues, including cell transplantation using hepatocytes and stem cells, the use of artificial livers, and, most recently, the use of bioartificial livers (BALs). Hepatocyte transplantation requires long-term immuno-suppression and is further limited by small numbers of donors, isolation difficulties, and low hepatocyte engraftment rates (Vacanti and Kulig, 2014). Mesenchymal stem cell (MSC) transplantation, despite being relatively safer, also has the potential to trigger thrombosis in some cases (Zheng et al., 2013). Artificial livers can be developed with a synthetic device using an extracorporeal perfusion system, but they are functionally limited to the removal of toxins. (Zhang et al., 2018). BALs combine an artificial liver with hepatocytes, using primary hepatocytes from pig livers or hepatoblastoma cell lines; BALs have a short lifespan because of limited primary hepatocyte proliferation and differences between the functions of hepatoma cell lines and hepatocytes (Vacanti and Kulig, 2014). These limitations require the development of new technologies overcome the remaining challenges facing existing BAL prototype materials, such as imparting equivalent liver functions, to meet the high demand for liver transplantation.

A liver organoid is defined as an *in vitro* liver reconstruction that mimics the microstructure and function of the liver *in vivo*. Reconstructing liver organoids requires cell components that replicate the liver's *in vivo* microenvironment as well as culture techniques that can support long-term liver function. Techniques for the co-culture of hepatocytes with non-parenchymal cells are continuously being developed to obtain a 3D microenvironment and structure that mimic the liver's *in vivo* microenvironment. Co-culture is carried out to obtain a 3D microenvironment, while culture methods are used to obtain a 3D structure. The culture medium must also be refined to ensure an optimal hepatocyte microenvironment (Monckton and Khetani, 2018).

The liver is a complex unit consisting mainly of parenchymal cells, which are hepatocytes, and non-parenchymal cells, including Kupffer cells, hepatic stellate cells (HSCs), and endothelial cells (Vacanti and Kulig, 2014). Hepatocytes are co-cultured with an HSC line (e.g., LX2 cells), umbilical cord-mesenchymal stem cells (UC-MSCs), and umbilical cord blood (UCB)-CD34 hematopoietic stem/progenitor cells with the aim of producing a microenvironment matching the liver's *in vivo* microenvironment. A previous study reported the formation of a vessel-like structure in a co-culture of hepatocytes with endothelial cells and HSCs, with increased urea and albumin secretion as well as Cyp3A4 expression (Wang et al., 2018). Meanwhile, in a co-culture of MSCs and an endothelial cell line (HUVEC EA. hy 926), increases in EGF-A expression and cell viability and the formation of a tubular structure in the presence of CD31 expression were reported (Arutyunyan et al., 2016). MSCs have strong proliferation and differentiation abilities, allowing them to produce a paracrine effect that supports the endogenesis required by endothelial cell progenitors ($CD34^+$) for neovascularization and biliary duct formation.

In this study, we combined four cellular components that have not been used before in combination for liver organoid reconstruction. We also used four culture media with basic supplementations to ensure that they could be applied in areas with limited resources. An

optimal density ratio of the four cellular components and an optimal culture medium are required for liver organoid reconstruction to ensure that viability and liver function are maintained for an extended period of time. The results of this study are expected to provide proof of concept that with an optimal density ratio of hepatocytes, LX2 cells, MSCs, and CD34$^+$ cells and an optimal medium, it is possible to reconstruct liver organoids.

2. Methods

2.1. Preparation of Co-Cultured Cells

2.1.1. Isolation of hepatocytes

Livers were dissected from adult male Sprague-Dawley rats (250–350 g, *n*=3), which were obtained from the Animal Research Laboratory at the Biomedical and Basic Health Technology Research Centre, Ministry of Health, Republic of Indonesia. All research animals were treated according to national and institutional guidelines. Primary hepatocytes were freshly isolated by cutting rat dissected livers into small fragments, followed by multi-step TrypLE Select (Gibco) digestion. Cells were mixed directly into cold Williams E medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and centrifuged for 4 min at 4°C. The supernatant was discarded, and the pellets were resuspended in cold culture medium. TrypLE Select digestion and harvesting were repeated 4–5 times to obtain large numbers of hepatocytes. The number and viability of the cells were determined using a trypan blue exclusion assay.

2.1.2. Isolation and culture of UC-MSCs

MSCs were isolated from human umbilical cord Wharton's Jelly using a multiple harvest explant method with some modifications (Pawitan et al., 2015) and then cultured in a 24 well culture plate using alpha-minimal essential medium (αMEM) supplemented with platelet lysate (local Indonesian Blood Bank), Glutamax (Gibco), and heparin (Pawitan et al., 2015). Cells were cultivated at 37°C in a 5% CO_2 incubator and harvested with TrypLE Select after the cells reached 70%–80% confluence. Expression of CD105, CD90, and CD73 was measured using flow cytometry following the International Society Cell and Gene Therapy criteria for MSCs.

2.1.3. Isolation of UCB-CD34$^+$ stem cells

CD34$^+$ hematopoietic stem/progenitor cells were isolated from human umbilical cord blood (UCB) using Ficoll-Hypaque solution and centrifugation to obtain the buffy coat. The buffy coat was subsequently washed by centrifugation at 650 × *g* for 10 min using a no-brake system. The supernatant was discarded, and the pellet was resuspended in phosphate buffered saline (PBS). Cell numbers were then counted using a trypan blue exclusion assay. CD34$^+$ cell isolation was conducted using the Miltenyi MACS isolation kit according to the manufacturer's protocol. The purity of the isolated CD34$^+$ cells was then analyzed using flow cytometry.

2.1.4. LX2 cell culture and passage

LX2 cells (Millipore SCC064) were cultured in high-glucose DMEM (Gibco) supplemented with 2% FBS according to the Millipore protocol (Cat. SCC064) at 37°C in a 5% CO_2 incubator.

2.2. Determining the Optimal Density Ratio of Hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34$^+$ Hematopoietic Stem/Progenitor Cells and Optimal Culture Medium

Suspensions of primary hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34$^+$ cells were co-cultured in 11 ratio sets and in four culture media. The first culture medium was a combination of four media containing cellular components. The second culture medium was Williams E medium supplemented with platelet lysate, insulin-transferrin-selenium

(ITS), and dexamethasone. The third culture medium was Williams E medium supplemented with FBS, ITS, and dexamethasone. The fourth culture medium was αMEM supplemented with platelet lysate (Figure 1). Co-culture was conducted by mixing a total of 5×10^3 hepatocytes, LX2 cells, UC-MSCs, and UCB-$CD34^+$ cells in 96 well ultra-low attachment culture plates. For hepatocyte monoculture, 5×10^3 hepatocytes were cultured in 6 well plates. Cells were incubated in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidity.

All co-culture ratio sets were analyzed to assess 3D spheroid formation using an inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan) after day 2 and compared with the hepatocyte monoculture. Ratio sets with a positive liver organoid appearance were further cultured for 5 days in all four culture media, after which viability was assessed (Figure 1). Co-cultures and hepatocyte monocultures were carried out for 2 days with a total 5×10^3 cells and 5 days with a total 3×10^4 cells in 96 well and 24 well ultra-low attachment culture plates, respectively. Co-cultures were analyzed from day 2 to day 5 to assess spheroid formation using an inverted microscope, and their viability was evaluated using a trypan blue exclusion assay. The co-culture with the highest viability was regarded as having the optimal cell ratio and culture medium (Patent Registration Number S00202003637).

2.3. Liver Organoids

Liver organoid cultures (n=3) were conducted using the optimal ratio and culture medium for 14 days, and cell viability, spheroid formation, and albumin and urea secretion were measured and immunohistochemistry and gene expression analyses were performed on day 2, day 7, and day 14 of co-culture (Figure 1).

2.3.1. Assessment of Albumin and Urea Secretion Liver Organoids

Albumin levels were measured using a Rat Albumin ELISA Kit according to the manufacturer's instructions (MBS282256), and the absorbance signal was measured with a spectrophotometer (Thermo Fisher Scientific, MA, USA). Urea levels were measured using a Urea Assay Kit (Sigma-Aldrich; MAK006) following the manufacturer's instructions.

2.3.2. Immunohistochemistry Analysis

Immunohistochemistry staining was performed to analyze albumin, GOT2, GPT, and CD31 expression in the liver organoid's pellet suspension. Spots from the cell pellet suspension were fixed on slide preparations, incubated in a hydrogen peroxide block, and then incubated in a protein block. Briefly, the samples were incubated overnight (4°C) with primary antibodies: mouse anti-human/rat albumin (monoclonal, 1:500; Abcam ab236466), mouse anti-human/rat GOT2 rat (polyclonal, 1:100; Invitrogen PA5-27572), mouse anti-human/rat GPT (polyclonal, 1:100; Invitrogen PA5-29600), and mouse anti-human/rat CD31 (polyclonal, 1:50; Invitrogen PA5-16301), after which they were incubated with secondary antibodies. Antibody binding was detected after incubation with DAB chromogen for 1 min at room temperature followed by PBS washing.

2.3.3. Gene expression analysis

RNA was isolated from the pellet suspension using GENEzol Reagent, and synthesis of RNA to cDNA was carried out using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo A1172K). Gene expression analysis was performed by RT PCR using primers for albumin (F: TGAACCGTCTGTGTGTGCTG; R: CTTGGGCTTGTGTTTCACCA), GPT1 (F: CCGTAATCCGCTTTGCTTTC; R: CGCCCATGTAGCCCTTAGAG), GOT1 (F: GGACCGACCCAACTGAAGAG; R: CGGTCGATTCCCCACTCTC), and GAPDH (F: ATCAACGGGAAACCCATCAC; R: TCTCGTGGTTCACACCCATC). The initial denaturation temperature was set at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and elongation at 60°C for 10 s, and final elongation at 72°C for 15 s. The

analysis was carried out using the Livak method or delta delta threshold (ΔΔCt) comparison.

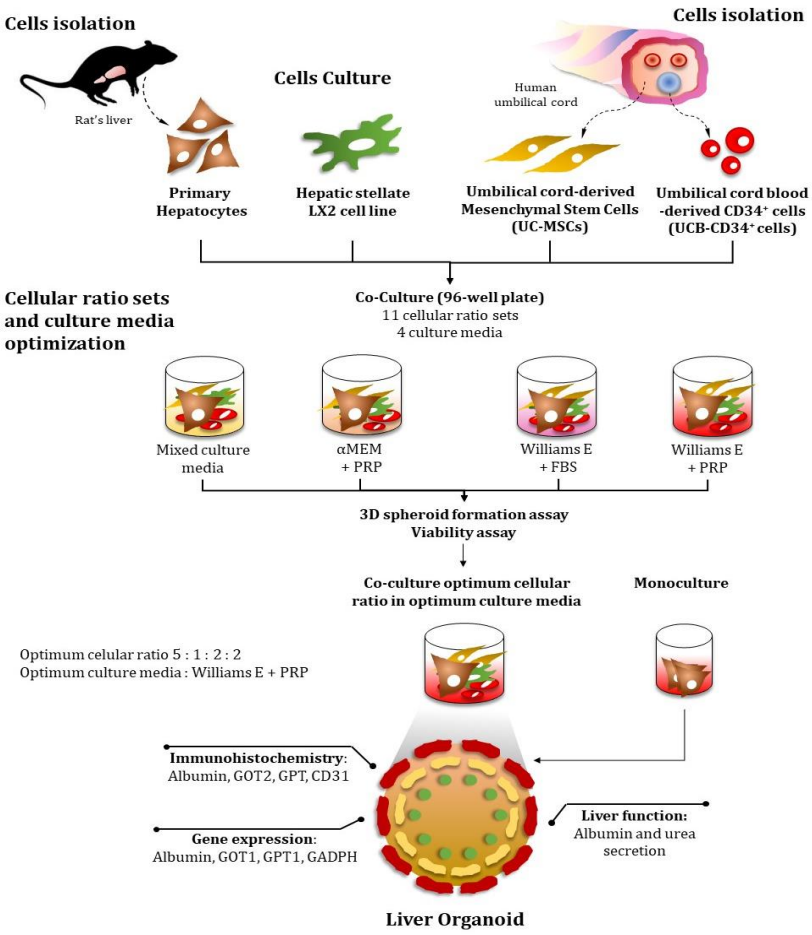

Figure 1 Experimental scheme for liver organoid formation using the 3D co-culture of hepatocytes, a hepatic stellate cell line, and stem cells

3. Results and Discussion

3.1. Optimal Cell Density Ratio and Culture Medium Determined via 3D Spheroid Formation and Viability Assays on 11 Sets of Cell Density Ratios and Four Types of Culture Medium

Three cell density ratio sets produced positive 3D spheroid formations, with two ratio sets in a specific culture medium and one ratio set in all culture media (Figure 2A). The three cell density ratio sets were 9:1:1:1 (in a mixture of four media containing cellular components, 16:2:1:1 (in Williams E medium supplemented with FBS, ITS, and dexamethasone; and in αMEM supplemented with platelet lysate), and 5:1:2:2 (in all culture media) for hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34$^+$ cells, respectively. These three cell density ratio sets were cultured for 5 days, and a single ratio (5:1:2:2) was determined to result in the highest viability in Williams E medium supplemented with platelet lysate, ITS, and dexamethasone. This ratio and culture medium were regarded as the optimal co-culture conditions for liver organoid reconstruction (Figure 2B).

3.2. Organoid 3D Spheroid Formation, Viability, Albumin and Urea Secretion, and Gene and Protein Expression Using the Optimal Ratio and Culture Medium

Based on the spheroid formation results indicating that 5:1:2:2 was the optimal cell ratio, we prolonged co-culture for 14 days in Williams E medium supplemented with platelet lysate, ITS, and dexamethasone. During the 14 day co-culture, we evaluated the

organoids on day 2, day 7, and day 14. We did not evaluate the organoids after more than 14 days, which was a limitation of this study. The results indicate that spheroid formation increased, as there was a larger number of spheroids on day 7 than on day 2 (Figure 3). The spheroids also remained solid until day 14. In contrast, spheroid formation was not observed in the hepatocyte monoculture (Figure 3).

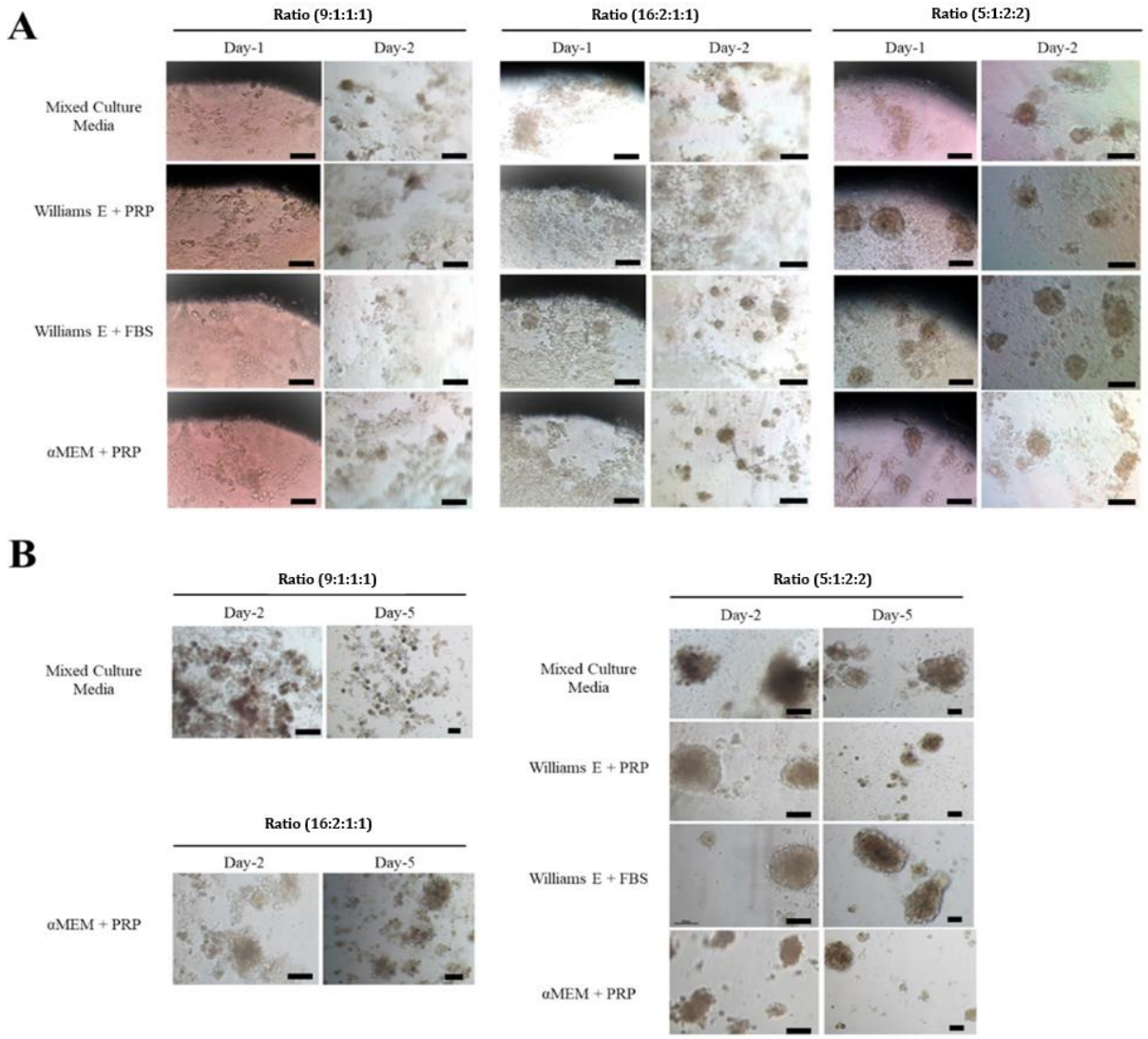

Figure 2 Spheroid formation in co-cultures at various ratios in four types of culture media on day 1, day 2, and day 5. (A) Three of the 11 ratio sets produced positive spheroid formations: ratio 9:1:1:1 in mixed culture media; ratio 16:2:1:1 in αMEM supplemented with platelet lysate; and ratio 5:1:2:2 in all types of culture media. (B) Spheroid formation in three ratio sets observed on day 2 and day 5. The ratio with the highest viability was 5:1:2:2 in Williams E medium supplemented with platelet lysate. Scale bars indicate 100 μm

The viability of hepatocytes in co-culture and monoculture significantly decreased over 14 days ($p < 0.05$ in co-culture). Hepatocyte viability in co-culture was higher than that in hepatocyte monoculture (Figure 4A). Co-culture maintained 24.86% hepatocyte viability on day 14, while no viable hepatocytes were found in the monoculture on day 14. These results indicate that hepatocyte viability was better in co-culture than in monoculture and that liver organoids maintained better viability and a longer culture duration than the hepatocyte monoculture.

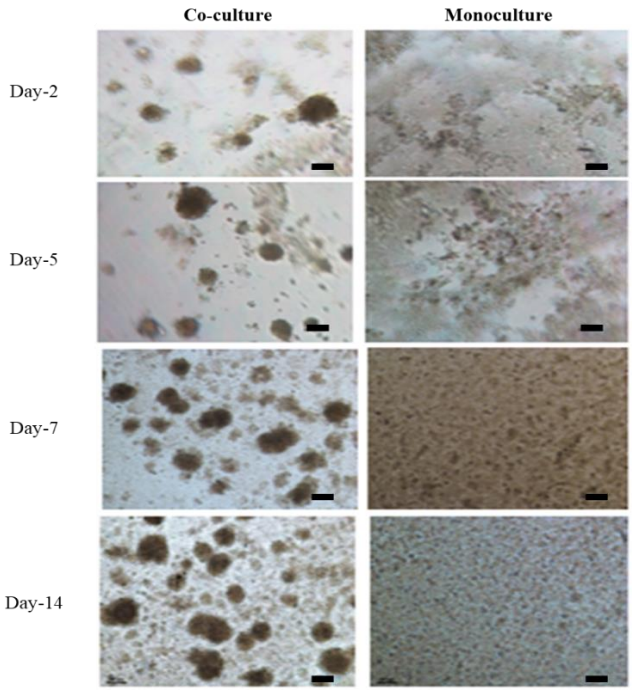

Figure 3 Comparison of spheroid formation between a co-culture and monoculture on days 2, 5, 7, and 14. No spheroids were formed in the monoculture. Scale bar indicates 100 μm

Our results are in accordance with those from a previous study (Table 1) that reported good viability in a co-culture of hepatocytes and AD-MSCs or UC-MSCs (Fitzpatrick et al., 2015). Therefore, liver organoid reconstruction using primary rat hepatocytes, LX2 cells, UC-MSCs, and UCB-CD34$^+$ cells under the conditions determined in this study can provide the appropriate cellular components to support hepatocyte viability.

Albumin protein concentrations from organoid supernatants were greater than those from monocultures and decreased from day 2 to day 7 of co-culture (Figure 4B), and no albumin secretion was detected on day 7 of monoculture (Figure 4B). We found low expression of the albumin gene and low secreted levels of albumin (Figure 4B) when hepatocyte viability decreased sharply in monoculture (Figure 4A). There was no detectable albumin gene expression or albumin secretion in non-viable hepatocytes on monoculture day 14 (Figure 4B). Albumin secretion levels were greater in co-culture than in monoculture, which is consistent with the results of a recent study (Table 1) on hepatocyte co-culture with sinusoidal endothelial cells and stellate cells in a hollow fiber bioreactor (Ahmed et al., 2017). The results of our study indicated that albumin gene expression increased in organoids while the albumin protein concentration in the organoid supernatant decreased. This phenomenon could be caused by the presence of other cells that also expressed albumin genes in co-culture or by direct MSCs trans-differentiation into hepatocyte-like and expressed albumin genes.

Urea levels in the supernatant of liver organoids were analyzed, and urea levels in co-cultures were greater than those in hepatocyte monocultures (Figure 4C). Urea levels in co-cultures exhibited the opposite trend of the levels in monocultures, as there was a continuous increase in co-cultures over time (Figure 4C). Our results are in accordance with those of a previous study (Table 1) that reported higher levels of urea and albumin secretion in hepatocytes co-cultured with endothelial cells, HSCs, and adipose cells than in a monoculture (Wang et al., 2018). Another study that examined the co-culture of HepaRG cells (cell line), LX2 cells, HUVECs, and monocytes (Table 1) also reported more urea secretion in the co-culture than in a monoculture (Rennert et al., 2015).

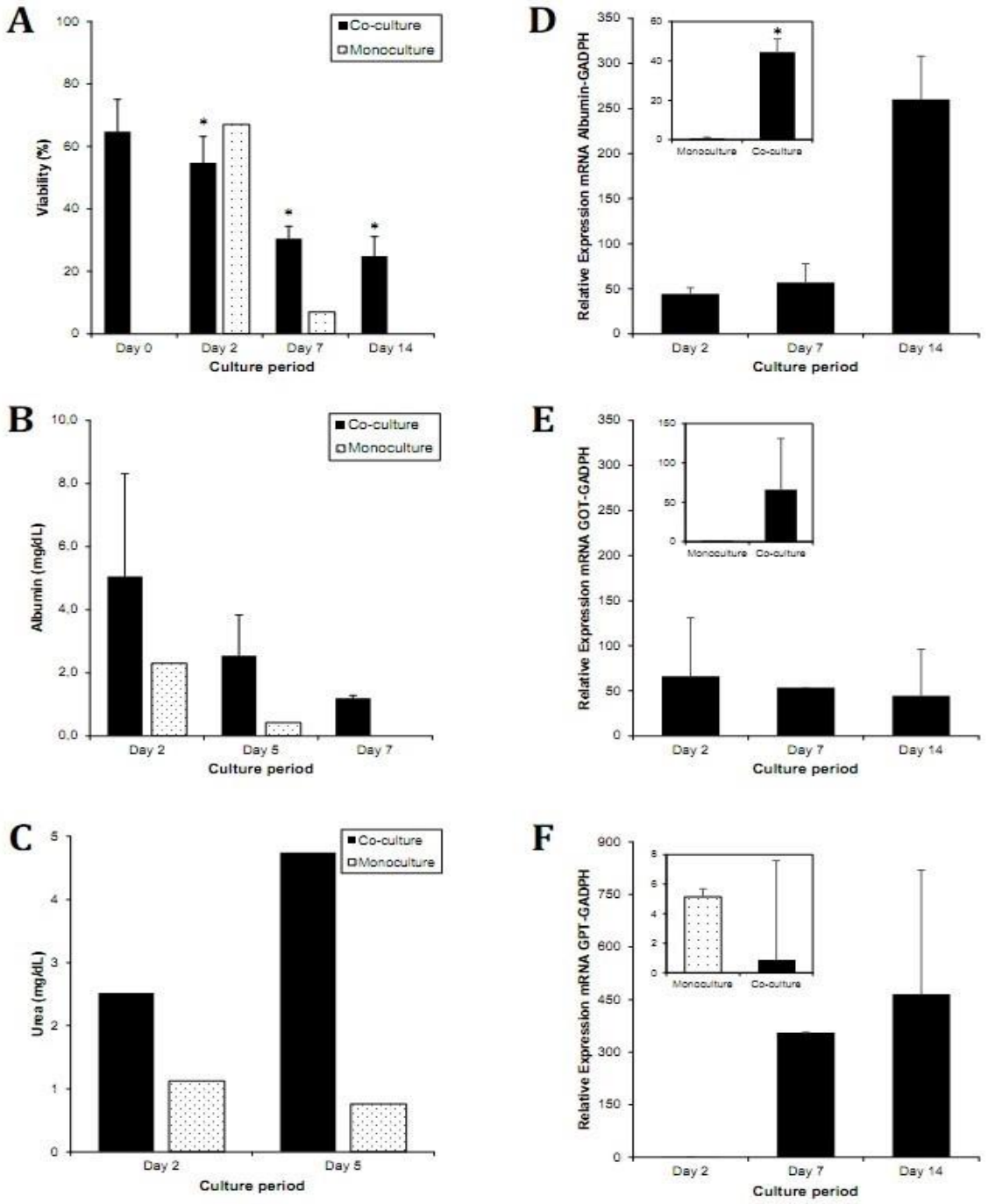

Figure 4 Viability and functions of hepatocytes in co-culture and monoculture on day 2, day 7, and day 14. (A) Viability. An asterisk (*) indicates a significant difference ($p < 0.05$). (B) Albumin secretion in the co-culture was greater than that in the monoculture, and no albumin secretion was detected on day 7 of monoculture. (C) Urea secretion in the 3D co-culture was greater than that in the monoculture on day 2 and day 5. Urea levels also increased from day 2 to day 5 in the co-culture. (D) Relative expression of albumin-GAPDH mRNA increased in co-cultures on day 2, day 7, and day 14, whereas no expression was detected in monocultures on day 7 and day 14. (E) Relative expression of GOT-GAPDH mRNA was high on day 2, day 7, and day 14. (F) Relative expression of GPT1-GAPDH mRNA increased in co-cultures on day 2, day 7, and day 14. Insets in Figure 4D–4F show a comparison of mRNA expression between monocultures and co-cultures on day 2

In our study, the secreted urea levels were higher in liver organoids than in hepatocyte monocultures. Interestingly, although hepatocyte viability decreased in co-culture (Figure

4A), urea secretion increased (Figure 4C). This phenomenon might have been caused by the induction of urea secretion in the liver organoids by the MSC secretome. Together, these findings suggest that a 5:1:2:2 density ratio of hepatocytes:LX2cells:UC-MSCs:UCB-CD34$^+$ cells supports the hepatocyte function of urea secretion.

The expression of the albumin (Figure 4D) and GPT1 genes (Figure 4F) increased from day 2 until day 14 of co-culture. In contrast, no expression of the albumin (Figure 4D) or GPT1 gene (Figure 4F) was detected on day 7 and day 14 of monoculture. The GOT1 gene was expressed through day 14 of co-culture, but no expression was detected on day 7 and day 14 of monoculture (Figure 4E).

Table 1 Comparison of studies on the co-culture of hepatocytes

Previous studies	Cellular Components	Medium	Result
Wang et al., 2018	Human liver cell line (LO2) + HUVECs + LX2 cells (ratio 700:150:3) and LO2 cells + HUVECs + ADSCs (ratio 14:3:3)	RPMI 1640 supplemented with FBS, HGF, and EGF.	Urea secretion and albumin expression were maintained in co-culture until day 7, better than those in monoculture.
Ahmed et al., 2017	Hepatocytes + sinusoidal endothelial cells + HSCs on hollow fiber membranes	Endothelial and stellate medium supplemented with FBS, IGF, EGF, and FGF	Urea secretion in co-culture decreased from day 5, and albumin secretion in co-culture was maintained until day 13 on hollow fiber membranes and until day 28 in a bioreactor, better than those in monoculture.
Rennert et al., 2015	HepaRG (cell line) + LX2 cells + HUVECs + monocytes	HUVECs + monocytes in endothelial cell culture medium supplemented with EGF; HepaRG + LX2 cells in Williams E medium supplemented with FBS	Urea secretion was better than that in monoculture
Bell et al., 2016	Hepatocytes + biliary cells + Kupffer cells + HSCs (Parenchymal:NPC = 2:1)	Williams E medium supplemented with chemically defined condition	Hepatocyte function and spheroid appearance were maintained until day 35
Fitzpatrick et al., 2015	Hepatocytes + UC-MSCs or AD-MSCs (ratios of 10:1, 6:1, and 3:1)	Williams E medium supplemented with FBS	Viability and albumin and urea secretion in co-culture were maintained until day 25, better than in those in monoculture

The expression of albumin, GOT2, and GPT protein was maintained in co-culture until day 14 (Figure 4D-4F), despite the decrease in the number of hepatocytes from day 2 to day 14 (Figure 4A). The number of CD31$^+$ cells on day 7 was higher than that on day 2 of co-culture, and CD31 was expressed through day 14; in contrast, it was not expressed in monoculture from day 2 to day 14. As CD31 is an endothelial marker, this result indicates the possibility of endothelial cell formation in the co-culture of hepatocytes, LX2 cells, MSCs,

and $CD34^+$ cells. This is consistent with the study conducted by Nakamura et al. (2016), who performed a $CD34^+$ cell transplant in mice with liver fibrosis and found that $CD34^+$ cells differentiated into endothelial cells in the presence of CD31 expression (Nakamura et al., 2016).

Hepatocyte cultures require a suitable medium and culture methods because hepatocytes can easily lose their phenotype and functions (Bale et al., 2016). Williams E culture medium is a suitable basal culture medium for co-culture because it supports hepatocyte culture. A previous study that used Williams E as a basal medium supplemented with chemically defined conditions (different from this study) reported the development of 3D spheroids with stable cell viability (Bell et al., 2016). These findings are consistent with the results of this study, in which solid 3D spheroids were maintained in co-culture for up to 14 days (Table 1).

The higher viability observed in organoids under co-culture than under monoculture conditions (Figure 4A) led to the higher expression of albumin (Figure 4D), GOT (Figure 4E), and GPT1 (Figure 4F) in co-culture than in monoculture. These results suggest that the presence of other cells might provide a better microenvironment for hepatocytes and that those cells support the hepatocytes' viability and function. The combination of hepatocytes, LX2 cells, UC-MSCs, and UCB-$CD34^+$ cells co-cultured in an optimal density ratio supported by an optimal culture medium provided the appropriate cellular components and microenvironment to mimic the *in vivo* liver microstructure. The identification of this optimal density ratio and culture medium should prove useful for constructing liver organoids. Further studies are needed to examine the functions of the organoids that are developed using this method, especially *in vivo* studies. To prevent rejection, the organoids should be encapsulated with non-immunogenic materials. These materials need to be developed (Kartohardjono et al., 2019), and there are several candidates, such as biocompatible hydrogel (Barleany et al., 2020) or a combination of maltodextrin and Arabic gum (Sahlan et al., 2019), that are promising for future development in this field.

4. Conclusions

Our results indicate the successful reconstruction of liver organoids using an optimum ratio of hepatocytes:LX2cells:UC-MSCs:UCB-$CD34^+$ cells. We also identified Williams E medium supplemented with platelet lysate, ITS, and dexamethasone as the optimum culture medium for reconstructing liver organoids, which is a simple and economical medium. The combination of these cellular components and culture medium provides a suitable microenvironment that mimics the *in vivo* liver microenvironment. This optimum ratio with a simple and economical medium can be used to develop and maintain liver organoid function for 14 days. As a future direction, these organoids can be used in BALs, although the organoids should be exchanged with new organoids every 14 days.

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