ORIGINAL ARTICLE

Optimized Microwell Array Device for Preparation of Hair Follicle Germ-like Aggregates

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Abstract
Hair follicle morphogenesis is triggered by reciprocal interactions between epithelial and mesenchymal layers in the hair follicle germ. Techniques to create follicle germ-like structures *in vitro* may be useful for understanding hair follicle morphogenesis and for creating transplantable tissue for hair regenerative medicine. Microwell arrays with oxygen-permeable materials have been proposed as a promising tool for *in vitro* fabrication of hair follicle germ. In this study, we investigated the effects of microwell morphology and culture process on the formation and function of hair follicle germ. Microwell geometries (flat, round-bottom, pen, and cone) significantly affected the formation of hair follicle germ and the expression of hair growth-related genes. Specifically, pen-type microwells enhanced the rate of follicle germ formation by promoting cell assembly. Even in typical round-bottom wells, centrifugal filling of cells into the microwells enhanced cell assembly and follicular germ formation rates. The higher the efficiency of two cell types to form two separate follicular germ, the higher the expression of hair growth-associated gene, *versican*. The highest expression of *versican* was observed when cells were centrifugally packed in round-bottom wells. Aside from hair follicle formation, these microwell array with various shapes may be useful for producing various other organoids and tissues.

Key words: *hair follicle germ, PDMS, epithelial-mesenchymal interactions, microwell structure, centrifugation*

Introduction

Epithelial-mesenchymal interactions trigger early morphogenesis in a variety of tissues, including hair follicles, stomach, kidney, lung, lacrimal gland, and teeth (Kim and Shivdasani, 2016; Pleniceanu *et al*., 2010; Shannon and Hyatt, 2004; Yao and Zhang, 2017; Thesleff, 2003). Recently, studies have reported *in vitro* approaches that allow for the creation of protoplast-like structures via self-assembly of epithelial and mesenchymal cells (Koike *et al*., 2019; Tanaka *et al*., 2018; Yamamoto *et al*., 2015; Toyoshima *et al*., 2012). Elucidating the key mechanisms underlying the process of protoplast self-assembly via epithelial-mesenchymal interactions is key to understanding the development of complex tissues. In addition, the generated protoplasts will be useful in drug evaluation and in the application of transplanted tissues in regenerative medicine (Clevers, 2016; Nakamura and Sato, 2018). Especially in the field of drug discovery, culture models that reproduce *in vivo* functions stand as an alternative to animal experiments as they fulfill the 3R principle (*i.e*. minimizing the use of animals in research by replacement, reduction, and refinement of animal testing). For this purpose, a technology is required to produce protoplasts in a controlled *in*

vitro environment with high efficiency and reproducibility.

Follicular morphogenesis is initiated by triggering the formation of the follicular germ, which is an aggregation and junction of epithelial and mesenchymal cells (Millar, 2002). Since the formation of the follicular germ activates epithelial-mesenchymal interactions, studies have attempted to reproduce this *in vitro* (Toyoshima *et al*., 2012; Ehama *et al*, 2007; Ohyama and Veraitch, 2013; Kageyama *et al*., 2018). For example, an organ culture method has been proposed in which epithelial and mesenchymal cell pellets are prepared separately by centrifugation and then joined using a pipette to induce epithelial-mesenchymal interactions. It has been reported that transplantation of this follicle germ-like structure into mouse back skin can efficiently regenerate hair follicles (Toyoshima *et al*., 2012). However, this technique is currently limited by the need to manually fabricate hair follicle germ (HFG) under a microscope. Thousands to tens of thousands of HFG are required for drug screening for drug discovery and for use as transplanted tissues in hair regenerative medicine. Therefore, there is a need for a technology that is not only highly efficient and reproducible but also capable of producing a large quantity of HFG at a time.

One method to form spheroids in large quantities is to agglutinate cells by culturing them on low-cell-adhesion treated flat petri dishes. However, in this method, it is difficult to control the size of spheroids due to variation in the number of cells when forming cell aggregates (Liu *et al*., 2021). Therefore, microwell plates that can reproducibly form spheroids of uniform size have been produced. When cell suspensions are poured into these plates and allowed to stand, a uniform number of cells enter each microwell by gravity, allowing the preparation of large quantities of uniform diameter spheroids formed with a fixed number of cells at a time. There are various microwell structures such as hemispherical, U-bottom, inverted pyramid, conical, and semispherical, and there are also various patterns of depth and opening width (Liu *et al*., 2021; Comley, 2017; Breslin and O'Driscoll, 2013; Tu *et al*., 2014). For example, EZSPHERE™, AggreWell™, and Elplasia plates are commercially available, but the appropriate microwell structure should be selected according to the application and scale of the culture (Kamatar *et al*., 2020).

We used microwell plates for the formation of follicular germ in a previous study (Kageyama *et al*., 2018). Epithelial and mesenchymal cells were mixed and seeded into laboratory-prepared microwell plates to form aggregates. The two cell types were initially randomly distributed within the aggregate, but within 3 days of culturing, they had spatially separated by self-organization, and a dumbbell-shaped HFG had formed as aggregate layers joined. The self-separated follicular germ showed highly efficient hair growth when transplanted into nude mice, which subsequently exhibited a normal hair cycle. This study used round-bottom microwells, and showed a relatively high rate of follicular germ formation, but structural variation was observed. Formation of more uniform follicular germ is considered important for evaluation models and drug screening. The effect of microwell structure on the structural uniformity and function of the formed HFG has not been investigated.

Centrifugal processes have also been investigated for spheroid formation by promoting cell aggregation (Breslin and O'Driscoll, 2013; Ivascu and Kubbies, 2006). For example, it was shown that spheroids of cancer and noncancer cells can be rapidly produced in 96-well plates with either round or conical bottoms (Ivascu and Kubbies, 2006). The application of this method to HFG culture may promote the formation of HFG at an early stage. As a result, maturation is expected to progress in the subsequent culture period. However, this process has not been investigated as yet.

Therefore, this study aimed to clarify the effect of the structure of microwells in the incubator and the centrifugation method on the structure and function of the HFG (Fig. 1). The results showed that the microwell shape and centrifugal seeding manipulation significantly affected the function of the follicular germ. The culture system established in this study provides a platform not only for understanding organogenesis and screening drug candidates but also for developing organ regeneration therapies.

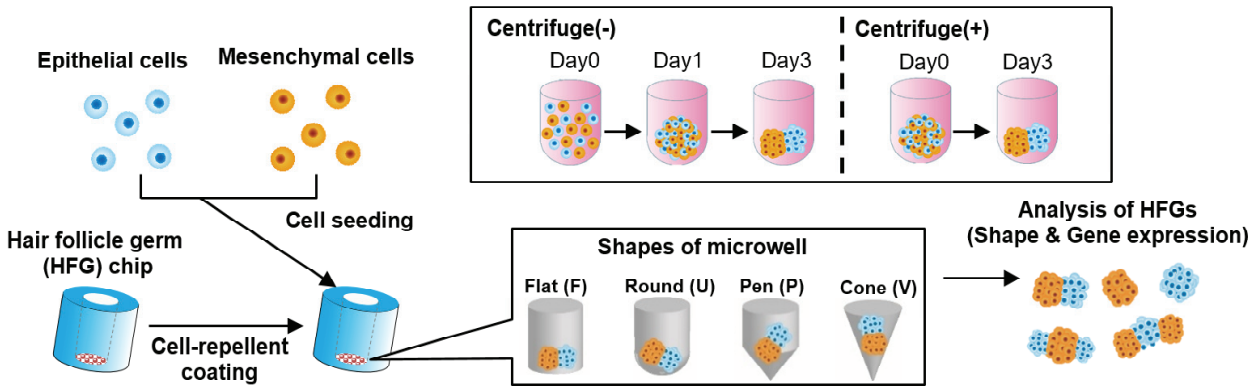

Figure 1 Schematics of microwell configurations for preparation of hair follicle germ-like aggregates. Microwell array cultures with either flat, round-bottom, pen or cone microwell geometries are prepared and seeded with mesenchymal and epithelial cells. Follicular germ shape and gene expression were evaluated for each microwell shape.

Materials and Methods

Materials and reagents

For HFG chip fabrication, we used a PMMA-Duralumin mold (Nissan Chemical Co., Japan), polydimethylsiloxane (PDMS) prepolymer solution and curing agent (SYLGARD 184 Silicone Elastomer Kit; Dow, USA), and a cell-repellent coating material (prevelex; Nissan Chemical Co., Japan).

The materials used for cell isolation, culture, and analysis were 40 mm mesh cell strainers (Corning, Corning, USA), phosphate-buffered saline (PBS; Thermo Fisher Scientific, USA), trypsin (Thermo Fisher Scientific, USA), penicillin-streptomycin mixed solution (Thermo Fisher Scientific, USA), Vybrant DiI cell-labeling solution (Thermo Fisher Scientific, USA), dispase II (Sigma Aldrich, USA), collagenase (Fujifilm Wako Pure Chemical Corporation, Japan)., epidermal keratinocyte growth medium-2 (KG2; Kurabo, Japan), Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, USA), fetal bovine serum (FBS; Sigma Aldrich, USA). Unless otherwise indicated, all other chemicals were purchased from Fujifilm Wako Pure Chemical Corporation, Japan.

Animals

Pregnant C57BL/6 mice were purchased from CLEA Japan (Japan). The animal study was approved by the Animal Care and Use Committee, Yokohama National University (Permit Numbers: 2020-04) All mouse care and handling were conducted in strict accordance with the requirements of the Animal Care and Use Committee of Yokohama National University, Kanagawa Institute of Industrial Science and Technology, and the Central Institute for Experimental Animals.

HFG chip fabrication

HFG chips with four microwell shapes (flat, round-bottom, pen, and cone) were fabricated via molding processes (Fig. 2B). Briefly, microwell array configurations (diameter, 1 mm; pitch, 1.3 mm; depth, 2 mm) in a 7 mm region were designed, and a positive mold was fabricated using PMMA-Duralumin substrate. A PDMS solution (10:1 mix of pre-polymer solution and curing agent) was poured onto the positive mold and cured in an oven (1 h, 100 °C). The thickness of the PDMS substrate at the floor of the microwells was set to 1.5 mm by adjusting the volume of the PDMS solution used.

Cell-repellent coating and analysis of wettability change

The surface of the HFG chip was cleaned with oxygen etching (5 mA, 3 min; SEDE-GE, Meiwa Fosis, Japan) and modified by dip coating with prevelex solution for 1 h to render it non-cell-adhesive. After baking at 50 °C for 24 h, HFG chips were washed with distilled water to remove excess coating materials. The substrate was exposed to 25 kGy of gamma irradiation for sterilization.

PDMS sheets were prepared and coated with prevelex using the same method as HFG chips. The changes in the surface wettability due to prevelex coating were quantified via water and air contact angles using a static contact angle goniometer (DMC-MC3, Kyowa Interface Science Co., Ltd, Tokyo, Japan). We conducted four independent repeats using each sample plate.

Spheroid formation and imaging

Human mesenchymal stem cells (Promocell, Germany) were used for the study of spheroid formation on HFG chips. After three days of culture, cells were harvested with 0.25% trypsin for 5 min at 37 °C and seeded on the polymer-coated round bottom HFG chips at 1×10^4 cells/well.

After one day of culture, the medium was aspirated, and replaced with an ionic liquid （HILEM IL1000; Hitachi High-Tech Co., Japan）diluted to 5% with distilled water. After drying at room temperature for three days, cross-sectional images were obtained with a scanning electron microscope (SEM; JSM-7400F, JEOL Ltd., Japan) at an acceleration voltage of 1.0 kV.

Effects of microwell depth

Human adipose-derived stem cells (ADSCs; Promocell, Germany) were used for the study of spheroid formation and microwell depth in HFG chips. After three days of culture, cells were harvested with 0.25% trypsin for 5 min at 37 °C, and 200 μL of cell suspension was seeded onto each polymer-coated round-bottom HFG chip (microwell depth; 1 mm and 2 mm) at 19×10^4 cells/well. After one day of culture, 150 μL of the medium was aspirated and 200 μL of new medium was added. Spheroids were observed using a phase-contrast microscope (CKX53, Olympus, Japan).

Centrifugation process for cell aggregation

ADSCs were used for the study of the promotion of cell aggregation using a centrifugation process. Cells prepared as mentioned above were seeded on the polymer-coated round-bottom HFG chips (microwell depth = 2 mm) at 19×10^4 cells/well. Within 5 min, centrifugation was performed at 1000 rpm for 1 min, then phase-contrast microscope observation was performed to observe cell aggregation.

Preparation of mouse epithelial and mesenchymal cells

Embryonic mice (E18) were extracted from C57BL/6 pregnant mice, and small pieces of their back skin were harvested. After aseptic treatment with 4.8 U / mL dispase II for 60 min, the epithelial and mesenchymal layers were separated using tweezers (Lichti *et al*., 2008). The epithelial layer was then treated with 100 U/mL collagenase type I for 80 min and 0.25% trypsin for 10 min at 37 °C. The dermal layer was treated with 100 U / mL collagenase type I for 80 min at 37 °C. Debris and undissociated tissues were removed using a 40 μm mesh cell strainer. After centrifugation at 1,200 rpm for 3 min, epithelial and mesenchymal cells were re-suspended in KG2 and DMEM, respectively. Freshly isolated cells were used for the experiments without passaging in culture. When the cells were mixed for co-culture, we used a mixed culture medium of DMEM and KG2 at a 1:1 ratio supplemented with 10% FBS and 1% penicillin-streptomycin.

HFG formation in various HFG chip microwell shapes

Mouse embryonic epithelial and Vybrant DiI-labeled mesenchymal cells were separately suspended at a density of 4.75×10^4 cells / 0.1 mL in DMEM / KG2 culture medium. An epithelial cell suspension and a mesenchymal cell suspension (of equal density) were then mixed and seeded in HFG chips with different microwell shapes (microwell depth = 2 mm). For the round-bottom HFG chips, centrifugation (1,000 rpm, 1 min) was performed after cell seeding. The self-organization of the two cell types in the HFGs was examined after three days using a fluorescence microscope (BZ-X810, Keyence, Japan).

Characterization of hair follicles by cross-sectional contact ratio.

Mouse mesenchymal cells were stained with Vybrant DiI-labeled solution to distinguish them from epithelial cells. HFG was cultured following the same protocol as described in section 2.7. After 3 days of culture, the hair follicles formed in each substrate were transferred to a 96 well plate and observed using a fluorescence microscope. The shape of hair follicles was analyzed by image analysis using ImageJ, as previously reported (Hoffecker and Iwata, 2014). The

Analysis was conducted to manually trace the perimeter of non-Vybrant-stained domains of epithelial cells as well as the perimeter of adjacent Vybrant and non-Vybrant domains. The ratio of contact perimeter to the epithelial cell perimeter was determined for each condition to yield the cross-sectional contact ratio, α.

Gene expression analysis

RNA was extracted from samples using an RNeasy mini kit (Qiagen, Netherlands), and cDNA was synthesized via reverse-transcription using a ReverTraAce RT-qPCR kit (Toyobo, Japan), according to the manufacturer's instructions. qPCR was performed using the StepOne Plus RT-PCR system (Applied Biosystems, Foster City, CA, USA), with SYBR Premix Ex Taq II (Takara-bio, Japan), and primers for the genes *versican* (GACGACTGTCTTGGTGG, ATATCCAAACAAGCCTG), *CD34* (TGGGTC AAGTTGTGGTGGGAA, GAAGAGGCGAGA GAGGAGAAATG), and *GAPDH* (AGAACAT CATCCCTGCATCC, TCCACCACCCTGTT G CTGT) (Kishimoto *et al*., 1999; Kageyama *et al*., 2019). All gene expression levels were normalized to that of *GAPDH*. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method and presented as the mean ± standard deviation of the four independent experiments. Statistical evaluation of numerical variables was conducted using Student's t-tests, and differences with p values of less than 0.05 were considered statistically significant.

Results and Discussion

Fabrication of microwells of different shapes

Culture vessels with microwells at the bottom have been fabricated for the culture of cell aggregates such as spheroids. The currently available shapes of microwells include round-bottom, inverted pyramid, V-bottom, and other similar shapes, mainly due to the convenience of fabrication. We have previously shown that HFG form spontaneously in culture vessels equipped with round-bottom microwells (Kageyama *et al*., 2018). These devices are made of PDMS to improve oxygen supply for spheroid culture. However, the effect of microwell geometry on the formation and function of follicular germ has not been investigated until now. In this study, we fabricated a culture device with PDMS microwell geometries of either flat, round-bottom, pen, or cone. The vessel can be inserted into a 24-well plate (Fig. 2A). The diameters of the top surface are all 1.0 mm, and 19 of them are regularly triangulated on the bottom with a diameter

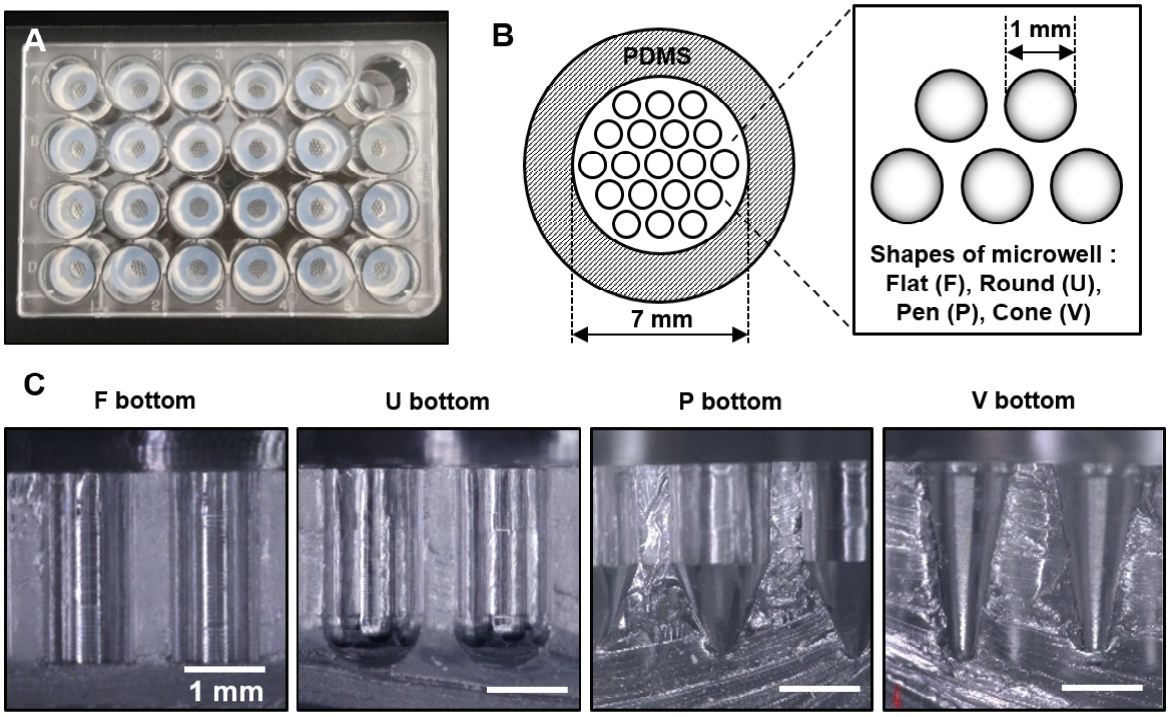

Figure 2 Fabrication of hair follicle germ (HFG) chips with different microwell shapes
a) HFG chips in a 24-well plate. b) Dimension of the HFG chips. c) Cross-section of each microwell type in the HFG chip. The depth of microwell was unified to 2 mm.

of 7.0 mm. Cross-sectional photographs of the microwells show that the microwell molds we developed were successful in forming microwells with each of the four shapes (Fig. 2C).

Wettability change of PDMS surface by prevelex coating

Biomolecular and cellular reactions on the substrate surface are closely related to surface wettability (Spriano *et al.*, 2017). In general, the higher the hydrophilicity of the surface, the stronger the suppression of cell adhesion. The hydrophilic and hydrophobic properties were evaluated before and after coating microwells with the adhesion-inhibiting material prevelex (Suzuki *et al.*, in press). As shown in Fig. 3A, the difference in water contact angle was small before and after coating. On the other hand, the bubble contact angle was significantly reduced by the prevelex coating. Since substrates for biomedical applications are generally used in wet environments, we considered that the bubble contact angle could be a better indicator of cell adhesion. The results suggest that PDMS substrates modified with prevelex can impart high hydrophilicity and prevent nonspecific adsorption of biomolecules and cells.

Spheroid formation in prevelex-coated round-bottom microwell vessels

Spheroid formation was evaluated by seeding cells onto the fabricated HFG chips. ADSCs seeded into prevelex-coated round-bottom microwells formed spheroids after one day of incubation, after which SEM observation was performed. For SEM observation of biological specimens, it is necessary to treat the specimens to make them conductive for electron beam scanning and resistant to vacuum conditions in the SEM specimen chamber. As shown in Fig.

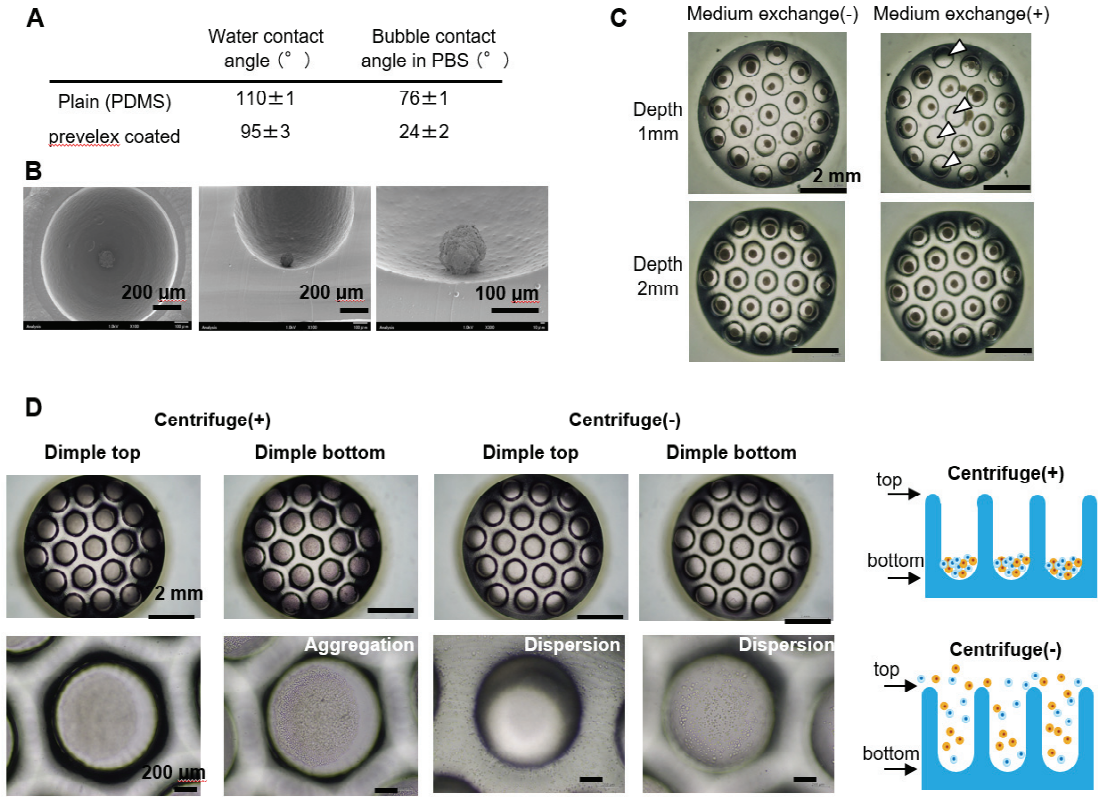

Figure 3 Cell-repellent coating and the evaluation of cell culture on hair follicle germ (HFG) culture chips.
a) Bubble and water contact angles with and without adhesion-preventing prevelex coating on polydimethylsiloxane substrates. The bubble contact angle is measured with a 2.0 µl air bubble in phosphate-buffered saline. The water contact angle is measured with a 2.0 µl pure water droplet in the air. Error bars represent the standard error calculated from four independent measurements.
b) Scanning electron microscopic images of human adipose-derived mesenchymal stem cell (ADSC) spheroids in the microwell of HFG culture chips. Cells are observed one day after seeding.
c) Microscopic images of ADSC spheroids formed in HFG chips (1 mm and 2 mm depths) before and after medium exchange. White arrows indicate microwell spheroid shedding.
d) Microscopic images immediately after seeding ADSCs into HFG chips in round-bottom microwells. The image was taken by focusing on the top and bottom of the microwell, either with or without centrifugation treatment.

3B, spheroids with a diameter of about 100 μm were formed at the bottom of the microwells, adjacent to the prevelex coating. Therefore, in subsequent experiments, all vessels were coated by prevelex.

The effects of microwell depth on spheroid formation

If the depth of the microwells is too shallow, their spheroids may detach and migrate from the wells due to the flow of medium during medium exchange or manipulation. This is undesirable due to observability issues and size variation caused by fusion with other spheroids. The depth of the wells is limited for ease of fabrication. We investigated the appropriate microwell depth by forming spheroids in HFG chips at different depths (1 mm and 2 mm) and examining their migration upon medium exchange. One ADSC spheroid was formed in each microwell at both 1 and 2 mm depths (Fig. 3C). Before and after medium exchange, some spheroids formed in the 1 mm deep microwells had migrated and were present in another microwell or the septum between microwells. On the other hand, in the microwells with a depth of 2 mm, no migration was observed and all spheroids remained in their respective microwells. The oxygen supply rate generally decreases with increasing microwell depth because the distance from the gas-liquid interface increases with increasing depth. However, in this device using oxygen-permeable PDMS, oxygen is supplied from the bottom of the culture medium, so there is no such concern, even in deep microwells. To keep the spheroids in the microwells, the depth of the microwells was set to 2 mm in the following experiments.

Examination of cell aggregation by centrifugal treatment

In our previous study, we found that HFG formation was most likely regulated by cadherin mediated cell-sorting (Kageyama *et al*., 2018). Therefore, if cadherin-mediated interactions are promoted by the proximity of both epithelial and mesenchymal cells, we predicted that they will have some effect on the formation of follicular germ. Centrifugation has previously been applied as a method for early cell assembly (Ivascu, 2006; Lichti *et al*., 2008). Therefore, we compared cell aggregation in HFG chips containing ADSC suspensions, which were either subjected to centrifugation or no centrifugation before culturing. Focusing on the bottom and top surfaces of the microwells, in centrifuged treatments few cells were present on the top surface of the microwells, and almost all cells had aggregated at the bottom (Fig. 3D). In contrast, in the no centrifugation treatments, cells were dispersed throughout the microwells (including the spaces between the microwells), with no aggregation at the bottom of the microwells. These results indicate that the cells assembled immediately after cell seeding using centrifugation. This suggests that centrifugation can promote cell-cell interaction.

Effects of microwell geometry on HFG formation

To evaluate the effect of microwell shape and centrifugation on HFG formation, epithelial and mesenchymal cells collected from mouse skin were seeded into four types of HFG chips having microwell shapes of either flat (F-type), round-bottom (U-type), pen (P-type), and cone (V-type), and HFG were cultured (Fig. 4A). For U-type microwells, centrifugation (±) was used. After germ formation, all microwell shapes contained one germl cell structure per well, except for the F-type, which contained multiple cell structures per well. When the shape of the structures was observed, we identified many dumbbell-shaped HFG in which the epithelial system and the mesenchymal system were separated. This feature is characteristic of the true HFG. The U-, P-, and V-type microwell shapes are considered to form follicular germ with high efficiency because cells tended to assemble at the bottom of the microwells.

The uniformity of the obtained cellular structures was then evaluated by image analysis (Fig. 4B). We found that a variety of structures were formed in the F-type microwells, with a large variation in shape. On the other hand, many dumbbell-shaped follicular germ with small α values were observed in all other microwell shapes. In particular, the P- and U-type microwells in combination with centrifugal treatment showed a high structural uniformity of the follicular germ. Considering the structure and volume of the microwells, we expected that cells assemble more easily in the order of F<U<P<V. Essentially, in narrow microwells, cells assemble more easily, facilitating cell-cell interaction and thus HFG formation. However, the volume of culture medium in

narrow microwells is lower, and circulation is more difficult, which may lead to nutrient deprivation. Therefore, we speculate that the narrow structure of microwells such as V-type microwells may not provide sufficient amounts of culture medium for the number of cells, resulting in damage to the cells and a decrease in the structural uniformity.

In the case of the U-shaped microwell structure, centrifugation improved the structural uniformity of the follicular germ compared to no centrifugation. The enhancement of initial cell aggregation during seeding may have contributed to the formation of the follicular germ.

Confirmation of correlation between gene expression and shape evaluation

We confirmed the correlation between the structure of the HFG and the expression levels of genes related to hair growth (Fig. 4C). The expression level of *versican* in mesenchymal cells (an index of hair growth efficiency) was particularly low in F-type microwells, in which dumbbell-shaped HFG did not form. We found a positive correlation between the expression of *versican* and the proportion of structures with a lower cross-sectional contact ratio (α) ($R^2 = 0.91$ for the plots without Uc). Among microwell types, the U-type microwell with centrifugation had the highest *versican* expression level. This may be due to the fact that centrifugation process promoted the cells to assemble quickly, and promoted the formation and maturation of HFG. The expression levels of *CD34*, a marker of hair follicle stem cells (Chacón-Martínez *et al*, 2017), were similar in all microwell types except for the F-type, indicating that they could retain their undifferentiated nature by forming follicular germ. Meanwhile, F-type wells likely did not form follicular germ and promoted dedifferen-

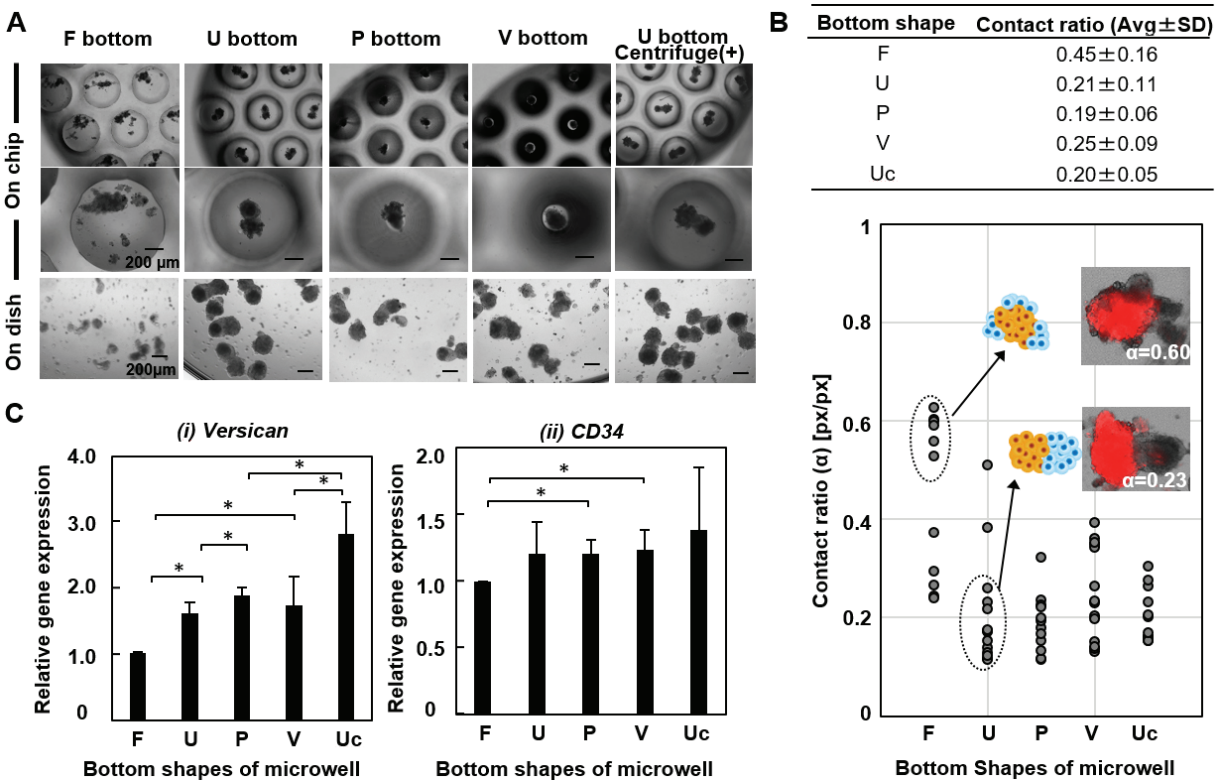

Figure 4 Preparation and analysis of hair follicle germ (HFG) formation.
a) HFG formation on HFG chips with flat, round-bottom, pen, or cone-shaped microwells, and with round-bottom microwells after centrifugation. Mesenchymal cells (5.0×10^3 cells) and epithelial cells (5.0×10^3 cells) were seeded in the HFG chip. The microscopic images of HFGs on chips, and HFGs transferred to a 96 well flat bottom plate after three days of culture.
b) Image analysis of coaggregate cross-sections was used to quantify the cohesive interaction between epithelial cells and Vybrant DiI stained mesenchymal cells in microwells of different shapes. Confocal cross-sections of day-3 coaggregates were analyzed to obtain the cross-sectional contact ratio (α), which was high in the case of core-shell configurations, low in the case of multisphere configurations, and intermediate for coaggregates exhibiting mixed configuration.
c) Expression of hair generation-related genes, normalized to that of *GAPDH*. Error bars represent the standard deviations calculated from three independent experiments. Numerical variables were statistically evaluated via Student's t-tests (*$p < 0.05$).

tiation. This suggests that after transplantation, differentiation of the hair follicles is influenced by the surrounding environment and can induce hair regrowth.

Conclusions

In this study, we examined the effect of incubator microwell shape and centrifugation in HFG culture and found a method to form uniformly structured HFG *in vitro*. In terms of microwell shape, the formation rate of follicular germ was higher in pen-shaped microwells, which promote cell aggregation and do not limit access to nutrients in the culture medium. The highest formation rate of HFG was observed in the round-bottom microwells where cells were initially assembled by centrifugation. Furthermore, the expression level of *versican*, an indicator of hair growth efficiency, was positively correlated with HFG formation rates. Microwell cultures with various shapes may be useful for the preparation of various organoids, including but not limited to the formation of HFG. The homogeneous HFG prepared by this method may be suitable as a model for studying the mechanism of hair regeneration.

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Conflict of Interest

K. Suzuki and Y. Hiroi are employees of Nissan Chemical Corporation. This work was partially funded by Nissan Chemical Corporation. This work was performed under the condition of an Invention Agreement among Yokohama National University, Kanagawa Institute of Industrial Science and Technology, and Nissan Chemical Corporation.

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