Self-organization of hepatocyte morphogenesis depending on the size of collagen microbeads relative to hepatocytes

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Abstract

Recent advances in microfabrication technologies have enabled us to construct collagen gel microbeads, which can be cultured with hepatocytes. However, little is known about the hepatocyte–collagen gel microbead interactions. Here, we aimed to clarify the effects of the balance between cell–cell and cell–collagen gel microbead interactions on hepatocyte morphogenesis and functions. The magnitude of cell–microbead interactions was controlled by changing the size of the microbeads, which were smaller than, comparable to, and larger than hepatocytes. These small, medium, and large microbeads were cultured separately with primary hepatocytes. Phase-contrast and time-lapse imaging revealed that the medium microbeads significantly induced the construction of 3D structures composed of the microbeads and hepatocytes in a self-organizing manner, whereas hepatocytes formed 2D monolayers with the small or large microbeads. These results suggest that only the medium microbeads induced the 3D tissue formation of hepatocytes. Furthermore, liver-specific functions, such as albumin secretion and ammonia clearance, were significantly upregulated in the 3D structures. These findings are critical to understand how to control the construction of 3D hepatocyte tissues with hydrogel microbeads in the context of biofabrication.

Introduction

Three-dimensional (3D) culture of hepatocytes is critical for the viability and functions of primary hepatocytes in vitro. Hepatocytes in 3D culture maintain their cuboidal cell shape, which is similar to that of those in vivo, resulting in the maintenance of cell polarity and function \cite{1–3}. Therefore, various techniques have been developed for culturing hepatocytes in 3D configurations. For example, hepatocytes formed multicellular aggregates, which are known as hepatocyte spheroids, when they were cultured in non-adherent culture devices such as spinner vessels \cite{4}, dishes with a positively charged surface \cite{5}, and concave microwells \cite{6}. Hepatocytes can also construct 3D tissues by stacking two-dimensional (2D) cell sheets \cite{1, 2, 7}. Furthermore, advances in microfluidic technologies have enabled us to construct 3D structures in microfluidic devices \cite{8, 9}.

Although hepatocyte functions are maintained in 3D culture models, most previous studies demonstrated 3D structures composed of hepatocytes alone, suggesting that cadherin-mediated signaling dominated hepatocyte functions in these 3D culture models \cite{10}. However, hepatocytes in vivo also contact with extracellular matrix (ECM), which is essential for the polarization of hepatocytes. This in vivo condition suggests that integrin-mediated signaling is also important to regulate hepatocyte structure and function in vitro because signal transduction via integrin.
mediates various cellular processes [11]. Therefore, the control of integrin-mediated signaling via cell–ECM interactions in 3D culture of hepatocytes is a challenge to achieve improved construction of 3D hepatic tissues in vitro.

Integrin-mediated signaling is critical for the maintenance of hepatocyte morphology and functions. Type I collagen is a major ECM component in the liver. When hepatocytes were cultured in collagen gel sandwich culture, they formed bile canaliculi and maintained liver-specific functions, such as albumin secretion and urea synthesis [12–17]. These results suggest that, in addition to cadherin-mediated signaling via cell–cell interactions, integrin-mediated signaling via cell–ECM interactions plays important roles in regulating hepatocyte polarity, functions, and morphogenesis. Recent advances in microfabrication technologies have enabled us to construct collagen gel microbeads, which can be cultured with hepatocytes. However, little is known about the hepatocyte–collagen gel microbead interactions. This study aims to clarify the effects of the balance between cell–cell and cell–collagen gel microbead interactions on hepatocyte morphogenesis and functions.

In the present study, we utilized collagen gel microbeads to control integrin-mediated signaling in primary hepatocyte culture. In particular, we controlled the magnitude of hepatocyte–collagen gel microbead interactions by changing the size of the microbeads. Specifically, collagen microbeads of three sizes, namely smaller than, comparable to, and larger than hepatocytes, were fabricated and cultured with hepatocytes. The use of various collagen microbeads in hepatocyte culture provide different growth surfaces for hepatocytes, which is another important aspect of 3D culture. Our findings demonstrated that hepatocyte morphology significantly changed depending on the size of the microbeads. In particular, only microbeads with a size comparable to that of hepatocytes induced the formation of 3D hepatocyte structures. Time-lapse imaging revealed the process whereby the hepatocytes and the microbeads attached to each other and finally formed 3D structures in a self-organizing manner. Furthermore, we confirmed that liver-specific functions, such as albumin secretion and ammonia clearance, were significantly upregulated in these 3D structures. These findings are critical for understanding how to control the formation of 3D hepatocyte tissues with hydrogel microbeads in the context of biofabrication.

Materials and methods

Fabrication of collagen microbeads

Collagen microbeads were fabricated based on the method reported by Nagai et al [18] with some modifications in surfactant concentration and rotation speed. Briefly, 10 ml of 10 mg ml⁻¹ type I collagen solution from porcine skin (Nippon Meat Packer, Ibaraki, Japan) was emulsified in 50 ml of liquid paraffin containing 1% Span 20, a surfactant, and stirred using a top stirring plate for 15 min at various rotation speeds at 4 °C. When the collagen microbeads were labeled with green fluorescence, 1 ml of 1 mg ml⁻¹ FITC-conjugated type I collagen from bovine skin (Collagen Research Center, Tokyo, Japan) was mixed with the collagen solution. Thereafter, 1 ml of water-soluble carbodiimide (WSC; Dojindo, Kumamoto, Japan), a crosslinking agent, at 50% in water (v/v) was added to the emulsified mixture and stirred at 4 °C for 60 min to form collagen microbeads. The samples were kept at 4 °C until this step for controlling viscosity of the collagen solution, which correlated to the size of fabricated microbeads. Thereafter, the emulsified mixture was kept in a humidified 5% CO₂ incubator at 37 °C overnight to stabilize the polymerization process of the collagen microbeads, which resulted in a top layer of liquid paraffin, a middle layer of water, and a bottom layer of collagen microbeads. The bottom layer of microbeads was collected and rinsed with 70% ethanol to remove liquid paraffin. The mixture was then centrifuged at 3500 rpm for 5 min. The supernatant was removed and the collagen microbeads were rinsed with 70% ethanol again. The collagen microbeads were then rinsed with phosphate-buffered saline (PBS) three times to remove ethanol, after which they were suspended in culture medium and stored at 4 °C until use.

Measurement of the size of collagen microbeads

The collagen microbeads suspended in Dulbecco’s modified Eagle’s medium (DMEM) were stirred overnight to disperse them homogeneously and their size was measured using phase-contrast images. The diameters of at least 300 microbeads were measured using AxioVision software.

Isolation of rat primary hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (250–350 g; Sankyo Labo Service, Tokyo, Japan) using the two-step liver-perfusion method. Collagenase-digested liver cells were suspended in L-15 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g/l galactose, 30 mg l⁻¹ L-proline, 0.5 mg l⁻¹ insulin, 10⁻³ M dexamethasone, and antibiotics. After the suspension had been centrifuged twice at 50 × g for 1 min, the pellet was suspended in L-15 medium and mixed with the same amount of Percoll solution, which consisted of 9 vol. of Percoll (Sigma-Aldrich, St. Louis, MO, USA) and 1 vol. of 10 × Hanks balanced salt solution without calcium or magnesium. The suspension was then centrifuged at 50 × g for 15 min to remove dead cells. Thereafter, the pellet was suspended in the medium and centrifugation was
again performed at 50 × g for 1 min. Finally, the obtained pellet was suspended in DMEM supplemented with 20 mM HEPES, 25 mM NaHCO₃, 0.5 mg l⁻¹ L-proline, 10% fetal bovine serum (FBS), 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 10 ng ml⁻¹ epidermal growth factor (EGF), and antibiotics. Cell viability of >90% was verified by the trypan blue exclusion test. All animal experiments were performed according to the Institutional Guidelines on Animal Experimentation at Keio University and approved by the Keio University Institutional Animal Care and Use Committee.

**Culture of rat hepatocytes with collagen microbeads**

Hepatocytes isolated from rats were mixed with collagen microbeads of three different sizes: smaller than, comparable to, and larger than hepatocytes. The mixture of hepatocytes and the small, medium, or large collagen microbeads was cultured in a 35 mm glass-bottomed dish. Hepatocytes were seeded at the density of 6 × 10⁶ cells/35 mm dish. Based on this seeding density, 6.0 × 10⁵ medium microbeads were used to give 1:1 hepatocyte:micrubead ratio. To investigate the effect of microbead size on hepatocyte morphogenesis, we also used the small and large microbeads. We assumed that the probability of the contact between hepatocytes and microbeads was dependent on the 2D projection area of seeded microbeads. When we used the small microbeads, the number of microbeads increased to give the same 2D projection area compared to the medium microbeads. In contrast, when we used the large microbeads, the number of microbeads decreased. The average diameters of the small, medium, and large microbeads were 12, 33, and 189 μm, respectively, which corresponds to the 36:27:8 ratio of the 2D projection area. Based on this ratio, 6.0 × 10⁵ hepatocytes were mixed with 4.5 × 10⁶ small microbeads, 6.0 × 10⁵ medium microbeads, or 1.8 × 10⁶ large microbeads, and seeded in a 35 mm dish. The culture medium was replaced every other day.

For the experiment with collagen coating, 300 μl of 10 mg ml⁻¹ type I collagen solution from porcine skin was added in a 35 mm glass-bottomed dish and air-dried. Thereafter, the culture dish was rinsed three times with PBS. Hepatocytes and collagen microbeads were then seeded into the culture dish.

**Phase-contrast imaging**

The hepatocytes and collagen microbeads cultured in a 35 mm glass-bottomed dish were observed every day until day 21 by phase-contrast microscopy. Time-lapse imaging was also carried out to observe the behavior of hepatocytes interacting with collagen microbeads in more detail. For the time-lapse imaging, cells were placed in a humidified, 5% CO₂ chamber at 37°C on a microscope stage, and photographed using a phase-contrast microscope equipped with a time-lapse device, which was controlled by image acquisition software, LuminaVision (Mitani Corp, Fukui, Japan). Images of hepatocytes and collagen microbeads were recorded at 15 min intervals for 48 h.

**Immunofluorescence staining**

Immunofluorescence staining was performed to investigate 3D structures composed of hepatocytes and collagen microbeads. Hepatocytes cultured with the small, medium, and large microbeads were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 30 min. The samples were then incubated with Block Ace (Dainippon Pharmaceutical, Suita, Japan) to block non-specific staining. Actin filaments and nuclei were then stained with Alexa Fluor 594-phalloidin (Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), respectively. For staining collagen microbeads, FITC-conjugated type I collagen was used in the process of microbead fabrication, as described above. The samples were rinsed with PBS and a z-axis series of fluorescence images was obtained using a confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany). The z-stacks of the confocal images were reconstructed in 3D using ImageJ (NIH, Bethesda, MD, USA).

**Quantitative analysis of 3D cell morphology**

Hepatocyte morphogenesis differed depending on the size of collagen microbeads, resulting in a different 3D cell morphology in each culture condition. Therefore, the 3D cell morphology was analyzed quantitatively, focusing on the shape of nuclei in cross sections of 3D stacked-up structures using ImageJ. Nuclei were stained with DAPI and photographed using a confocal laser-scanning microscope, as described above. The thickness of a nucleus was measured as the cell height in a cross-sectional image. A z-axis projection of the z-stack images was reconstructed and the nucleus area in the projection image was measured. A circularity value was calculated to quantify the degree of nuclear deformation in a cross section along the major axis of the nucleus in the z-axis projection image. Specifically, the circularity was calculated using the area and perimeter of the nucleus by the following formula:

\[
\text{Circularity} = 4\pi \left( \frac{\text{Area}}{\text{Perimeter}^2} \right).
\]

A circularity value of 1.0 indicates a perfect circle. A value approaching zero represents an increasingly elongated ellipse.

**Measurement of albumin secretion**

Hepatocytes were cultured with the small, medium, or large microbeads, or without microbeads for control experiments until day 21. The albumin secretions produced by the cells were measured. Culture media from the three dishes were collected separately into
microtubes 48 h after replacement of the medium. The media were then centrifuged at 8000 × g for 10 min and the supernatant was transferred to new microtubes. Quantification of the secreted rat albumin was carried out using an enzyme-linked immunosorbent assay (ELISA) with the two-antibody-sandwich method. Sheep anti-rat albumin antibody (1.0 μg/well; Bethyl Laboratories, Montgomery, TX, USA) was coated onto each well of a 96-well plate (Nunc, Roskilde, Denmark). After incubating with blocking solution (0.05 M sodium carbonate, pH 9.6), samples were loaded to the wells and incubated for 1 h. Rat albumin (Bethyl Laboratories) was used as a standard. Horseradish peroxidase-conjugated sheep anti-rat albumin antibody (2.5 ng/well; Bethyl Laboratories) was then added to each well. Tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used for the enzyme-substrate reaction, which was stopped by applying 2 M H2SO4. A microplate reader was used to measure the absorbance of the samples at a wavelength of 650 nm.

Measurement of ammonia clearance rate
To evaluate ammonia clearance by hepatocytes cultured with collagen microbeads, the medium was replaced with fresh culture medium containing 2 mM ammonium chloride. After incubation for 4 h, the ammonia concentration in the culture medium was measured using a kit (ammonia-test Wako; Wako Pure Chemical Industries, Tokyo, Japan). The ammonia clearance rate was calculated based on the decrease in ammonia concentration. A microplate reader was used to measure the absorbance of the samples at a wavelength of 450 nm.

Statistical analysis
Cell culture experiments were repeated at least twice to confirm the repeatability of the results. Data are presented as means ± SEM. Tukey’s test with ANOVA was used to analyze the significance of differences, with P < 0.05 being considered to indicate statistical significance.

Results

Fabrication of collagen microbeads
The size of collagen microbeads can be controlled by adjusting the rotation speed or surfactant concentration, as previously reported [18]. In the present study, we used a constant surfactant concentration, 1% Span 20, and changed the rotation speed to control the size of collagen microbeads because this method can cover a wider range of sizes than can be achieved by changing the surfactant concentration. The results indicated that the size of collagen microbeads increased as the rotation speed decreased (figures 1(A) and (B)). After testing several rotation speeds, we selected 500, 750, and 1000 rpm to fabricate microbeads of three different sizes, namely, smaller than, comparable to, and larger than hepatocytes (~20 μm), respectively. Representative phase-contrast images showed that collagen microbeads were successfully fabricated with the desired sizes (figure 1(B)). The peaks of the size distributions of collagen microbeads fabricated at 500, 750, and 1000 rpm were 10, 20, and 210 μm, respectively (figure 1(A)). These were the small (~10 μm), medium (~20 μm), and large (~210 μm) microbeads used in the subsequent experiments.

Biocompatibility of fabricated collagen microbeads
Next, we used the small, medium, and large collagen microbeads for hepatocyte culture. These microbeads were mixed with hepatocyte suspensions, seeded in 35 mm dishes, and cultured for 3 days to test the biocompatibility of the fabricated collagen microbeads. A live/dead staining test demonstrated that most of the cells showed green fluorescence, which indicated CellTracker-positive live cells, regardless of the microbead size (figure 1(C)). In contrast, a few cells showed red fluorescence, which indicated PI-positive dead cells. Quantitative analysis of the live/dead fluorescence images revealed that the viabilities for the small, medium, and large microbeads were all >90% (figure 1(D)), suggesting that the fabricated microbeads are biocompatible.

Different morphogenesis of hepatocytes with the small, medium, and large microbeads
Upon confirming their biocompatibility, we then cultured hepatocytes with these microbeads until day 21 to investigate their morphogenesis. Interestingly, hepatocyte morphogenesis changed dramatically depending on the size of the microbeads (figure 2). When hepatocytes were cultured with the small microbeads, they gradually spread on the substrate and formed 2D cell sheets (figure 2, Small). By contrast, when hepatocytes were cultured with the medium microbeads, they spread on the substrate on day 3, but aggregated and formed 3D structures during days 7–21 (figure 2, Medium). Consequently, most of hepatocytes formed large 3D structures with the medium microbeads on day 21 although the thickness of the 3D structures was not homogeneous (arrowheads, figure 2, Medium). When hepatocytes were cultured with the large microbeads, they attached on the surface of the microbeads on day 3. During days 7–21, some hepatocytes spread on the substrates and formed 2D cell sheets, while others attached on the surface of the large microbeads (asterisks, figure 2, Large). Consequently, hepatocytes formed 2D cell sheets with partial 3D structures only around the microbeads (figure 2, Large). Time-lapse movies clearly demonstrated the differences in hepatocyte morphogenesis depending on the size of the
Hepatocyte–microbead interactions were further investigated by time-lapse microscopy. In the case of the small microbeads, representative time-lapse images showed that the beads were pulled by hepatocytes within 15 min when they attached to each other. Subsequently, the beads seemed to be embedded in hepatocytes, while the shape of hepatocytes did not change before versus after attachment to the microbeads (supplemental movies 1–3 are available online at stacks.iop.org/BF/11/035007/mmedia).

Hepatocyte–microbead interactions were further investigated by time-lapse microscopy. In the case of the small microbeads, representative time-lapse images showed that the beads were pulled by hepatocytes within 15 min when they attached to each other. Subsequently, the beads seemed to be embedded in hepatocytes, while the shape of hepatocytes did not change before versus after attachment to the
In the case of the medium microbeads, the beads were pulled by hepatocytes within 420 min when they attached to each other. In contrast to the case of the small microbeads, hepatocytes changed their shape to surround the medium microbeads. In the case of the large microbeads, the beads were not pulled by hepatocytes significantly within 420 min.

Differences in hepatocyte morphogenesis upon culture with the small, medium, and large microbeads were investigated further by confocal imaging. When hepatocytes were cultured with the small microbeads, the cells formed 2D cell sheets. The cross-sectional image revealed that nuclei showed a flattened shape (arrowheads, figure 4(A), Small). We also found that the small microbeads attached to the cell sheets while some beads located within the cells. By contrast, when hepatocytes were cultured with the medium microbeads, the cells formed 3D structures (figure 4(A), Medium). The cross-sectional image revealed that the 3D structures consisted of hepatocytes and microbeads. In addition, nuclei exhibited a round shape (arrowheads, figure 4(A), Medium), which suggested that the hepatocytes maintained a cuboidal shape. When the hepatocytes were cultured with the large microbeads, the cells formed both partial 3D structures and 2D cell sheets (figure 4(A), Large). The cross-sectional image revealed that some hepatocytes around the large microbeads maintained a 3D shape with round nuclei (arrowheads, figure 4(A), Large).

To show clearly that the cell configuration differed depending on the size of the microbeads, nucleus circularity in the cross-sectional images was quantified. The results indicated that the circularity value of the medium microbeads was significantly higher than those of the small and large microbeads (figure 4(B)).

Changes in hepatocyte functions depending on the size of collagen microbeads

Hepatocyte functions were then measured to investigate the relationship between hepatocyte functions and hepatocyte morphogenesis depending on the size of collagen microbeads. First, we measured albumin secreted by hepatocytes using an ELISA kit. When hepatocytes were cultured without microbeads, the amount of albumin secretion peaked on day 6 and then gradually decreased until day 21 (figure 5(A)). Similarly, when hepatocytes were cultured with the small microbeads, the amount of albumin secretion peaked on day 12 and then gradually decreased until day 21, although hepatocytes maintained higher levels of albumin secretion during days 9–15. By contrast, when hepatocytes were cultured with the medium and large microbeads, the amount of albumin secretion increased until day 18. Hepatocytes cultured with the

Figure 3. Representative phase-contrast images showing cell-bead interactions. Arrowheads indicate a microbead. Small: A microbead was pulled by a hepatocyte within 30 min. Medium: A microbead was surrounded by hepatocytes, resulting in the movement of the microbeads toward hepatocytes within 420 min. Large: A microbead was not significantly pulled by hepatocytes within 420 min. All images are shown at the same magnification. Scale bar, 100 μm.

microbeads (figure 3, Small). In the case of the medium microbeads, the beads were pulled by hepatocytes within 420 min when they attached to each other. In contrast to the case of the small microbeads, hepatocytes changed their shape to surround the medium microbeads (figure 3, Medium). In the case of the large microbeads, the beads were not pulled by hepatocytes significantly within 420 min (figure 3, Large).
medium microbeads showed significantly higher albumin secretion (asterisks, figure 5(A)).

To investigate hepatocyte functions further, we also measured the ammonia clearance rate focusing on long-term culture. This rate was significantly higher on day 21 in culture of hepatocytes with the medium microbeads (asterisks, figure 5(B)). Similar to the results of albumin secretion, hepatocytes with the large microbeads showed the second greatest ammonia clearance rate, followed by those with the small microbeads, and then those without microbeads.

**Hepatocyte morphogenesis with medium microbeads cultured on a collagen-coated substrate**

According to the results above, hepatocytes formed 3D structures when they were cultured with the medium microbeads. We hypothesized that hepatocyte–microbead adhesions dominated over hepatocyte–substrate adhesions under these culture conditions. Therefore, to enhance the hepatocyte–substrate adhesions, we tested hepatocyte morphogenesis when the cells were cultured on a collagen-coated substrate. Under these conditions, hepatocytes attached on the substrate on day 3 (figure 6(A), day 3). Thereafter, hepatocytes attached to the medium microbeads on day 7 (figure 6(A), day 7), which is similar to the results without collagen coating, as shown in figure 2 (Medium, day 7). However, hepatocytes spread on the collagen-coated substrate during days 14–21 (figure 6(A), days 14, 21). Confocal images of the cells on day 14 revealed that hepatocytes formed relatively thin tissues with a thickness of 1 or 2 cells (figure 6(B)).

**Discussion**

**Fabrication of three different sizes of collagen microbeads relative to hepatocytes**

In the present study, we fabricated a wide range of collagen microbeads, which were smaller than, comparable to, and larger than hepatocytes, to control the magnitude of cell–ECM interactions. Initially, we
attempted to fabricate collagen microbeads of a size comparable to that of hepatocytes using a microfluidic 3D T-junction device. This device has a junction of two channels, a continuous-phase channel and a dispersed-phase channel, which generates droplets of collagen solution [19]. Although this method is useful to fabricate collagen microbeads ~200 μm in size, it is difficult to fabricate collagen microbeads of ~20 μm due to high viscosity of the collagen solution. Therefore, we utilized a water-in-oil emulsion method to fabricate smaller (<20 μm) collagen microbeads.

During the process of microbead fabrication, we focused on two critical parameters to manipulate the size of microbeads, namely surfactant concentration and rotation speed. First, we used a surfactant concentration of 0.5%–1.5% with rotation at 1200 rpm, and confirmed that the size of the fabricated microbeads decreased with increasing surfactant concentration. However, it was difficult to fabricate microbeads of >100 μm in size only by changing the surfactant concentration. Therefore, we then varied the rotation speed to acquire a wider range of microbeads with a surfactant concentration of 1.0%. The medium microbeads, the size of which was ~20 μm, were successfully fabricated with rotation at 750 rpm. The small microbeads, the size of which was ~10 μm, could be fabricated by increasing the rotation speed up to 1000 rpm. By contrast, the large microbeads, with a size of ~210 μm, could be fabricated by decreasing the rotation speed down to 500 rpm. The size distribution of the large microbeads became wider, which might have been due to the unstable emulsion conditions. However, we could fabricate the large microbeads without changing conditions, such as the concentration of surfactant and a crosslinking agent. Consequently, we were able to fabricate collagen microbeads of three different sizes, namely, smaller than, comparable to, and larger than hepatocytes, only by changing the rotation speed.

Cell–collagen gel microbead interactions depending on the size of microbeads
The most important finding of this study is that hepatocytes spontaneously formed 3D structures with the medium microbeads, whereas they formed 2D structures with the small or large microbeads. This clearly demonstrates that the magnitude of cell–collagen gel microbead interactions has critical effects on the 3D tissue formation of hepatocytes.

Each medium microbead initially attached to one of the hepatocytes in our culture and was pulled by it. The microbead then attached to neighboring cells, which led to the formation of cell–microbead clusters mediated by both cell–cell and cell–microbead adhesions. Thus, the traction force between the medium microbeads and hepatocytes might become stronger than hepatocyte–substrate adhesion. Consequently, hepatocytes detached from the substrate and formed 3D structures with the medium microbeads. We tested only one seeding density of the medium microbeads at 1:1 cell:bead ratio. Although hepatocytes connected to multiple microbeads in this condition, the extent of the connection between hepatocytes and microbeads decreases with decreasing seeding density of the microbeads. Therefore, the seeding density of microbeads is also an important factor for regulating 3D tissue formation of hepatocytes.
By contrast, the existence of the small microbeads had no significant effects on hepatocyte morphogenesis. Since the small microbeads are much smaller than hepatocytes, the microbeads failed to mediate cell–cell and cell–microbead adhesions. Time-lapse images revealed that the small microbeads seemed to be absorbed by the hepatocytes. In addition, confocal images revealed that some microbeads located within a cell. Consequently, hepatocytes predominantly attached to the substrate, which led to the formation of 2D monolayers. The small microbeads might be endocytosed by hepatocytes, and there might be a maximal size for endocytosis. This size-dependent interaction between hepatocytes and microbeads is important to control hepatocyte morphogenesis in 3D culture. In addition, phagocytosing capability of rat hepatocytes was reported in a previous study [20]. Further investigation will be needed to clarify these phenomena in the context of cell–microbead interactions.

On the other hand, hepatocytes attached to the surface of the large microbeads. However, the microbeads and hepatocytes failed to form 3D structures. Since these microbeads were much larger than the hepatocytes, the microbeads were not pulled by the hepatocytes and failed to mediate cell–cell and cell–microbead adhesions. Consequently, hepatocytes predominantly attached to the substrate and formed 2D monolayers, while the cells adjacent to the microbeads maintained their 3D shape. We tested only one seeding density of the large microbeads. As shown in phase-contrast images, the seeding density of the large microbeads was relatively low. As the distance between each microbead was longer than a single cell scale in this condition, hepatocytes might fail to connect to multiple microbeads, which can also explain the formation of 2D monolayers in the case of the large microbeads. However, hepatocytes failed to construct 3D structures detached from the substrate even in the region where the large microbeads closely distributed. This suggest that the size of microbeads is a more important factor than their seeding density in terms of 3D tissue formation of hepatocytes. However, it is important to consider the balance between these regulating factors.

The differences in morphogenesis depending on the size of microbeads are described in schematic illustrations (figure 7). Lauffenburger and Griffith [21] proposed a principle that can explain multicellular organization through integrin and cadherin-mediated cell–ECM and cell–cell interactions, respectively. Specifically, cells form 2D structures when cell–ECM adhesion dominates over cell–cell cohesion. This principle is consistent with the hepatocyte morphogenesis demonstrated in the present study.

According to this principle, we hypothesized that the balance between the cell–cell/microbead interactions and cell–substrate interactions is critical for 3D tissue formation. To verify this hypothesis, we cultured hepatocytes and the medium microbeads on the substrate coated with collagen. Because the substrate was coated with collagen, the cell–substrate interaction was enhanced. As we expected, hepatocytes formed 2D monolayers on the collagen-coated substrate regardless of the size of the microbeads. These results suggested that the cell–substrate adhesion dominated over the cell–cell/microbead adhesion, even when hepatocytes were cultured with the medium microbeads, due to the enhanced cell–substrate interactions. Therefore, it is critical to consider the balance between the cell–cell/microbead interactions and the cell–substrate interactions when attempting to control hepatocyte tissue formation.

**3D tissue formation and liver-specific functions**

In the present study, hepatocytes cultured with the medium microbeads formed 3D structures and maintained their 3D cell shape, which was quantified using the characteristic of nucleus circularity. This is important given the well-known relationship between cell shape and cell functions [22–25]. Indeed, a simple experiment has demonstrated the close relationship between the shape of single hepatocytes and their
functions. When hepatocytes were cultured on a confined surface, they maintained a cuboidal shape and exhibited low proliferative activity and high differentiation activity [26]. Our previous studies also demonstrated that the maintenance of 3D cell shape is important for the expression of liver-specific functions of hepatocytes. Specifically, when cell sheets of hepatocytes were stacked to form 3D structures, the cells maintained their 3D shape and their differentiated functions were significantly upregulated [1, 2, 27]. Furthermore, liver-specific functions were upregulated when hepatocytes were cultured as composite spheroids with cell-sized collagen microbeads compared to hepatocytes cultured on a collagen-coated dish [28]. The findings of these previous studies are consistent with our results. Specifically, in the present study, hepatocytes cultured with the medium microbeads maintained their 3D cell shape, which corresponds to upregulated liver-specific functions such as albumin secretion and ammonia clearance.

In addition, expression of liver-specific genes such as albumin and ornithine transcarbamylase was upregulated in composite spheroids at 1:1 cell:bead ratio compared to the spheroids without beads [28]. However, expression of these genes in composite spheroids at 1:4 cell:bead ratio was comparable to the spheroids without beads, suggesting that the cell:bead ratio is an important factor for hepatocyte functions. On the other hand, a previous study reported comparison between hepatocyte spheroids and those with alginate microbeads at 1:1 cell:bead ratio [29]. They reported that both albumin secretion and ammonia reduction were improved in the spheroids with alginate microbeads compared to the spheroids without alginate microbeads. Since the alginate microbeads did not establish biologically relevant cell adhesion, the alginate microbeads in hepatocyte spheroids might play a role of conduits for better transports of oxygen and nutrients. In the present study, hepatocytes constructed 3D structures when cultured with the medium microbeads at 1:1 cell:bead ratio. According to the previous reports above, liver-specific functions of the 3D structures in the present study appeared to be improved compared to hepatocyte spheroids without microbeads.

In conclusion, we aimed to clarify the effects of the balance between cell–cell and cell–collagen gel microbead interactions on hepatocyte morphogenesis and function. To control the magnitude of cell–microbead interactions, three different sizes of collagen microbeads, which were smaller than, comparable to, and larger than hepatocytes, were successfully fabricated only by changing the rotation speed. We demonstrated that hepatocyte morphogenesis significantly changed depending on the size of the microbeads. Specifically, only microbeads of a size comparable to that of hepatocytes induced the formation of 3D hepatocyte structures, which led to the maintenance of liver-specific functions. These findings are critical for understanding hepatocyte morphogenesis under the different balances between cell–cell and cell–microbead interactions in the context of biofabrication with hydrogel beads. In addition, it is important for hepatocyte culture to use collagen

![Figure 7. Schematic illustrations of hepatocyte morphogenesis depending on the size of collagen microbeads relative to hepatocytes.](image)

When the beads are much smaller than hepatocytes, hepatocytes dominantly spread on the substrate, which leads to the formation of 2D monolayers. When the beads are comparable in size to hepatocytes, hepatocytes and beads attach to each other, which dominates over cell–substrate adhesion. Consequently, hepatocytes and beads are organized into 3D tissues. When the beads are much larger than hepatocytes, the beads fail to mediate cell–cell adhesion, which leads to the formation of 2D monolayers.
microbeads of appropriate size and seeding density in terms of 3D tissue formation. Furthermore, future investigation will be needed to clarify more detail polarity of hepatocytes such as bile canalicular formation and extension of microvilli on sinusoidal domain. However, the culture condition, which allowed 3D hepatocyte morphogenesis with collagen microbeads in the present study, can be applied for the other 3D culture models of hepatocytes such as bioreactors and multilayered constructs.

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