The Effect of Micropatterned Pores on the Formation and Movement of Small Hepatocyte Colonies*

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Abstract

Cell response on microporous membranes is important since the membranes are often used for the applications of tissue engineering. We previously developed the 3D stacked-up culture of hepatic progenitor cells, which are small hepatocytes (SHs), where the cells were cultured on microporous membranes for reconstructing layered tissues in vitro. Since membranes with randomly distributed micropores were used in the culture, the size and distribution of the micropores remained to be optimized. Here we prepared microporous membranes with different pore sizes and spacing to investigate the SH morphogenesis on the membranes. Laser-ablation technique was used to fabricate micropores in desired patterns. Phase-contrast microscopy revealed that the formation of SH colonies was correlative to the micropores. Furthermore, SH colonies exhibited collective cell movements along the micropatterned pores. The correlation enhanced with increasing sizes (>10 µm) and decreasing spacing (<40 µm) of micropores. Scanning electron microscope images showed that SHs located at the colony edge extended protrusions invading into the micropores. Furthermore, immunofluorescent staining revealed that the focal complexes localized intensively around the micropores, suggesting that the SH morphogenesis was regulated by the focal complexes. This study demonstrated that the surface topography of microporous membranes plays an important role in the formation and movement of SH colonies.

Key words: Collective Movement; Focal Complex; Micropatterning; Microporous Membrane; Small Hepatocyte

1. Introduction

Three-dimensional (3D) culture is important for creating physiological tissues in vitro. Liver is a complex 3D tissue with functional hepatocytes that reside in the 3D microenvironment (1). To reconstruct the 3D microenvironment in vitro, several culture models have been reported where hepatocytes were cultured with non-adherent substrates (2), micro-carriers (3), 3D peptide gels (4), microfabricated bioreactors (5), and microfluidic devices (6). In these 3D culture models, hepatocytes formed 3D structures such as hepatocyte spheroids. Although hepatocyte spheroids could maintain liver-specific functions, these structures failed to form well-defined structures because they are cell aggregates. Consequently, we previously developed the 3D stacked-up culture that is a method to stack cell monolayers cultured on polycarbonate membranes (PCMs) to mimic
the layered 3D structures *in vivo* (7).

In the 3D stacked-up culture, we cultured small hepatocyte (SHs) (8), which are hepatic progenitor cells, on PCMs with 10 µm-micropores (7). The cells formed colonies expanding in a random manner on the microporous PCMs since the micropores were randomly distributed on the membrane. The 3D culture model using the PCMs was useful for reconstructing liver-like tissues. However, the effects of the pore size and distribution on the formation of SH colonies remain to be elucidated. Since surface topography of the biomaterials influences the attachment and spreading of individual cells, the size and distribution of micropores presumably influence behaviors of SH colonies.

Although little is known about the effect of surface properties on the behavior of cell colonies, it has been suggested that physical and geometric properties of a culture surface influenced a wide variety of cell properties at the single-cell level. Cells protruded filopodia resulting in forming morphology of spherical shape because of limitation of cell spreading by the micropores larger than 3.0 µm in diameter (9,10). Cells also adhered, proliferated, and exhibited a 3D shape when attaching inside the cavities of 30 or 100 µm in diameter, whereas nanoscale topography on the surfaces had little effect on cell morphology (11,12). In addition, the spacing between micropores was a critical parameter in cell movements (13). These studies indicated that both size and spacing of mechanical obstacles (e.g., micropores) were important factors in surface properties for regulating the morphologies of fibroblasts, osteoblast-like cells, and leukocytes. However, little is known about these effects on hepatocytes, especially at the multi-cell level.

Here we investigated the effect of substrate surface topographies on the formation of SH colonies using microporous PCMs with different pore sizes and distributions. The laser-ablation technique was used to fabricate micropores in desired patterns. Phase-contrast and time-lapse microscopy revealed that the formation and movement of SH colonies were influenced by the micropatterned pores. Scanning electron microscope (SEM) images showed that SHs located in the peripheral regions of the colonies extended protrusions invading into micropores. Furthermore, immunofluorescent staining revealed that the focal complexes localized around micropores in SH colonies, suggesting that the formation and movement of SH colonies are regulated by the micropatterned pores through the formation of focal complexes around the micropores. In addition, our results demonstrated that both size and patterning of micropores were critical factors in the formation and movement of SH colonies.

**2. Materials and Methods**

**2.1 Isolation and culture of rat SHs**

Male Sprague-Dawley rats (Nippon Bio-Supp. Center, Tokyo, Japan), weighing 250–450 g were used to isolate SHs by the two-step liver-perfusion method with some modification (14). A collagenase-digested liver cell suspension was sequentially passed through filters of 250 and 70 µm, and centrifuged at 50×g for 1 min. The supernatant was centrifuged at 50×g for 5 min, and the pellet was suspended in L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES (Dojindo, Kumamoto, Japan), 1.1 g/l galactose (Katayama Chemical, Osaka, Japan), 30 mg/l L-proline, 0.5 mg/l insulin (Sigma-Aldrich, St. Louis, MO), 10⁻⁷ M dexamethasone (Wako Pure Chemical, Tokyo, Japan) and antibiotics. The pellet was re-centrifuged at 50×g for 5 min. Thereafter the pellet was suspended in the medium and centrifuged at 150×g for 5 min. After this procedure was repeated once again, the pellet was suspended in the medium and centrifuged at 50×g for 5 min. Finally, the pellet was suspended in DMEM (Sigma-Aldrich), supplemented with 20 mM HEPES, 25 mM NaHCO₃ (Wako Pure Chemical), 30 mg/l L-proline, 0.5 mg/l insulin, 10⁻⁷ M dexamethasone, 10% FBS, 10 mM nicotinamide (Sigma-Aldrich), 1 mM ascorbic
acid 2-phosphate (Wako Pure Chemical), 10 ng/mL epidermal growth factor (BD Biosciences) and antibiotics. The number of viable cells was counted using the trypan blue-exclusion test.

The cells were inoculated on random-patterning microporous PCMs (rPCMs; Nuclepore®, Whatman, Clifton, NJ) with a thickness of 8 µm, a size of 12.0 µm and a density of $1 \times 10^5$ pores/cm². The membranes were placed in a 35-mm dish (Corning Glass Works, Corning, NY), and coated with rat-tail collagen (250 µg of dried tendon/0.1% acetic acid). The cells were seeded on the membrane in a 35-mm dish at a cell density of $4.5 \times 10^5$ cells/dish and placed in a humidified, 5% CO₂/95% air incubator at 37°C. Culture medium was changed to remove dead cells 3 h after seeding and was replaced every other day. After day 4 (96 h after seeding), 1% dimethylsulfoxide (DMSO; Sigma-Aldrich) was added to the culture medium.

2.2 Analysis for the effect of pore size on SH colony formation

SHs were cultured on rPCMs with 5.0, 10.0 and 12.0 µm-micropores (Table 1). To quantify the correlation between the edge of SH colonies and micropore distribution, the number of micropores that overlap with the colony edge was counted. The cells were fixed with cold absolute ethanol for 15 min and immunostaining for cytokeratins was carried out to identify the outline of SH colonies. SHs were labeled with a rabbit anti-cytokeratin wide spectrum screening (1:100, Dako, Denmark) and detected by the avidin-biotin peroxidase complex method (Histofine®; Nichirei, Tokyo, Japan). 3,3’-Diaminobenzidine (Vector Laboratories, Burlingame, CA) was used as a substrate. The sample was then counterstained with hematoxylin, and photographed with a microscope (TE200; Nikon, Tokyo, Japan) equipped with a CCD camera (AxioCam MRc5; Carl ZEISS, Hallbergmoos, Germany).

The number ($N_{\text{experiment}}$) of micropores that overlap with the edge of SH colonies was counted using the cytokeratins-stained images (e.g., red circles, Fig. 1A). The stained images were binarized to clarify the outline of SH colonies (Fig. 1B) and the perimeter of the colonies was measured using Scion Image (Scion Corporation, Frederick, MD). The number ($N_{\text{experiment}}$) was then divided by the perimeter of the colonies to get the number per unit length ($N_{\text{experiment}}'/\text{unit length}$). As a control, the binarized images were superimposed on the image of the membrane (Fig. 1C) and the number ($N_{\text{control}}$) of micropores that overlap with the edge of the binarized images was counted (e.g., red circles, Fig. 1D) and divided by the perimeter of the colony to get the number per unit length ($N_{\text{control}}'/\text{unit length}$). The ratio $N_{\text{experiment}}'/N_{\text{control}}'$ was calculated to quantify the correlation between micropore distribution and SH colony formation. We analyzed 30 SH colonies cultured for 9–11 days on rPCMs with each micropore size (5.0, 10.0, and 12.0 µm).

2.3 Fabrication of patterning micropores on a PCM

PCMs (Special Clear Cyclopore®; Whatman) with a thickness of 9 µm and 0.6 µm micropores were used for fabrication of patterning 10 µm micropores. The patterning micropores were formed on the membranes by the laser-ablation technique (Laserx, Aichi, Japan). Excimer laser beam (KrF; $\lambda = 248$ nm) was radiated against the membrane through a mask with desired micropore patterning. The mask was designed to fabricate micropores patterning 200–600 µm circles with different spacing (20, 30, 40, 50 and 100 µm). The micropatterned PCMs (mPCMs) were rinsed with absolute ethanol, autoclaved at 121°C for
20 min, and stored at room temperature until use.

2.4 Morphology observation of SH colonies
SH colonies on rPCMs and mPCMs at day 10–12 were photographed by a phase-contrast microscope. Actin filaments were also stained with fluorescent phalloidin to observe the morphology of the SH colonies. The cells on mPCMs at day 12 were fixed with cold absolute ethanol for 15 min, washed with phosphate buffered saline (PBS) three times, and incubated with BlockAce (Dainippon Pharm., Tokyo, Japan) for 1 h. After washing with PBS, the sample was incubated with Alexa Fluor 594-conjugated phalloidin (1:50; Invitrogen) for 3 h. Nuclei were counterstained with 6-diamino-2-phenylindole (DAPI). The corresponding fluorescent and phase-contrast images were recorded with the Axiovert 200M microscope equipped with a CCD camera (Carl ZEISS).

2.5 Time-lapse microscopy and quantitative analysis of colony movements
SH colonies cultured on mPCMs at day 6–20 were placed in a humidified, 5% CO₂/95% air chamber (Sankei Co., Tokyo, Japan) at 37°C, and photographed using a phase-contrast microscope (Nikon) equipped with a CCD camera (CoolSNAP HQ, Roper Scientific, Trenton, NJ). Colony movements were recorded every 5 min and sequential images were analyzed using the MetaMorph® imaging system (Universal Imaging Corporation, Downingtown, PA). To analyze the fluctuation of colony movements, the outline of an SH colony was traced at 2-h intervals for 24 h using the time-lapse images. The extension of colonies was quantified by measuring colony area within regions of interest (ROI) set on each time-lapse image. ROI were set around micropatterned pores to investigate the effect of the pores on colony extension.

2.6 Scanning electron microscopy of SH colony morphologies
To observe the fine structure at the interface between SH colonies and micropatterned pores, cells on mPCMs were washed with PBS and fixed with 2% glutaraldehyde (Chiyoda Jyunyaku, Tokyo, Japan) in 0.1 M phosphate buffer for 1 h at 37°C. After washing with PBS, the sample was post-fixed with 1% osmium tetroxide (Chiyoda Jyunyaku) for 1 h. The sample was then dehydrated through a sequential ethanol series (20–100% at an increment of 10%) for 10 min each. The sample was displaced with butanol (Wako Pure Chemical) three times, freeze-dried, sputter coated with gold, and examined with an SEM (FE-SEM S-4700; Hitachi High-Technologies, Tokyo, Japan).

2.7 Immunofluorescent staining for the focal complex protein
Cells cultured on mPCMs were fixed with cold absolute ethanol for 15 min, washed with PBS three times, and incubated with BlockAce for 1 h. After washing with PBS, the samples were incubated with mouse anti-vinculin antibody (1:800; Sigma-Aldrich) for 3 h, followed by Alexa Fluor 488-conjugated anti-mouse IgG antibody (1:200; Invitrogen). Alexa Fluor 594-conjugated phalloidin (Invitrogen) was used to detect actin filaments. The z-axis series of fluorescent images were obtained at 0.850-µm-intervals with a confocal laser-scanning microscope (LSM5 Pascal; Carl ZEISS) and analyzed using Imaris (Bitplane AG, Zurich, Switzerland).

3. Results

3.1 Effect of pore size on SH colony formation
SHs formed colonies on rPCMs with 12 µm-micropores within a week and the colonies gradually extended with time in culture. In some colonies, the edge of the colonies was coincident with micropores. First, the cells were cultured on rPCMs with different pore
sizes (5.0, 10.0, and 12.0 µm in diameter) to investigate the effect of pore size on SH colony formation. To quantify the correlation of the colony edge and micropores, we measured the number of micropores that overlap with the edge of the colonies (Fig. 1A). The number was then divided by the perimeter of the colony to calculate the number per unit length \(N_{\text{experiment}}\). As a control, the colony outline was superimposed on the image of rPCM and the number of micropores that overlap with the superimposed colony was also measured. Similarly, the number was then divided by the perimeter to calculate \(N_{\text{control}}\) (Fig. 1B–D). The ratio \(N_{\text{experiment}}/N_{\text{control}}\) represents the correlation between micropore distribution and SH colonies. As shown in Fig. 1E, the \(N_{\text{experiment}}/N_{\text{control}}\) values were larger than 1.0 with all pore sizes, suggesting that the micropores larger than 5 µm can be recognized by cells and affect the colony morphogenesis. In addition, the values increased as the pore size increased. The values for 10 (1.51±0.06) and 12-µm (1.59±0.10) micropores were significantly larger than that for 5-µm micropores (1.16±0.04). The values for 10 and 12-µm micropores were significantly higher than that for 5-µm micropores.

![Fig. 1](image)

**Fig. 1** The effect of pore size (5.0, 10.0 and 12.0 µm) on SH colony formation. A: A bright-field image of an SH colony on a rPCM with 12.0-µm-micropores. The cells were stained for cytokeratins to visualize the SH colony. The number \(N_{\text{experiment}}\) of micropores that overlap with the edge of the SH colony was counted (red circles) and divided by the perimeter of the colony to get the number per unit length \(N_{\text{experiment}}\). B: The binarized image of A. The perimeter of the colony was calculated by the binarized image. C: A bright-field image of a rPCM with 12.0-µm-micropores. D: The superimposed images of B and C. The number \(N_{\text{control}}\) of micropores that overlap with the edge of the binarized colony was counted (red circles) and divided by the perimeter of the colony to get the number per unit length \(N_{\text{control}}\) as a control. E: The ratio \(N_{\text{experiment}}/N_{\text{control}}\) was calculated and graphically shown for each pore size (5.0, 10.0 and 12.0 µm). The ratio increased with increasing pore size of rPCMs. All error bars represent s.e.m. (n=30). * p < 0.01 vs. 5 µm.

### 3.2 Fabrication of micropatterned pores on PCMs

To further investigate the correlation between the micropore distribution and SH morphogenesis, micropores were fabricated in desired patterns. Bright-field micrographs of the mPCMs revealed that micropatterned pores were successfully fabricated on a PCM (Fig. 2A). SEM images confirmed that 10 µm micropores were fabricated through the 9 µm-thickness PCMs (Fig. 2B).
3.3 SH colony formation on rPCMs and mPCMs

When SHs were cultured on flat substrates such as culture dishes, they tended to form circular colonies because the cells proliferated and moved isotropically (Fig. 3A). However, when the cells were cultured on rPCMs, they also formed colonies but the outline of the SH colony showed polygonal shape as the cells tended to attach to micropores (arrowheads, Fig. 3B, D). On the other hand, when the cells were culture on mPCMs, the cells formed colonies along with the micropatterned pores (arrowheads, Fig. 3C, E). The colony edge stayed around the micropatterned pores, although a portion of the colony overpassed the pores.
SH colony on a culture dish at day 12. B, D: Phase-contrast (B) and corresponding bright-field (D) images of an SH colony on an rPCMs at day 11. Note that the edge of the colony overlap with micropores (arrowheads). C, E: Phase-contrast (C) and corresponding bright-field (E) images of an SH colony on an mPCM at day 10. The cells formed a colony along with the micropatterned pores (arrowheads). All images were taken at the same magnification. Scale bar: 200 µm (E).

Correlation between the outline of SH colonies and micropatterned pores was also examined by immunofluorescent microscopy. Actin filaments were stained to visualize the outline of SH colonies since actin filaments intensively localize at cell–cell borders when the cells form colonies. The results showed that some SHs formed a colony of which the outline was completely along with the micropatterned pores. (Fig. 4) When the cells were attached to the surface within the micropatterned pores (200 µm-circle), they formed a colony within the circle. As the colony expanded, the edge cells became attached to the micropatterned pores and the colony stayed along with the circle for several days. Intensive actin filaments were observed along the micropatterned pores as well as cell–cell borders (Fig. 4A–C). The enlarged fluorescent image also showed that the cells near the micropatterned pores protruded filopodia into the pores (arrowheads, Fig. 4D).

Fig. 4  SH colony formation regulated by the micropatterned pores. Cells were fixed at day 12 and stained for actin filaments (red) and nuclei (blue). A–C: Corresponding fluorescent (A), phase-contrast (B), and merged (C) images. SHs formed a colony of which outline was completely along with the micropatterned pores. D: An enlarged fluorescent image for actin filaments. The cells at the edge of the SH colony protruded filopodia into the micropatterned pores (arrowheads). Dotted lines indicate micropores. Scale bars: 100 µm (C).

To further investigate the interaction between SHs and micropores, SH colonies formed on mPCMs were examined using an SEM. SEM images clearly showed that the colony was formed correlating with micropore distribution (Fig. 5A). The cells in the peripheral region of the colony protruded filopodia into micropores (Fig. 5B). These results are consistent with the results of phase-contrast and fluorescent microscopy.
3.4 SH colony movement on mPCMs

To investigate how SHs formed colonies along with the micropatterned pores, the movements of SH colonies were observed by time-lapse microscopy. The time-lapse movies revealed that SH colonies actively migrated on mPCMs while they continued to expand with time in culture. The movement of SH colonies was affected by the micropatterned pores. SH colonies moved in a random manner on flat surface, but some colonies occasionally moved toward the micropatterned pores. When a colony reached the micropatterned pores, the edge cells failed to overpass the pores, resulting in the inhibition of the movement. A representative result is shown in Fig. 6. SHs formed a colony within the micropatterned pore circle at day 8 (0 h, Fig. 6A). The colony occasionally moved toward the micropatterned pores (17 h, Fig. 6B). The colony failed to move across the micropatterned pores, resulting in inhibited colony movement (24 h, Fig. 6C). The colony stayed within the micropore circle for the next 10 days (Fig. 6D). These results were quantified by measuring the colony area within two ROI (#1 and 2, Fig. 6A) set on the near (ROI #1) and far (ROI #2) side of the micropatterned pores. As the colony approached the micropatterned pores, the area in ROI #1 increased over time until 17 h (arrowhead b, Fig. 6E). However, the area stopped increasing after the colony reached the micropatterned pores while the area in ROI #2 remained small (arrowhead c, Fig. 6E), indicating that the colony failed to overpass the micropatterned pores.
phase-contrast images of the SH colony at day 8 (A: 0 h, B: 17 h, C: 24 h) and day 18 (D). The process of
the SH colony movement was followed by several steps, 1) an SH colony expanded randomly (A), 2) a
colony occasionally moved toward micropatterned pores and became attached to the pores (B), and 3) The
colony failed to move across the micropatterned pores, resulting in inhibited colony movements (C). This
movement was kept even after 10 days of culture (D). E: The corresponding graph that quantified SH
colony movements. Two ROI were set on images (#1, 2; A–C) and the area of the SH colony within the ROI
were measured for each time-lapse image and graphically shown in E. The area within #1 increased until 17
h, but the increment became slight after the colony attached to the micropatterned pores (between
arrowheads b and c). Arrowheads (b) and (c) correspond to the images B and C. The area within #2
remained below 100 µm² in the experimental period. Scale bar: 50 µm.

The time-lapse movies also revealed that the direction of the SH colony movement
changed when the colony encountered the micropatterned pores. We analyzed 9 colonies
focusing on the movement, and SH colonies changed their direction when encountered
micropatterned pores in 7 experiments. A representative result is shown in Fig. 7. An SH
colony actively moved on a PCM without micropores, resulting in fluctuation of colony
outlines over time (Fig. 7A, B). However, a colony moving toward the micropatterned pores
on an mPCM changed movement direction after the colony attached to the micropores and
moved along the micropatterned pores (Fig. 7C, D).

![Fig. 7 Dynamical analysis of SH colony movements along micropatterned pores. A, B: Phase-contrast and
corresponding line images of an SH colony on a PCM without micropores at day 10. The colony movement
was analyzed by tracing outlines of the colony at 2-h intervals for 24 h. Superimposed line images of the
colony outlines represent the colony movement. Arrows indicate direction of the colony movement. C, D:
Phase-contrast and corresponding line images of an SH colony on an mPCM at day 15. Note that the SH
colony moves along micropatterned pores on the mPCM. Scale bar: 50 µm.](image)

The regulation of SH morphogenesis by the micropatterned pores was often observed
on mPCMs with small spacing of micropores. SHs were cultured on mPCMs with different
spacing (20, 30, 40, 50 and 100 µm) to investigate the spacing of micropores on SH
morphogenesis. SH colony formation and movements along the micropatterned pores were
frequently observed as the spacing decreased. Most SH colonies moved across the
micropatterned pores with spacing of >40 µm (data not shown). Some colonies, however,
overpassed the micropatterned pores regardless of the spacing of micropores.
3.5 SH colony adhesion around the micropatterned pores

To visualize the focal complex location around the micropatterned pores, double immunofluorescent staining for actin filaments and vinculin was carried out for the cells on an mPCM with micropatterned pores at day 12 (Fig. 8). Intensive expression of vinculin was observed coinciding with micropores (arrowheads, Fig. 8B) as well as at the edge of the colony (arrows, Fig. 8B). Actin filaments localized at cell–cell borders while some actin stress fibers were observed in cells in peripheral region of the colony (Fig. 8A).

![Fig. 8](image)

The localization of vinculin was further examined using a confocal laser-scanning microscope. Sequential z-plane images showed that actin filaments located at the top surface of cells (Fig. 9A) while vinculin located at the bottom surface of the cells (Fig. 9B). In addition, intensive localization of vinculin and actin filaments was observed coinciding with the location of micropores (Fig. 9C). The cross-section and stereoscopic images clearly showed that vinculin and actin filaments localized inside micropores (arrowheads, Fig. 9D, E). The protrusion was observed especially at the peripheral region of the colony.

![Fig. 9](image)
4. Discussion

4.1 SH colony formation and movements in correlation with micropores

Geometric properties of culture surface have been shown to be important for regulating the behavior of fibroblasts, osteoblasts, and neutrophil at the single-cell level (9–13). In this study we demonstrated that the geometric properties play a significant role in SH morphogenesis at the multi-cell level as well. SHs were cultured on microporous PCMs with different pore sizes and spacing to investigate cell behaviors on the membranes. Phase-contrast and time-lapse imaging techniques revealed that the formation and movement of SH colonies were correlative to the micropatterned pores. Our results demonstrated that both size and spacing of micropores are important for the SH morphogenesis on microporous PCMs.

The pore size is an important factor of the geometric properties of microporous PCMs. In previous studies, the effect of pore size on cell behaviors was investigated at the single-cell level. Spreading of osteoblast-like cells (10) and fibroblasts (9) were progressively inhibited with increasing pore sizes (0.2–8.0 µm) on PCMs. We demonstrated that these phenomena could also be extended to the multi-cell level. In the present experiment, as a pore size increased, SHs tended to form colonies coinciding with the micropores on PCMs, suggesting that spreading of SH colonies was also inhibited with increasing pore sizes (5.0–12.0 µm) on PCMs. The “N_experimental/N_control” value, which represents the correlation between micropores and colony outlines, was significantly higher on PCMs with >10 µm-micropores than that with 5 µm-micropores (Fig. 1). This is probably due to surface discontinuities produced by the micropores. When cells are cultured on PCMs with smaller micropores, the cells can override the surface discontinuities, resulting in no correlation between the cell movement and micropore distribution. On the other hand, when cells are culture on PCMs with larger micropores, the cells fail to override the micropores because of the large discontinuities, resulting in cell movement along the micropores.

The spacing between adjacent micropores is another important factor of the geometric properties of microporous PCMs. Some evidence suggests that spacing of mechanical obstacles is a critical parameter of cell motility. Neutrophil motility was sensitive to the spacing (6–14 µm) between 2 µm-holes on a culture surface (13). The motility and proliferation of fibroblasts were also sensitive to the spacing (20–40 µm) between 7–25 µm-pits on a culture surface (15). In this study, spacing between micropores was successfully fabricated on PCMs in the range from 20 µm to 100 µm by the laser-ablation technique. The correlation between SH morphogenesis and micropore distribution was often observed on the PCMs with smaller spacing micropores. SHs formed colonies along the micropatterned pores with <40 µm spacing. Time-lapse movies revealed that the movements of SH colonies were also correlative to the micropatterned pores. In contrast, the cells formed colonies regardless of the micropores with >50 µm spacing because the cells could protrude onto the space between the micropores to expand the colonies.

The spacing between adjacent micropores appeared to be important, especially in the movement of SH colonies. Time-lapse microscopy revealed that cells at the colony edge shifted from pore to pore. The cells appeared to search for a place to shift by extending protrusions while they were trapped on micropores with focal complexes. The cells then moved to the adjacent micropores when the protrusions encountered the adjacent micropores. When the spacing between micropores was smaller, cells could easily find the place to shift, and move using micropores as anchor points. On the other hand, cells failed to shift the adjacent micropores with larger spacing. Similar movements have been reported for a single endothelial cell with stress fibers anchored at the periphery of adhesive area where focal adhesions formed, and spread from the area to the adjacent one (16).
4.2 SH colony formation and movements regulated by focal complexes

Time-lapse microscopy revealed that SH colonies tended to move along micropatterned pores. Since SEM and fluorescent images showed that the cells in the peripheral region of the SH colonies protruded filopodia-like structures into the micropores, the cell–substrate interaction around micropores might induce the movement along the micropatterned pores. Filopodia are protruding cell structures composed of parallel bundles of actin filaments and various actin-associated proteins, and they play important roles in cell migration by acting as sites of signal transduction (17). In fact, the immunofluorescent staining of vinculin revealed that focal complexes localized in the peripheral region of SH colonies coinciding with micropores.

Cell migration is a highly integrated multi-step process (18,19). The initial response of a cell is to polarize and extend protrusions (e.g., filopodia) in the direction of migration. The protrusions are then stabilized by adhering to the substrate. The initial integrin–substrate adhesions become capable of exerting migration force with the recruitment of vinculin, a marker for focal complexes, which are precursors of focal adhesions (20). The cells generate traction force at the adhesion sites and detach from the substrate at the cell rear to move forward. The movement of SH colonies in the present study can be explained by the process. The cells in the peripheral region of SH colonies protrude filopodia into micropores when the colony approach the micropatterned pores. Focal complexes are formed at the filopodia and the cell–substrate adhesion is stabilized by the focal complexes around the micropores. As a result, the movement of the SH colony was limited by strong cell–substrate bond around the micropores, resulting in the movement along the micropores.

Interestingly, SHs formed colonies on mPCMs and moved as groups rather than as single cells. When the cells in a peripheral region of an SH colony encountered the micropatterned pores, the colony moved along the micropores. Although only the peripheral cells received the geometric information of the micropatterned pores, the other cells in the colony kept the cell–cell contacts, resulting in collective cell movements. This movement is called "collective cell migration", which is an important cell behavior in tissue formation (21,22). Although cell migration has been studied extensively at the single-cell level, the mechanism of the multi-cellular movement only begins to be understood. In collective migration, there are two distinct types of epithelial cells; one is the leader cell that appears at the colony edge, and anothers are follower cells that move with the accompanying neighboring cells (23,24). Substrate rigidity is also important for collective migration since the collective cell movements were observed on an elastic gel substrate but not on a rigid substrate (25). In the present experiments, we demonstrated that the geometric properties of the substrate were also important in the collective cell movements. The leader cells at a colony edge might be formed correlating with the micropatterned pores. The follower cells within the colony gave rise to collective cell movements in a self-organized manner.

While many studies have been focusing on regulation of cell behaviors by chemical properties, physical properties of culture surfaces also influence cell behaviors. In particular, cell behaviors on different surface geometries are important since porous scaffolds are often used for the application of tissue engineering. Our findings indicate that >10 µm-micropores can be recognized by SHs and the spacing between the micropores plays a significant role in the formation and movement of SH colonies. The cells exhibited collective cell movements correlating with the micropores. These findings will be useful for designing geometric properties of scaffold materials that achieve the preferred cell guidance. This study may contribute not only to SHs but also to the other epithelial cell types.
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