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# Preparation of hair beads and hair follicle germs for regenerative medicine

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ABSTRACT

Hair regenerative medicine is a promising approach for hair loss, during which autologous follicular stem cells are transplanted into regions of hair loss to regenerate hairs. Because cells transplanted as a single cell suspension scarcely generate hairs, the engineering of three-dimensional (3D) tissues before transplantation has been explored to improve this process. Here, we propose an approach to fabricate collagen-enriched cell aggregates, named hair beads (HBs), through the spontaneous constriction of cell-encapsulated collagen drops. Mouse embryonic mesenchymal cells or human dermal papilla cells were encapsulated in 2-μl collagen microgels, which were concentrated > 10-fold in volume during 3 days of culture. Interestingly, HB constriction was attributed to attraction forces driven by myosin II and involved the upregulation of follicular genes. Single HBs with epithelial cells seeded in U-shaped microwells formed dumbbell-like structures comprising respective aggregates (named bead-based hair follicle germs, bbHFGs), during 3 days of culture. bbHFGs efficiently generated hair follicles upon intracutaneous transplantation into the backs of nude mice. Using an automated spotter, this approach was scalable to prepare a large number of bbHFGs, which is important for clinical applications. Therefore, this could represent a robust and practical approach for the preparation of germ-like tissues for hair regenerative medicine.

## 1. Introduction

Hair loss generally occurs due to various causes such as genetics, aging, hormonal imbalances, autoimmune reactions, and anti-cancer drug medications, and this is linked to the loss of stem cells responsible for normal hair formation and hair cycling [1]. Current treatments for hair loss mainly rely on drugs and autologous hair transplantation, but both approaches are associated with some difficulties such as the limited effects of drugs and the inability to increase hair numbers in the scalp [2].

Hair follicle formation during embryonic development and postnatal hair cycling is governed by a series of interactions between epithelium-derived follicular stem cells (e.g. follicular epithelial stem cells and bulge epithelial stem cells) and mesenchymal-derived follicular stem cells (e.g. dermal papilla (DP) cells, follicular dermal cells) [3–5]. Recent studies revealed that co-transplantation of these stem cells results in the efficient generation of hair follicles and hairs [6–8]. Follicular epithelial stem cells differentiate and eventually form a hair shaft. DP cells provide signals to follicular epithelial stem cells, specifying the size, shape, and pigmentation of hair shafts [9]. However, the hair induction ability of both cell types is gradually lost after isolation from *in vivo* tissues and during expansion culture [10,11]. Thus, various approaches to maintain this ability have been examined, including the use of growth factors and signaling molecules. For example, the loss of DP cell hair induction ability was alleviated by fibroblast growth factor-2 [10,12], Wnt [13,14], and bone morphogenetic protein [15]. Another approach is to fabricate 3D tissues prior to transplantation [16–19]. DP cells form aggregates when cultured in hanging drop or non-cell adhesive culture conditions and exhibit improved expression of DP-specific markers. Recent studies have shown that a compartmentalized hair follicle germ (HFG), which was fabricated by integrating two respective 3D aggregates of mesenchymal and epithelial cells *in vitro*, could efficiently regenerate hair follicles on the back skin of mice [20]. This is a sophisticated approach to recapitulate embryonic development, but the challenge associated with this approach is the preparation of a large number of HFGs necessary for a human treatment (> 3000 HFGs/patient). This is because the two aggregates were manually merged with a pipette under a microscope with this approach.

We have recently reported an approach to prepare a large number of HFGs [21]. In our approach, a mixture of epithelial and mesenchymal cells was seeded in a lab-made microwell array plate and allowed to form aggregates in microwells. The two types of cells were initially randomly distributed in individual aggregates, but spontaneously and spatially separated from each other and formed a compartmentalized

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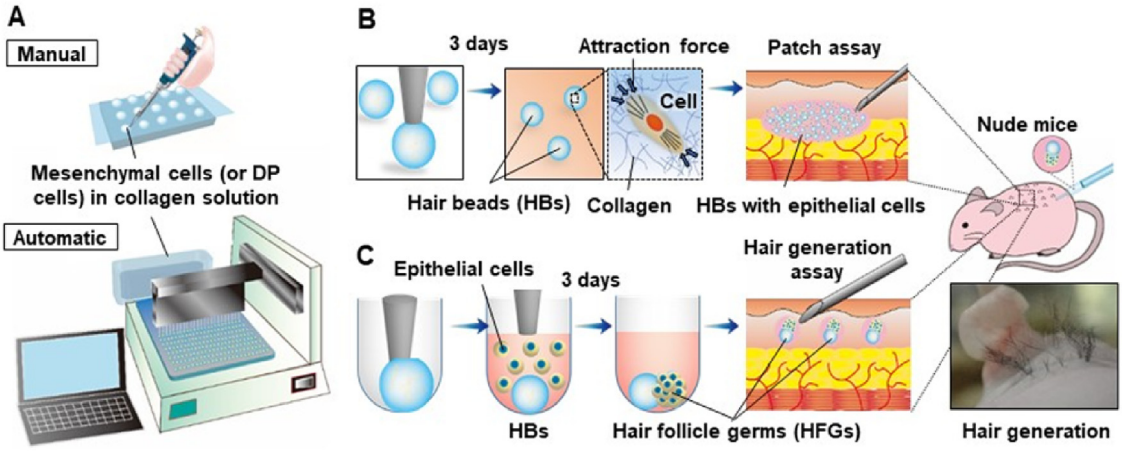


**Fig. 1. Schematic of the preparation of hair beads (HBs) and hair follicle germs (HFGs).** (A) Manual and automatic approaches to fabricate 2-μl collagen microgels containing mesenchymal cells. DP, dermal papilla cells. (B) Formation of HBs through the spontaneous constriction of collagen microgels by cell attraction forces, and the evaluation of their trichogenous activity using the patch assay. (C) Formation of an HFG composed of an HB and epithelial cell aggregate in a U-shaped microwell, and evaluation of trichogenous activity using hair generation assays.

HFG. Because the cells self-organized into compartmentalized HFGs, this approach was scalable for the simultaneous preparation of > 5000 HFGs. In the present study, considering that during embryonic development, an epithelial aggregate invaginates into a collagen-rich mesenchymal layer and triggers subsequent morphogenetic changes and hair follicle generation [5,22], we hypothesized that a collagen-rich mesenchymal cell aggregate might be a beneficial component of HFGs. Collagen-rich mesenchymal cell aggregates, named hair beads (HBs), were prepared through the contraction of mesenchymal cell-encapsulated collagen microgels (Fig. 1A). We then examined whether HBs could induce improved trichogenous functions *in vitro* and *in vivo* as compared to HFGs prepared with previous approaches (Fig. 1B). Furthermore, we investigated whether the HB approach could be used for the mass preparation of HFGs using an automated micro-dispenser (Fig. 1C). The fabricated HFGs were also evaluated for their ability to generate hairs on the back skin of nude mice. This could represent a practical germ-like tissue preparation approach for hair regenerative medicine.

## 2. Materials and methods

### 2.1. Materials and reagents

The reagents used for cell isolation, culture, and analysis were as follows: cell strainer (40-μm mesh) from BD Biosciences (USA); phosphate-buffered saline (PBS), trypsin, penicillin-streptomycin mixed solution, 1% bovine serum albumin (BSA), Triton X-100, Vybrant DiI cell-labeling solution, collagen I monoclonal antibody, vimentin polyclonal antibody, and goat anti-rabbit IgG (H + L) Alexa Fluor 488 highly cross-absorbed from Thermo Fisher Scientific Inc. (USA); cytokeratin 14 polyclonal antibody and CD34 polyclonal antibody from Abcam (UK); dispase II, ethidium bromide, 4% formaldehyde in PBS, 10% formaldehyde neutral buffer solution, *p*-nitrophenyl phosphate, *p*-nitrophenylate, and *t*-butyl alcohol from Wako Pure Chemical Industries, Ltd. (Japan); 3.0 mg/mL collagen type I-A solution, Ham's F12 medium and reconstitution buffer solution (0.05 N NaOH, 200 mM HEPES, and 2.2% $NaHCO_3$) from Nitta Gelatin (Japan); non-cell adhesive dish (Primesurface® 35 mm dish) and non-cell-adhesive round-bottom 96-well plate (Primesurface® 96U plate) from Sumitomo Bakelite, Ltd. (Japan); epidermal keratinocyte growth medium-2 (KG2) from Kurabo (Japan); human DP cells (passage 2), follicle dermal papilla cell growth medium kit (DPCGM) from Promocell (Germany); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 25% glutaraldehyde, fast blue RR salt, 3-hydroxy-N-(3-nitrophenyl)-2-naphthamide (Naphthol AS-BS), and 4,6-diamino-2-phenylindole (DAPI) from Sigma Aldrich (USA); rhodamine-phalloidin from Cytoskeleton Inc. (USA). All other chemicals were purchased from Wako Pure Chemicals, unless otherwise indicated.

### 2.2. Animals

Pregnant C57BL/6 mice were purchased from CLEA (Japan). ICR nude mice (5-weeks-old) were purchased from Charles River (Japan). The care and handling of mice conformed to the requirements of the animal care and use committee of Yokohama National University.

### 2.3. Preparation of mouse epithelial and mesenchymal cells and human DP cells

Embryonic mice (E18) were extracted from a C57BL/6 pregnant mouse and small pieces of their back skin were harvested under a surgical microscope (Mantis Elite, Vision Engineering, UK). After aseptic treatment with 4.8 U/ml dispase II for 60 min, the epithelial and mesenchymal layers were separated using tweezers [23]. The epithelial layer was then treated with 100 U/ml collagenase type I twice for 40 min and 0.25% trypsin for 10 min at 37 °C. The dermal layer was treated with 100 U/ml collagenase type I twice for 40 min at 37 °C. Debris and undissociated tissues were removed with a cell strainer. After centrifugation at 1000 rpm for 3 min, the epithelial and mesenchymal cells were re-suspended in KG2 and DMEM, respectively. The freshly isolated cells were used for experiments without passaging in culture. When these 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 (DMEM/KG2).

Human DP cells were maintained with DPCGM and the medium was changed every 2–3 days. Cells at passage 4 were used for experiments. For the co-culture of DP cells with mouse epithelial cells, a mixed culture medium of DPCGM and KG2 at a 1:1 ratio (DPCGM/KG2) was used.

### 2.4. Preparation of HBs

A collagen solution was prepared by mixing 0.8 ml of collagen type I-A, 0.1 ml of 10-fold concentrated Ham's F12 medium, and 0.1 ml of reconstitution buffer solution on ice. The resulting concentration of the collagen solution was 2.4 mg/ml at pH 7. Mouse mesenchymal cells or human DP cells were then gently mixed in the collagen solution on ice. The collagen solution containing the cells was spotted as 2-μl droplets

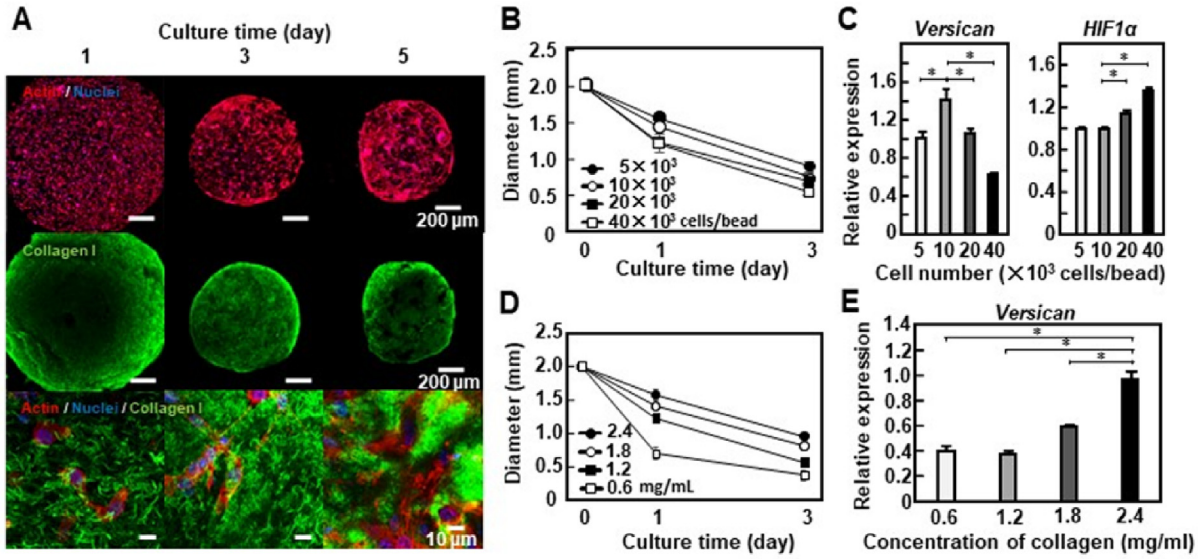


**Fig. 2. Characterizations of hair beads (HBs).** (A) Fluorescence microscopic images of HBs. Actin (red), nuclei (blue), and collagen type I (green) were visualized at 1, 3, and 5 days of culture. (B) Quantitative analysis of changes in the diameter of collagen microgels. Cells were encapsulated at four different densities in 2.4-mg/ml collagen microgels. (C) Dependence of *versican* and *HIF1α* gene expression on cell density. (D) Changes in the diameter of microgels with four different collagen concentrations. Cells were encapsulated in microgels at $1 \times 10^4$ cells/ml. (E) Dependence of *Versican* expression on collagen concentration. Error bars in (B) and (D) represent the standard error of the means calculated from eight images for each condition. In (C) and (E), all samples were normalized to levels of the reference gene *GAPDH*. Error bars represent the standard error of means calculated from three independent experiments for each condition. Numerical variables were statistically evaluated by a Student's t-test. $*p < 0.05$ was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on a flat surface using an electromotive pipette (Multipette® E3, Eppendorf, Germany) or a micro-dispenser (EDR-24LS, Biotech, Japan) and incubated for 30 min at 37 °C for gelation (Fig. 1A). The microgels, termed hair beads (HBs), were suspended and incubated in culture medium in a non-cell adhesive dish. After 1, 3, and 5 days of culture, immunohistochemical staining for type I collagen and rhodamine-phalloidin/DAPI staining were conducted to characterize HBs with a confocal laser scanning microscope (LSM 700, Carl Zeiss, Germany). The diameter of HBs was quantified from phase-contrast images using Image J software.

The dependence of mouse mesenchymal cell trichogenous functions on cell density and collagen concentration in HBs was investigated using four different cell densities (0.5, 1, 2, and $4 \times 10^4$ cells/HB) in 2.4 mg/ml collagen gels, and with four different collagen concentrations (0.6, 1.2, 1.8, and 2.4 mg/ml) and $1 \times 10^4$ cells/HB. Alkaline phosphatase (ALP) activity was quantified at 3 days of culture. The expression of a trichogenous gene (*versican*) was evaluated by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis at 3 days of culture (as described in a following section). For comparisons, the cells were cultured as spheroids on a non-cell-adhesive round-bottom 96-well plate and/or as a monolayer on a conventional culture dish, and the same evaluations were carried out.

### 2.5. Effect of myosin II ATPase inhibitor on HB formation

The effects of the myosin II ATPase inhibitor blebbistatin on the constriction of microgels and *versican* expression were evaluated. Mouse mesenchymal cells and DP cells ($1 \times 10^4$ cells/HB) were encapsulated in collagen microgels (2.4 mg/ml) suspended in culture medium containing 30 μM blebbistatin and then seeded on a 96-well round-bottom well plate. After 1 and 3 days of culture, changes in microgel diameters were observed using a phase-contrast microscope. The microgels at 3 days of culture were fixed with 4% formaldehyde in PBS, stained with rhodamine-phalloidin and DAPI, and observed using a confocal laser scanning microscope. The relative expression of *versican* was evaluated by RT-PCR.

### 2.6. Patch assays

The hair induction ability of HBs was quantified using the patch assay [11]. HBs were prepared by encapsulating $1 \times 10^4$ cells per HB in 2.4 mg/ml collagen microgels and culturing them in a 35 mm non-cell-adhesive dish for 3 days. A total of 30 HBs containing mouse mesenchymal cells or 50 HBs with human DP cells were mixed with a suspension of freshly-isolated mouse embryonic epithelial cells ($3.0 \times 10^5$ cells/ml or $5.0 \times 10^5$ cells/ml, respectively), and this was then centrifuged (1000 rpm, 3 min) for aggregation. Under anesthesia, the pellet was transplanted into a pocket (~5 mm diameter) that was surgically created on the dorsal skin of ICR nude mice. For comparison, spheroids with the same number of cells were prepared in a non-cell-adhesive round-bottom 96-well plate, centrifuged to generate pellets, and transplanted into mice with the epithelial cells. Four weeks after transplantation, transplanted sites were observed using a digital microscope (VHX-1000; Keyence Co., Japan) and digital camera (Tough; Olympus Co., Japan). A small piece of skin was treated with 100 U/ml collagenase at 37 °C for 2 h to dissociate the generated hair shafts from the skin. Then, the number of hair shafts per transplantation site was counted. The diameters of hair shafts were examined using a scanning electron microscope (Miniscope®; HITACHI) at 10–15 kV.

### 2.7. Preparation of HFG-like aggregates

To distinguish cell types, mouse mesenchymal cells were fluorescently labeled with Vybrant DiI and suspended in a 2.4-mg/ml collagen solution at a density of $5 \times 10^3$ cells/μl. The collagen solution was spotted with a pipette as 2-μl droplets on a non-cell-adhesive round-bottom 96 well plate and incubated for 30 min at 37 °C for gelation. Then, $1 \times 10^4$ epithelial cells in 100 μl of DMEM/KG2 culture medium were seeded into the well and cultured for 3 days to fabricate bbHFGs. For comparisons, a mixture of epithelial and fluorescently-labeled mesenchymal cells at the same density was seeded into the well and cultured for 3 days to fabricate ssHFGs as previously reported [21]. In addition, epithelial cells and fluorescently-labeled mesenchymal cells

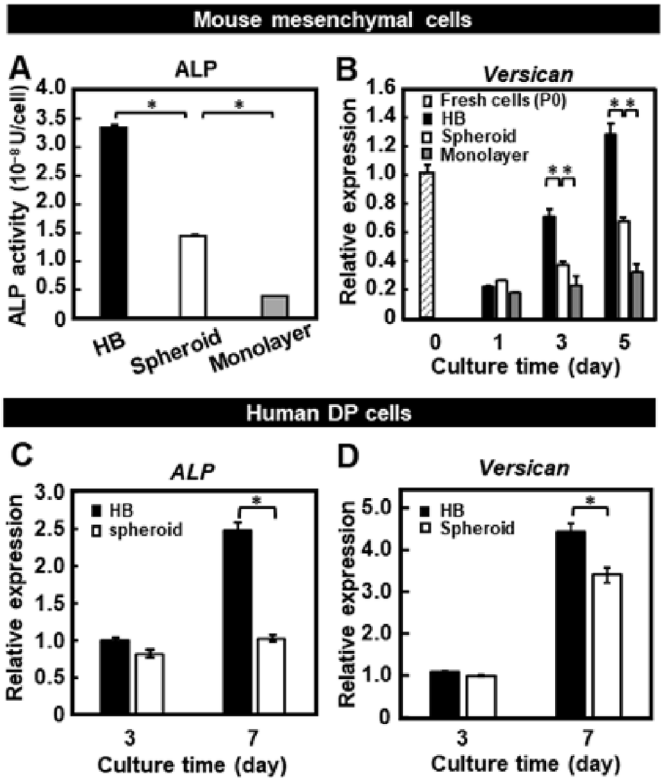


**Fig. 3. Comparison of the expression of hair induction marker genes in hair beads (HBs) and spheroids.** (A) Alkaline phosphatase (ALP) activity in mouse mesenchymal cells in the three different cultures on 3 days of culture. (B) Changes in *versican* gene expression in mouse mesenchymal cells. (C, D) Changes in *ALP* and *versican* gene expression in human dermal papilla (DP) cells. All RT-PCR data were normalized to levels of the reference gene *GAPDH*. Error bars represent the standard error of means calculated from three independent experiments for each condition. Numerical variables were statistically evaluated by a Student's t-test. $^{*}p < 0.05$ was considered significant.

were suspended in a collagen solution simultaneously, spotted as 2-μl droplets, and cultured for 3 days to prepare microgels containing the two cell types (termed HBs(e + m)). The expression of genes associated with hair follicle induction (*versican*, *Wnt10b*) was evaluated by RT-PCR analysis.

### 2.8. Hair generation assays

Single bbHFGs, ssHFGs, and HBs(e + m) were transplanted into each shallow stab wound on the back skin of nude mice, prepared using a 20 G ophthalmic V-lance (Alcon, Japan). Transplantation sites were then rubbed with an ointment. The mice were subsequently maintained under specific pathogen-free conditions with feeding ad libitum for up to 3 weeks depending on the experiment. Transplanted sites were observed every 2–3 days and images of generated hairs were taken with a digital microscope (VHX-1000, Keyence) and digital camera (Tough, Olympus). The number of hairs per transplanted site was evaluated based on these images after 3 weeks of transplantation. Cuticles of generated hair shafts were observed with a scanning electron microscope at 5–10 kV without any treatment.

### 2.9. ALP activity assays

To quantify ALP activity, cultures were washed with PBS and treated with 0.2% triton-X solution. Cells were then lysed with an ultrasonicator for 30 s. The cell lysates were centrifuged at 1300 rpm for 10 min, and 20 μl of supernatant was mixed with 100 μl of *p*-nitrophenyl phosphate solution. After incubation at 37 °C for 30 min, converted *p*-nitrophenylate was measured at an absorbance of 405 nm using a microplate reader (Sunrise, TECAN, Switzerland).

### 2.10. Histological and immunohistochemical staining

For histological staining, samples were fixed with 10% neutral-buffered formaldehyde solution overnight at room temperature and frozen sections (8-μm) were prepared. Then, the samples were stained with Meyer-hematoxylin and eosin (HE) Y solutions.

For immunohistochemical staining, samples were fixed with 10% formaldehyde neutral buffer solution overnight at room temperature and frozen sections (8-μm) were prepared. Then, the samples were treated with a blocking solution containing 1% BSA and 0.01% Triton X-100 in PBS for 1 h at room temperature, and subsequently incubated overnight with primary antibodies in blocking solution at 4 °C. The samples were further incubated with Alexa Fluor® 488-labeled goat anti-rabbit IgG antibodies in blocking solution for 3 h at room temperature. Fluorescence microscopic images were captured with a confocal laser scanning microscope.

### 2.11. Gene expression analysis

Total RNA was extracted from samples using the RNeasy mini kit (Qiagen, Netherlands) according to the manufacturer's protocol. cDNA was then synthesized by reverse transcription using the ReverTra Ace® qPCR RT kit (Toyobo, Japan). Quantitative PCR (RT-PCR) was performed with a real-time PCR system (StepOnePlus, Applied Biosystems, USA) using SYBR® Premix Ex Taq™ II (Takara-bio, Japan). Gene expression was evaluated using the following primers: Versican (mouse): GACGACTGTCTTGGTGG and ATATCCAAACAAGCCTG; HIF1α (mouse): CAGCTTCCTTCGGACACATAAG and CCACAGCAATGAAACC CTCCA; ALP (mouse): TCGGAACAACCTGACTGACCC and CTGCTTGG CCTTACCCTCATG; HEY1 (mouse): CACTGCAGGAGGGAAAGGTTAT and CCCCAAACTCCGATAGTCCAT; IGFBP5 (mouse): ATGAGACAGGA ATCCGAACA and TCAACGTTACTGCTGTCGAA; Wnt10b (mouse): CCAAGAGCCGGGCCCGAGTGA and AAGGGCGGAGGCCAGACCG; CD34 (mouse): TGGGTCAAGTTGTGGTGGGAA and GAAGAGGCGAGA GAGGAGAAATG; GAPDH (mouse): AGAACATCATCCCTGCATCC and TCCACCACCCTGTTGCTGTA; Versican (human): CCAGCAAGCACAAA ATTTCA and TGCACTGGATCTGTTTCTTCA; ALP (human): ATTGACC ACGGGCACCAT and CTCCACCGCCTCATGCA; GAPDH (human): GCA CCGTCAAGGCTGAGAAC and TGGTGAAGACGCCAGTGGA. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method and presented as the mean ± standard deviation of four independent experiments.

## 3. Results and Discussion

### 3.1. Preparation of HBs through dynamic microgel contraction

Various extracellular matrix components and their substitutes have been investigated to improve the survival, proliferation, and hair induction activity of DP cells *in vitro* [24–26]. Among them, collagen was shown to be one of the most effective components, probably because it is the most abundant protein in tissues including the skin [27–29]. In our preliminary experiments, mouse embryonic mesenchymal cells and human DP cells cultured on a collagen gel showed recovered activity of ALP (Fig. S1), which is a ubiquitous enzyme, but also a specific follicular indicator of the dermal layer [30]. Thus, in this study, we examined an approach to prepare tissue grafts by encapsulating follicular mesenchymal cells in collagen microgels (Fig. 1).

Collagen microgels (2 μl/microgel) containing mouse embryonic mesenchymal cells significantly contracted during 3 days of culture. The diameter decreased from 2.0 mm to 0.5 mm at its minimum when the initial collagen concentration was 0.6 mg/ml, indicating an ~64-fold enrichment in collagen concentration and cell density, given that

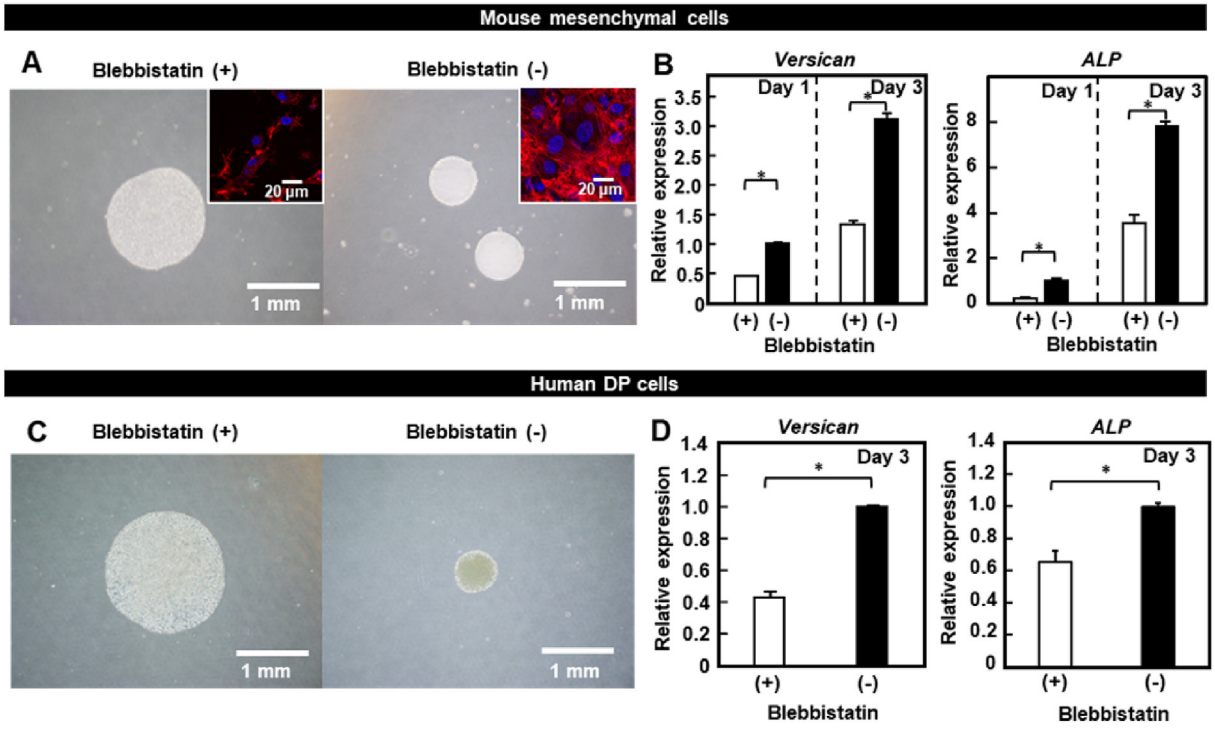


**Fig. 4. Relationship between microgel constriction and *versican* expression on mouse mesenchymal cells and human dermal papilla (DP) cells.** (A, C) Phase-contrast images of hair beads (HBs) after 3 days of culture with/without the myosin II inhibitor blebbistatin. The inserts show a magnified view of HBs visualized with rhodamine-phalloidin (red) and DAPI (blue). (B, D) Comparisons of *versican* and *ALP* expression with/without the inhibitor. All RT-PCR data were normalized to levels of the reference gene *GAPDH*. Error bars represent the standard error of means calculated from three independent experiments for each condition. Numerical variables were statistically evaluated by a Student's t-test. $^*p < 0.05$ was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

HBs are spherical (Fig. 2A). Further, the compaction was slightly dependent on the number of cells encapsulated (Fig. 2B). Although we expected that *versican* gene expression might be augmented with increasing numbers of cells encapsulated in HBs, due to the effects of cell–cell interactions, this parameter peaked at $10 \times 10^3$ cells/bead (Fig. 2C). This is most likely because of oxygen shortages with higher cell numbers since the gene expression of hypoxia induced factor 1α (*HIF1α*) was upregulated with higher cell numbers (Fig. 2C). The results are consistent with our previous report that *versican* and *HIF1α* gene expression levels are positively and negatively dependent on oxygen concentration, respectively [21]. The dependence on the initial collagen concentration for compaction and *versican* gene expression was also examined at the fixed cell density of $10 \times 10^3$ cells/bead. Changes in diameter were clearly dependent on the initial collagen concentration (Fig. 2D). The most significant change was observed at the lowest concentration of 0.6 mg/ml, whereas the highest *versican* expression was observed at the highest concentration of 2.4 mg/ml (Fig. 2E). Extrapolating from the change in diameter, the collagen concentration in HBs increased from 2.4 mg/ml to ~10 mg/ml during the 3 days of culture. Based on these results, HBs prepared with 2.4 mg/ml collagen and $10 \times 10^3$ mouse mesenchymal cells/bead were used for the following experiments.

The ALP activity of mouse mesenchymal cells cultured in HBs was compared to that in spheroid and monolayer cultures after 3 days of culture (Fig. 3A). ALP activity in HBs was 2- and 10-fold greater than that in spheroid and monolayer cultures, respectively. *Versican* gene expression in mouse mesenchymal cells in HBs increased over time and was significantly greater than that in spheroid and monolayer cultures after 3 days, which eventually was restored to levels observed in freshly isolated cells at 5 days of culture (Fig. 3B). These results suggest that HB culture might preferentially promote the differentiation of dermal cells into DP cells or recover the pre-existing DP cell properties better, although further studies are necessary to distinguish and quantify these notions. Similar results were obtained using human DP cells (Fig. 3C and D). *ALP* and *versican* gene expression in human DP cells cultured in HBs were greater than those in spheroid culture at 7 days of culture. These results suggested that the HB approach provides a more suitable environment for recapitulating the *in vivo* dermal papilla niche, compared to that with typical spheroid culture.

### 3.2. Effects of cell attraction forces on collagen contraction and trichogenous gene expression

Cell attraction forces driven by the motor protein myosin II play an important role in epithelial invagination during embryonic development of the hair follicle [31]. To investigate relationships between the spontaneous contraction of microgels and the recovery of trichogenous gene expression, the myosin II ATPase inhibitor blebbistatin was added to the culture medium. The results showed that in the presence of blebbistatin, the spontaneous contraction of microgels was inhibited, whereas in its absence, the cells contracted the microgels to form a dense meshwork of actin filaments (Fig. 4A, C). Furthermore, the expression levels of *versican* and *ALP* in both mouse mesenchymal cells and human DP cells were significantly downregulated in the presence of blebbistatin (Fig. 4B, D). These results suggest that the spontaneous formation of collagen-rich HBs can be attributed to cell attraction forces, which are important for the augmented hair induction ability of the cells.

### 3.3. Patch assays using HBs

In total, 30 HBs with mouse mesenchymal cells or 50 HBs with human DP cells were mixed with mouse epithelial cells, and subcutaneously transplanted into a pocket surgically created on the back

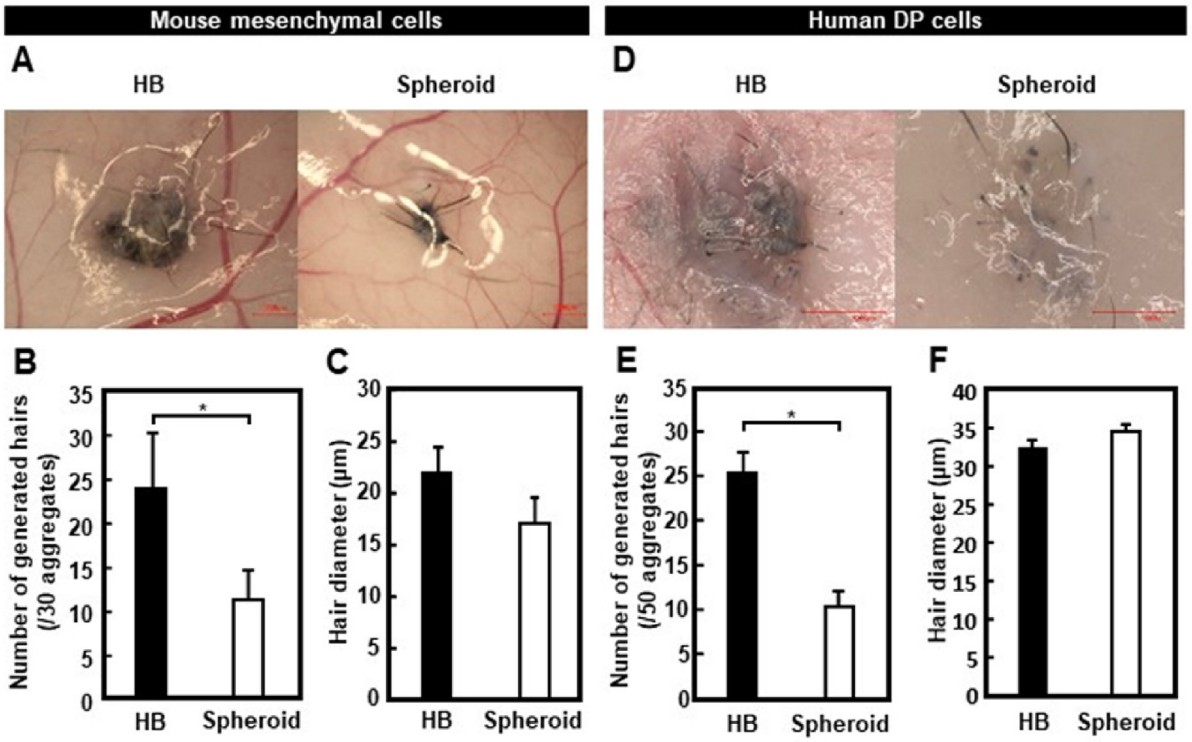


**Fig. 5. Patch assays using mouse mesenchymal cells and human dermal papilla (DP) cells.** (A, D) Appearance of hair clusters generated 3 weeks after the transplantation of hair beads (HBs). For this, 30 HBs ($1 \times 10^4$ cells/bead) of mouse mesenchymal cells (A) or 50 HBs of human DP cells (D) were transplanted with mouse epithelial cells ($3 \times 10^5$ cells) into a slab of nude mouse skin. (B, E) Number of generated hairs. The number of hairs generated per 30 HBs of mouse mesenchymal cells (B) or 50 HBs of human DP cells (E) was counted under a microscope after dissociating the clusters. (C, F) Diameter of generated hairs. The diameter of hairs generated per 30 HBs of mouse mesenchymal cells (C) or 50 HBs of human DP cells (F) was quantified based on scanning electron microscope images. The values and error bars were calculated based on at least four experiments per sample. Numerical variables were statistically evaluated by a Student's t-test. *$p < 0.05$ was considered significant.

skin of nude mice [11]. As a comparison, spheroids prepared with the same number of mouse mesenchymal cells or human DP cells were transplanted with mouse epithelial cells into the mice. A clump of black hair shafts generated in the hypodermis was observed 3 weeks after transplantation under all conditions (Fig. 5A, D). The hair shafts then were isolated from the clump and counted. Hair numbers generated by HBs were more than double those generated by spheroids for both mouse and human cells (Fig. 5B, E). Moreover, the diameters of hair shafts generated by HBs using mouse mesenchymal cells were comparable to those on the adult mouse body (~20 μm) and were slightly larger than those produced by spheroids (Fig. 5C). However, there was virtually no difference in hair diameters between HBs and spheroids prepared using human DP cells (Fig. 5F). A previous study reported that approximately 12 hairs/50 aggregates (calculated from Ref. [17]) could be generated in the patch assay when human DP spheroids were transplanted into mice. This value is equivalent to that obtained with spheroids in the present study and supports the fact that HBs are superior to spheroids in terms of the number of regenerated hairs.

### 3.4. Preparation of HFGs using HBs

For *in vivo* development, the formation of HFGs, consisting of epithelial and mesenchymal cell aggregates, triggers the morphogenesis of hair follicles via enhanced homogeneous/heterogeneous cell–cell interactions [32]. Efficient hair follicle regeneration has been reported by preparing HFG-like cell aggregates *in vitro* and subcutaneously transplanting them into mice [20,21]. Considering that thousands of grafts are necessary for each patient for the clinical application of hair regenerative medicine, an approach that can not only recover the hair induction activity of cells but could also be used to prepare large numbers of grafts is desired. Thus, we investigated whether the HB approach was feasible to prepare HFGs and further amenable to scaling.

Using a handheld electromotive pipette, we spotted a 2-μl collagen droplet containing mesenchymal cells in each well of a non-cell-adhesive round-bottom 96-well plate. Immediately after a 30-min incubation (for gelation), a cell suspension containing epithelial cells was introduced into the well (Fig. 6A(i)). During 24 h of culture, epithelial cells formed a spherical aggregate and attached to the collagen microgels of each well. Over the following 2 days of culture, as shown in Fig. 2A, the collagen microgel contracted and an HFG-like aggregate (bbHFG) was formed. For comparison, we fabricated cell aggregates with two different approaches. As shown in our previous study, when the two types of cells were mixed and seeded in a well, they initially formed an aggregate in which the two cell types were randomly distributed at day 1 of culture; however, they then spatially separated from each other and self-organized into an HFG (ssHFG) during 3 days of culture (Fig. 6A(ii)). Another simple and scalable approach is to prepare a collagen solution containing epithelial and mesenchymal cells and spot the droplets. The contraction of microgels was observed over 3 days of culture, similar to that observed with the encapsulation of mesenchymal cells only, resulting in the formation of HBs (HBs (e + m)). Of note, in the HBs(e + m) group, the two types of cells remained randomly distributed (Fig. 6A(iii)). HE staining showed that epithelial and mesenchymal cell aggregates could form distinctive two-compartmentalized internal structures in the bbHFG and ssHFG groups, although multiple cyst structures were formed inside the aggregates of the HBs(e + m) group at 3 days of culture (Fig. 6B). We further analyzed the structures by staining for vimentin, a DP marker, and cytokeratin 14, a marker for the basal layer of the epidermis and the outer root sheath cells [33]. Vimentin-positive cells were observed in the

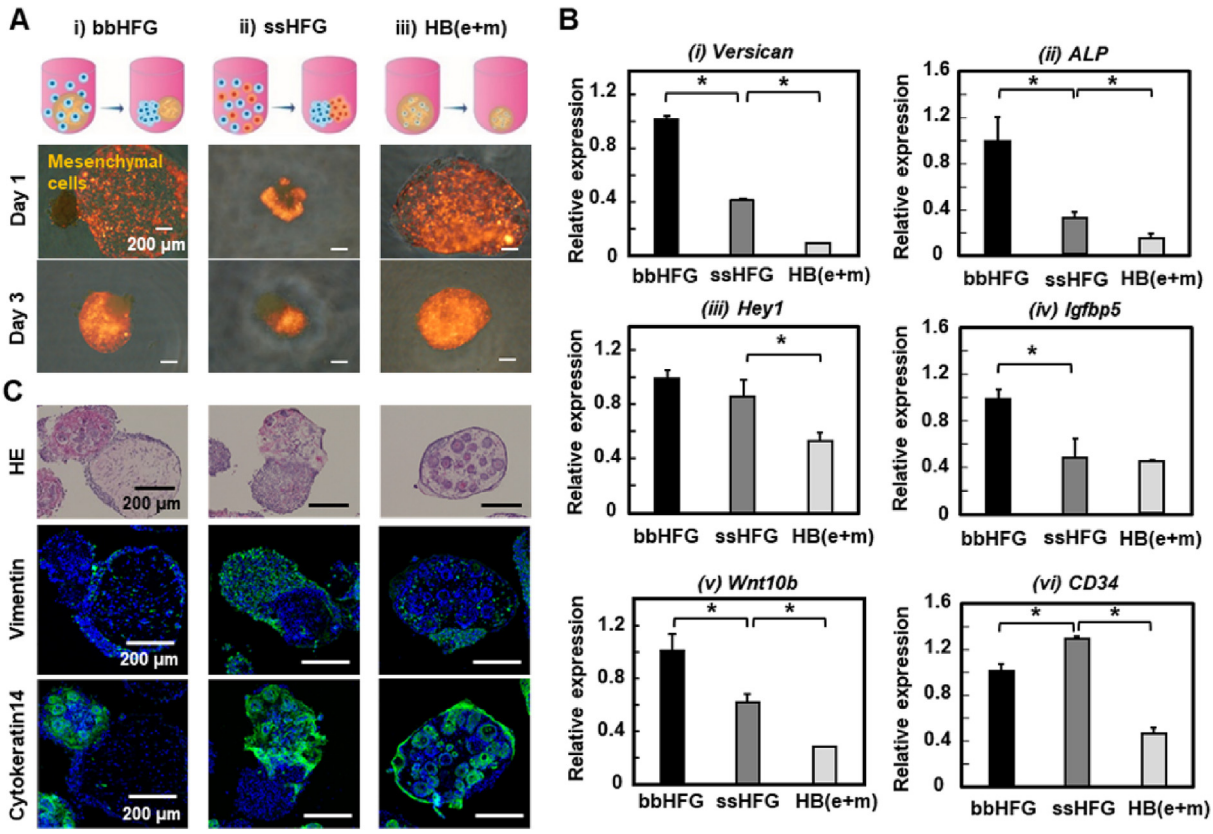


**Fig. 6. Hair follicle germs (HFGs) prepared using hair beads (HBs) and epithelial cells.** (A) Changes in the spatial distribution of mesenchymal cells in HFGs based on three preparations: i) bead-based HFGs (bbHFGs) were prepared by mixing HBs and epithelial cells; ii) self-sorted HFGs (ssHFGs) were prepared by mixing epithelial and mesenchymal cells; iii) HBs(e + m) were prepared by encapsulating epithelial and mesenchymal cells in a microgel. To distinguish the two cell types, mesenchymal cells were stained with Vybrant Dil cell-labeling solution (red). Fluorescent and phase-contrast images are merged in the images. (B) Comparison of *Versican*, *ALP*, *Hey1*, *Igfbp5*, *Wnt10b*, and *CD34* gene expression at 3 days of culture. All data were normalized to levels of the reference gene *GAPDH*. Error bars represent the standard error of means calculated from three independent experiments for each condition. Numerical variables were statistically evaluated by a Student's t-test. $*p < 0.05$ was considered significant. (C) HE staining and immunohistochemical staining of cross-sections of HFGs at 3 days of culture. Nuclei (blue), vimentin (green), and cytokeratin 14 (green) were visualized. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mesenchymal aggregates of the ssHFG group more so than in the bbHFG and HB(e + m) groups, whereas cytokeratin 14-positive cells were observed in all groups. Further studies such as time-course analysis and staining with multiple markers are necessary to reveal differentiation dynamics and morphogenesis of the HFGs. We then compared the expression of trichogenous gene markers, *versican*, *alp*, *hey1*, *igfbp5*, *wnt10b*, and *CD34*, among the three cell aggregate preparations (Fig. 6C). The expression of almost all genes (except *CD34*) in bbHFGs was greater than that in ssHFGs and HBs(e + m). These results suggest that collagen-rich microenvironments and the aggregation of each cell type are both crucial for the formation of a stem cell niche for HFG.

Using our approach, spheroid formation with epithelial cells and HB formation through the contraction of mesenchymal cell-encapsulated microgels occurred spontaneously, facilitating scale-up using a micro-dispenser system. As shown in Fig. 1A, collagen microgels were spotted on a flat surface with an automated micro-dispenser for a large number of HB preparations (Suppl. Movie 1). *Versican* gene expression was comparable between manually and automatically prepared HBs (data not shown). We further demonstrated that the automatic micro-dispenser was applicable for the preparation of a large number of bbHFGs using a round-bottom 96-well plate (Suppl. Movie 2). It only took ~10 min to spot 5000 drops of collagen, and after a 30-min incubation for gelation, it only took ~10 min to seed a suspension comprising the second cell type in the wells. We thus consider that these processes are sufficiently simple and practically feasible for clinical applications.

### 3.5. Transplantation of bbHFGs

Single bbHFGs, ssHFGs, and HBs(e + m) prepared with mouse mesenchymal cells and human DP cells were respectively transplanted into the back skin of nude mice to evaluate hair generation. Three weeks after transplantation, black hairs were observed at the sites of bbHFG and ssHFG transplantation (Fig. 7A, D). However, almost no hair was observed with HBs(e + m) for both mouse mesenchymal and human DP cells. Considering that HBs of these cell types had hair inductivity in the patch assay when transplanted with mouse epithelial cells (Fig. 5), the preparation of aggregates comprising homogeneous cell types and close cell–cell interactions (at least for mesenchymal cells) could be vital for hair regeneration. Between bbHFG and ssHFG groups, there was no significant difference in the hair generation efficiency, which was defined as the number of hair-generating sites divided by the number of transplanted sites (Table S1), whereas the number of generated hairs with bbHFGs was greater than that with ssHFGs (Fig. 7B, E). Scanning electron microscope images of the generated hair shafts revealed typical morphological characteristics of hairs including the hair cuticle, which was comparable to natural hairs from C57BL/6 adult mice (Fig. 7C, F). The hair follicles generated 3 weeks after the transplantation of bbHFGs were further examined with HE and immunohistochemical staining of bulge and DP regions (Fig. 7G). Typical morphological features of the root-sheath and DP were observed including CD 34- and vimentin-positive cells. These results indicate that bbHFGs might be promising microtissues for hair

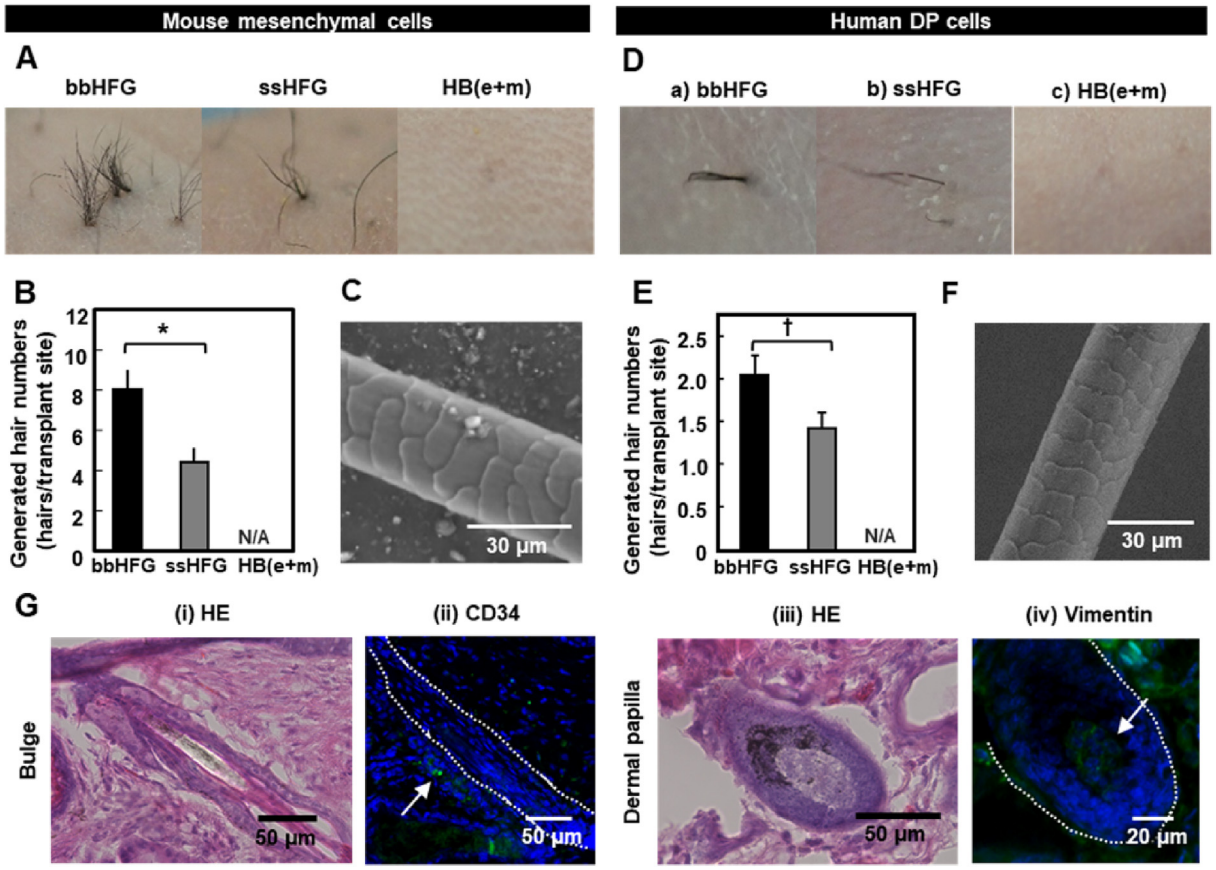


**Fig. 7. Hair generation assays using different hair follicle germs (HFGs).** (A, D) Hair shafts were generated on the back skin of mice 3 weeks after transplantation. Single bead-based HFGs (bbHFGs), self-sorted HFGs (ssHFGs), and HBs(e + m) (prepared by encapsulating epithelial and mesenchymal cells in a microgel) containing mouse mesenchymal cells (A) and dermal papilla (DP) cells (D) were transplanted. (B, E) The number of generated hair shafts. Values and error bars were calculated from at least 25 HFG transplantations for each condition in four independent experiments. Numerical variables were statistically evaluated by a Student's t-test. The p-values are represented by $^{*}p < 0.05$ and $^{\dagger}p < 0.1$. (C, F) Scanning electron microscopic images of generated hair shafts 3 weeks after the transplantation of bbHFGs. (G) HE staining and immunohistochemical staining of hair follicles 3 weeks after the transplantation of bbHFGs. The bulge region was visualized with HE staining (i) and CD34 staining (ii). Nuclei (blue); CD34 (green). Dermal papilla was visualized with HE staining (iii) and vimentin staining (iv). Nuclei (blue); vimentin (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regenerative therapy.

In this study, chimeric bbHFGs composed of human DP cells and mouse embryonic epithelial cells were examined. However, further investigation using both cells of human origin, preferably from a patient with hair loss, will be performed next. Because human epithelial stem cells such as hair follicle stem cells lose their trichogenic nature more rapidly than DP cells in expansion culture [34], we expect that the present bbHFG approach will also be effective to recover the trichogenic nature of expanded human epithelial stem cells. In addition, the conjugation of growth factors with collagen gels might further increase hair generation efficiency [10,35], which will be a promising option that could be combined with the present approach.

## 4. Conclusions

A key issue in the field of hair regenerative medicine is the preparation of microtissues, which should possess high trichogenic ability upon transplantation. Moreover, the preparation approach should be scalable to > 5000 tissues. The present study demonstrated that HBs prepared by encapsulating mouse embryonic mesenchymal cells or human DP cells in a drop of collagen gel could provide a preferable microenvironment for these cells. Compared to conventional spheroid culture, *versican* and *ALP* expression were upregulated in HBs and the transplantation of HBs into nude mice resulted in almost a two-fold increase in the number of hair shafts generated. The HB approach was further combined with spheroids formed from epithelial cells, resulting in HFG-like constructs (bbHFGs) that generated hairs more efficiently than previous approaches. Because bbHFGs were spontaneously formed through cell–cell adhesion and cell attraction forces in collagen gels, this preparation is considered scalable using an automatic micro-dispenser. Although further studies using cells derived from individuals with hair loss are necessary, this technology might present a new avenue for the fabrication of microtissues for hair regenerative therapy.

## Conflicts of interest

There are no conflicts of interest to declare.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2019.05.003.

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