이학석사 학위논문

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지도교수 최 태 현

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위 원 장 유 형 곤 (인)
부 위 원 장 최 태 현 (인)
위 원 조 정 현 (인)

Abstract

Development of *de novo* human hair microfollicle (hHMF) *in vitro* with primary cultured dermal papilla cells and outer root sheath cells

Gee Ho Park
Interdisciplinary program in Stem Cell Biology
The Graduate School
Seoul National University

The development of the human hair follicle (HF) is a consequence of the intricate interactions between mesenchyme and epithelium during the embryonic stage. Mesenchymal-epithelial interaction mechanism has been applied in tissue engineering and regenerative medicine researches recently. Although many studies have been attempted to reconstruct HF using

mesenchymal-epithelial mechanism, the regeneration of the complete HF in vitro is still challenging. In this study, we extracted dermal papillae (DP) cells and outer root sheath (ORS) cells from human scalp and used them to develop into human hair microfollicles (hHMF). Cultured DP cells were used as mesenchymal cells and ORS cells were used as epithelial cells to induce mesenchymal-epithelial interactions and de novo hHMF were developed by incubation in 3D hanging drop (HD) culture for 5 days. We extracted DP and ORS cells with a simple and effective method from the scalp of young patients and characterized cells by attaching specific antibodies through immunofluorescence staining. DP cells and ORS cells were cultured according to specific timeline that we established. Spontaneous localization of the single cells was observed after 5 days of HD incubation. The morphology of hHMF resembled HF in that hHMF has uni-directional structure and the DP spheroid resides in the hair bulb-like area. Also, immunofluorescence analysis of hHMF showed the expression of specific DP and ORS cell markers in the specific site of structure. This efficient hHMF development technique provides a potential to demonstrate the mechanism of HF development in vitro. Finally, an advanced culture system will allow screening of hair-related drugs in patients with hair loss or hair implantation with hHMF. Our findings are expected to bring new insights into tissue engineering and hair regeneration research.

Keywords : Hair follicle, Dermal papilla cell, Outer root sheath cell, Hanging drop culture, Tissue engineering, Regenerative medicine
Student Number **:** 2016-22741

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1. Introduction

Alopecia is a common and profound defect that may connect to mental illness due to loss of confidence although it is not a hazardous disease (Cash, 2001). Not only alopecia patient lose hair, but also ordinary people are exposed to hair loss due to extreme stress, aging, adverse effect of drugs, burnt wounds and many other reasons. Approximately, 50% of men and 25% of women under go hair loss at the age of 50 (Vary, 2015). To date, medical treatment (e.g. Minoxidil, Propecia) of hair loss has such limitations as the surgical transplantation of hair that the numbers of hair follicles (HF) are restricted. To overcome the limitation, many researchers are attempting to regenerate the HF *in vitro*.

HF is the smallest organs composed with interaction of epithelium and mesenchyme cells at the embryonic stage. Morphogenesis of the nascent HF requires dermal papilla (DP) cells which receive signals from epithelial cells in the epidermis (Fuchs, 2007; Yang and Cotsarelis, 2010; and Driskell et al., 2011). DP cells are mesenchymal cells that play an essential role in the induction of hair by interacting with epithelial cells (Paus and Corsarelis, 1999). Epithelial cells arise from hair follicle stem cell (HFSC) in the bulge region of outer root sheath (ORS) and these cells differentiate into matrix cells, inner root sheath cells and hair shaft (Botchkarev and Kishimoto, 2003; Yoo et

al., 2007; Inoue and Yoshimura, 2013). Many researchers have attempted to regenerate HF with epithelial mesenchymal interaction using primary HF cells. Moreover, due to the unique characteristics of hair follicle consisting cells, the cultured primary cells are used in HF regeneration and diverse therapeutic applications.

A number of studies have reported a methodology for isolating DP and ORS cells from human scalp and culture techniques preserving unique characteristics of the cells (Messenger, 1984; Inoue and Yoshimura, 2013; Gledhill et al., 2013; Chan et al., 2015). Recently, it was reported that DP cells lose trichogenic ability when cultured with 2D monolayer and lose overall characteristics of DP cells as the cells were subcultured. However, DP cells retain its hair inductivity when cultured in 3D environment (Oyama et al, 2012; Higgins et al, 2013). Therefore, the study on the culture method using the 3D environment is increasing not only in cancer organoid culture but also in the regeneration of HF. In fact, 3D culture of DP cells and epithelial cells showed a successful HF regeneration when cultured *in vivo* (Toyoshima et al., 2012; Kang et al., 2012; Lin et al, 2016). These studies used a low cell-binding plate or hanging drop (HD) culture to reassemble spheroids to enhance hair inductivity of 2D cultured cells. Nonetheless, these studies have such limitations that bioengineered hair germs produced hair shaft with assistant of murine *in vivo* environment. As yet, in order to develop a complete HF *in*

vitro, it is difficult to implement the *in vivo* environment and to preserve the pluripotency of the stem cells. There are a few studies reported on regeneration of HF *in vitro* (Qiao et al., 2008; Lindner et al., 2011; Lee et al., 2018). Reconstitution of homogenous human HF *in vitro* would be necessary to study the mechanism of HF development and to provide additional therapeutics for patients with hair loss.

In this study, we have isolated DP cells and ORS cells, corresponding to mesenchymal and epithelial cells, to reconstruct HF from human scalp. With efficient 3D hanging drop culture, we observed spontaneous localizations of DP and ORS cells develop into human hair microfollicle (hHMF) structure *in vitro* without additional growth factors (GF) or extracellular matrix (ECM). Here, we established an efficient methodology of hHMF development and suggests that our research provide new insights in tissue engineering and hair regenerative studies.

2. Materials and Methods

2.1 Isolation of human DP and primary culture

Intact scalp biopsy of human patient was received from Seoul National University Hospital under approval of Institutional Review Board (IRB No. 1108-098-374). The hair follicles (HF) were plucked from the scalp with micro forceps under stereoscopic microscope (OMAX, Korea). Bulb region of the HF was transected with surgical blade and dermal papilla was micro-dissected with insulin syringe (BD, USA). Isolated DP were then moved to 6-well plate about 5 to 10 DP per well. The DP medium (DPM) were cultured in Dulbecco modified Eagle's medium (DMEM-high glucose) containing 20% inactivated FBS and 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% humidity. After 7 to 10 days of incubation, DP were appeared to be attached and proliferate on the plate. Medium was changed every 2–3 days and passaged after 15 days.

2.2 Outer root sheath keratinocyte primary cell culture

The HF without bulb region was incubated in 1.2 U/mL of dispase II (Sigma, USA) for 3 h in RT. Vigorous pipetting and vortexing were conducted during incubation. After the Incubation, the HF were centrifuged in 1300 rpm for 3 min. 0.2 % of collagen was coated on

the 6 well plate and removed after 10 min of incubation. Centrifuged samples were moved to collagen coated plates and incubated for 2 days. The ORS cells were cultured in Keratinocyte Growth Medium 2 (KGM; Promocell, USA). After 3 days of incubation, ORS cells were observed to be stretched out from the ORS. Cells were fed every 1-2 days with rinsing with phosphate buffer saline (PBS).

2.3 Characterization of DP cells and ORS cells

DP and ORS cells were grown in confocal dish (SPL, Korea). Cells were then fixed in 4% (wt/vol) paraformaldehyde (PFA) for 15 min. After PBS rinsing, cells were treated with 0.1% Triton X-100 for 5min. Blocking was implemented with 1% (wt/vol) BSA for 1 h at RT. The following primary antibodies were used: mouse anti-Nestin (1:100, Santa Cruz), mouse anti-Versican (1:100, Santa Cruz), mouse anti-ITGA8 (1:100, Abcam), mouse anti-Cytokeratin14 (1:100, Abcam), rabbit anti-Cytokeratin 5 (1:100, Abcam), rabbit anti-Vimentin (1:100, Abcam), rabbit anti-β-catenin (1:100, Abcam) and rabbit anti-ALP (1:100, Abcam). The confocal dish was rinsed with PBS and incubated with secondary antibodies: Alexa Fluor 488 anti-rabbit (1:100, Abcam) and Alexa Fluor 647 anti-mouse (1:100, Abcam) for 1 h at RT. After rinsing with PBS, cells were exposed to Vectashield with DAPI (Vector's lab, USA) and visualized on a Leica TCS SP8 (Leica, Germany) confocal microscope.

2.4 Hanging drop 3D culture

Cultured DP and ORS cells (passage 1 and passage 2) were detached with TrypLE Express™ (gibco, USA). DP and ORS cells were seeded at concentration of 1×10^4 cells/well in hanging drop culture plate (HDP; Perfecta3D®, USA) or cover of petri dish on day 25-30 and incubated at 37°C in an atmosphere of 5% CO_2. Ten microliters of DPM were added to HDP everyday until day 5. PBS was inserted to the reservoir to maintain humid environment. Cautious handling is required to sustain droplets in the HDP.

2.5 Immunofluorescence analysis of hHMF

Developed hHMFs were collected from HD plate, removed to confocal dish and incubated in 4% PFA overnight. Same methods of fixation, permeabilization and blocking were used as *2.3*. Primary antibodies: mouse anti-Nestin (1:100, Santa Cruz), mouse anti-Versican (1:100, Santa Cruz), mouse anti-AE13 (1:100, Abcam), rabbit anti-ALP (1:100, Abcam), rabbit anti-Cytokeratin 15 (1:100, Abcam), rabbit anti-Cytokeratin 5 (1:100, Abcam), rabbit anti-Vimentin (1:100, Abcam) and rabbit anti-β -catenin (1:100, Abcam) were incubated in RT for 1 hr. After PBS rinsing, secondary antibodies: Alexa Fluor 488 anti-rabbit (1:100, Abcam) and Alexa Fluor 647 anti-mouse (1:100, Abcam) were incubated for 1hr in RT. Cells were exposed to Vectashield with DAPI (Vector's lab, USA) and observed on a Leica TCS SP8 (Leica, Germany) confocal microscope.

3. Results

3.1 Efficient and simple isolation method of DP cells and ORS cells from human scalp

Numerous methodology of isolating DP and ORS has been reported. Figure 1 shows the brief procedure of isolating DP from human scalp. Human scalp was first quickly rinsed with DPM to minimize the bacterial growth, then the large piece of the sample was cut into small pieces (Fig. 1A). Then by pinching hair shaft and ORS together with a micro forceps, HF were easily plucked out from the scalp. Adipose tissue and connective tissues were removed from HF by scalpel (Fig. 1B). Cleaned HF were collected (Fig. 1C) and the HF were aligned in one droplet of PBS then the bulb region of the HF were cut simultaneously (Fig. 1D). If the volume of the PBS exceeds, the isolated DP can be easily lost. Upper portion of the HF were incubated in isolation enzyme for ORS cell culture. Using insulin syringes, the bulb epithelium was dissected and opened (Fig. 1E). The water droplet-shaped DPs were collected and moved to 6-well plate. At this stage, it is important that the bulb region stays wet in liquid. Figure 1F shows freshly isolated DP in 6-well plate. Five to ten DPs were incubated in 6-well plate for 7 to 10 days. To demonstrate ORS cell isolation efficiency, three isolation enzymes were compared in Figure 2. ORS cells incubated in dispase II showed more efficiency

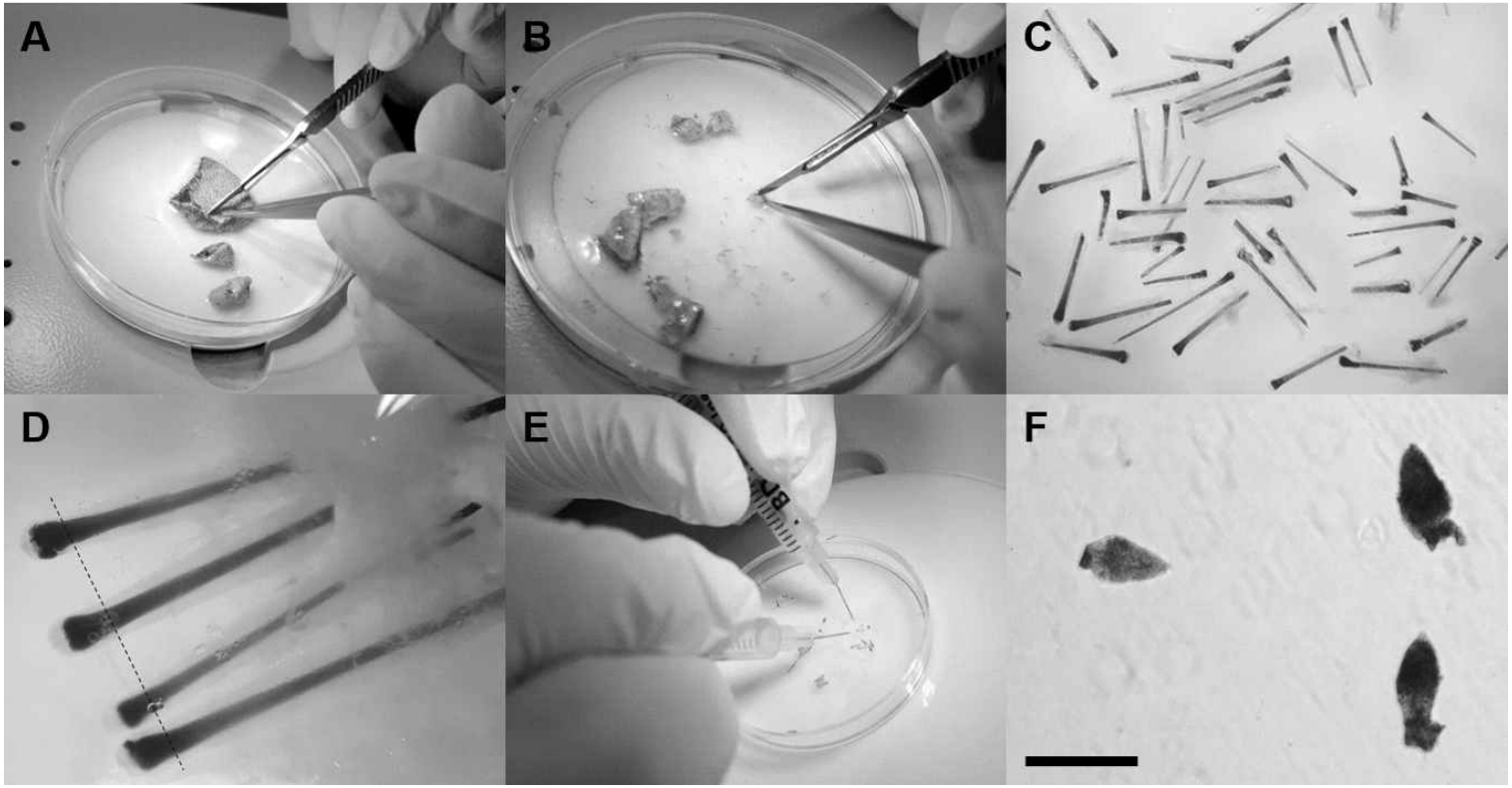

Figure 1. Photographs of hair follicles and dermal papilla isolation procedure. A) Human scalp was washed with saline and excised into small pieces. B) Subcutaneous fat and connective tissues were removed. C) Trimmed hair follicles were collected. D) Aligned hair follicles were cut with scalpel along a dotted line. E) Upper part of hair follicles were moved to dispase II and hair bulbs were dissected with insulin syringe to isolate dermal papilla. F) Isolated dermal papilla were incubated for 7 days at 37°C (Scale bar = 500 ㎛).

in isolating ORS cells than 0.2% collagenase type II and 0.5% trypsin-EDTA (Figure 2D). Thus, we selected dispase II for our ORS cell isolation method.

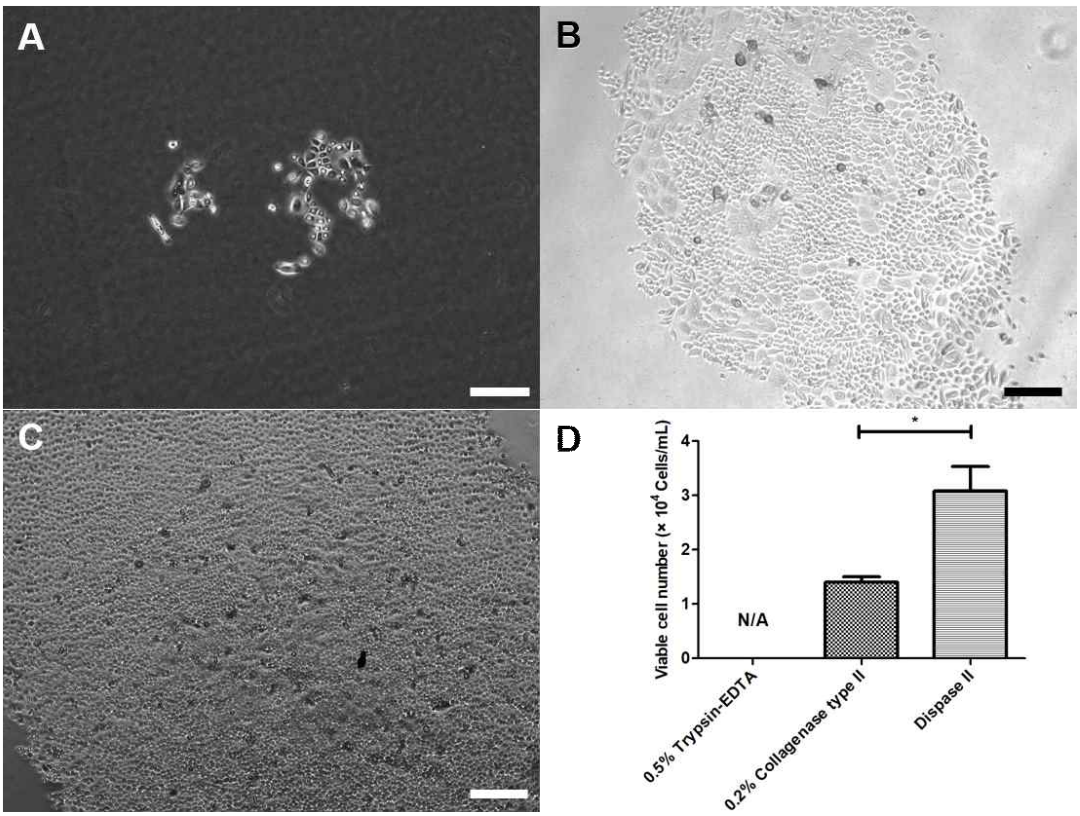

Figure 2. Isolation of ORS cells depending on enzymes. A) ORS cells isolated using 0.5% Trypsin-EDTA, B) 0.2 % Collagenase type II and C) Dispase II (Scale bar = 200 ㎛). D) Isolation yield of ORS cells depending on enzyme treatments (*$p < 0.05$).

Isolated DP and ORS were observed with microscopy as shown in Figure 3. Three isolated DPs were attached to the plate after 8 days of incubation (Fig. 3A). After the attachment, PBS rinsing was conducted and medium was changed. The DP cells stretch out from the DP and grew exponentially after first media change. Figure 3B represents DP cell growth 2 days after the first medium change.

Medium were changed every 2 days and at day 15, DP cells were subcultured to 100 pi cell culture dish. For ORS cell culture, upper part of the HFs were incubated for 4 hours in dispase II. Vigorous and extreme pipetting is required at this stage, while pipetting assure the HF are not attached to the pipette tip. After centrifugation, the HF were mixed in KGM and cultured in collagen coated 6-well plate. Unlike DP, the ORS adheres to the plate within 2 days. The ORS cells were spread out from the attached HF after 2 days (Fig. 3C). After 2 days of incubation, the ORS cells were rinsed with PBS and medium was changed. Figure 3D represents ORS cell growth 2 days after the first medium change. KGM were changed every 2 days same as DPM and subcultured at day 15. Careful handling is required when culturing ORS cells because no antibiotics are contained in KGM.

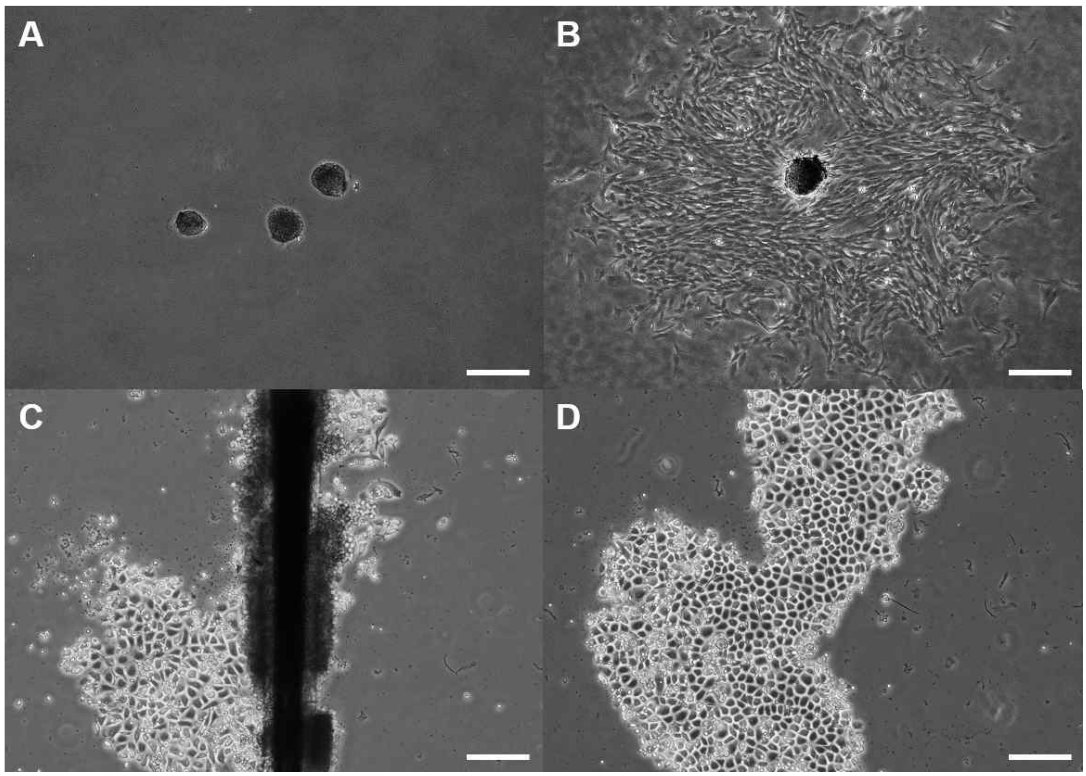

Figure 3. Microscopic image of DP cells and ORS cells at passage 0. A) Isolated dermal papilla from human scalp. B) DP cells spread out from isolated dermal papilla. C) ORS cells spread out from human hair follicle. D) ORS cells grown after rinsing with PBS. Scale bar represents 200 ㎛.

3.2 Characterization of human DP cells and ORS cells

Isolated cells were characterized by immunofluorescence staining. We selected alkaline phosphatase (ALP), integrin alpha 8 (ITGA8) and nestin as DP cell markers, and keratin 5 (K5), keratin 14 (K14) and beta-catenin as ORS cell markers. ALP and versican is a well known follicular papilla marker for many years and nestin is known to be expressed in skin-derived precursor reside in DP cells which can be a marker for DP cells. Passage 1 DP cells were positive for ITGA8 and nestin (Fig 4A). Also, ALP and vimentin were observed on DP cells (Fig 4B and 4C), but keratin 14 showed negative which was used as negative control. Keratin 5 and 14 is a marker for ORS in human HF and beta-catenin shows expression in ORS cells through wnt/beta-catenin signaling in hair morphogenesis. For passage 1 ORS cells, keratin 5 and 14 were visualized (Fig 4D). Moreover, beta-catenin and vimentin presented positive whereas nestin was negative (Fig 4E and 4F). Our results demonstrate that cells isolated from human hair follicle express specific DP cell markers and ORS cells markers, in other words we have confirmed our DP cells and ORS cells isolation method.

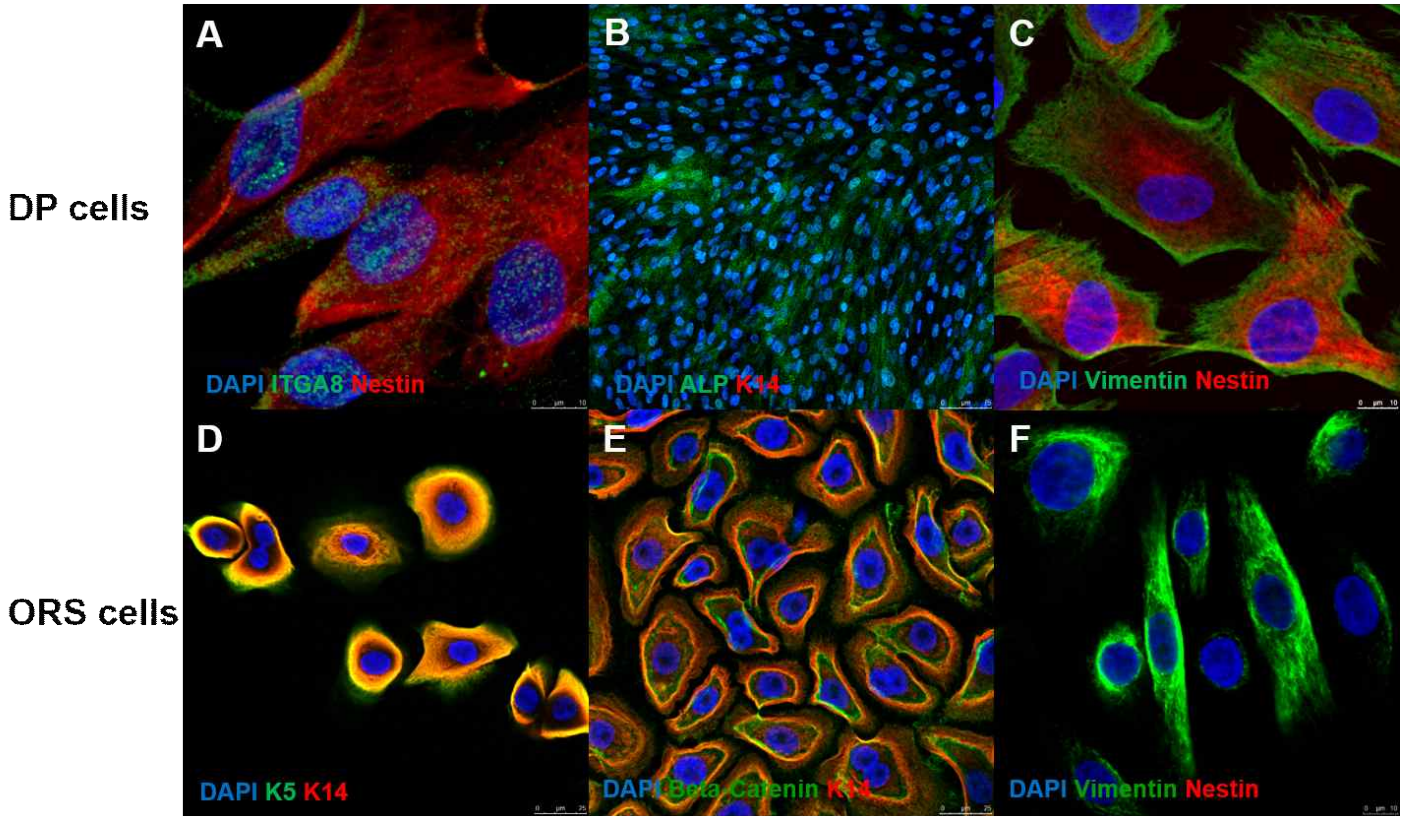

Figure 4. Characterization of DP cells and ORS cells. A) Integrin alpha 8 (ITGA8; green) and Nestin (red) positive in DP cells. B) Alkaline Phosphatase (ALP; green) positive, but Keratin 14 negative in DP cells C) Vimentin (green) and Nestin (red) stained in DP cells. D) Keratin 5 (green) and Keratin 14 (red) positive in ORS cells. E) â-catenin (green) and Keratin 14 (red) were observed on ORS cells. F) Vimentin (green) positive, but Nestin (red) negative on ORS cells. Scale bars represent 10㎛ (A, C, F), 25㎛ (D, E), and 75 ㎛ (B).

3.3 Development of hair microfollicle with hanging drop culture.

In order to establish the HD culture method, at first, the morphology and the condition of the subcultured cells were examined under light microscopy. Morphology of DP cells were observed as polygonal spindle shape like fibroblasts (Fig 5A). As cells were subcultured, the size, length and shape of the cells became bigger, longer and sharper (Fig 5A, 5B and 5C). For ORS cells, cobble stone shape was observed like keratinocytes (Fig 5D). Enlarged, vacuolated morphology of senescence cells and keratinized cells was increased as cells were subcultured (Fig 5D, 5E, and 5F). The freshest and healthiest cells (passage 1) among subcultured cells were introduced into the 3D culture. Next, two methods of 3D culture were conducted by using concave well and HDP. A photo of six pieces of concave wells and medium drops hang on the petri dish cover are shown in Figure 6A and 6C. After seeding DP cells, spheroids were made after day 1 in concave well and HDP (Fig 6B and 6D). Although both methods has made spheroids successfully, concave well 3D culture method required more highly pipetting skills and consumed more time. When changing medium, the spheroids in the wells flew around and aggregated with each other, and the bubbles were also trapped easily in each wells which make spheroids culture difficult (Data not shown). Whereas for HD culture, the medium was simply added with multi-channel pipette in each drop. Thus, we chose HD culture method to develop the hair

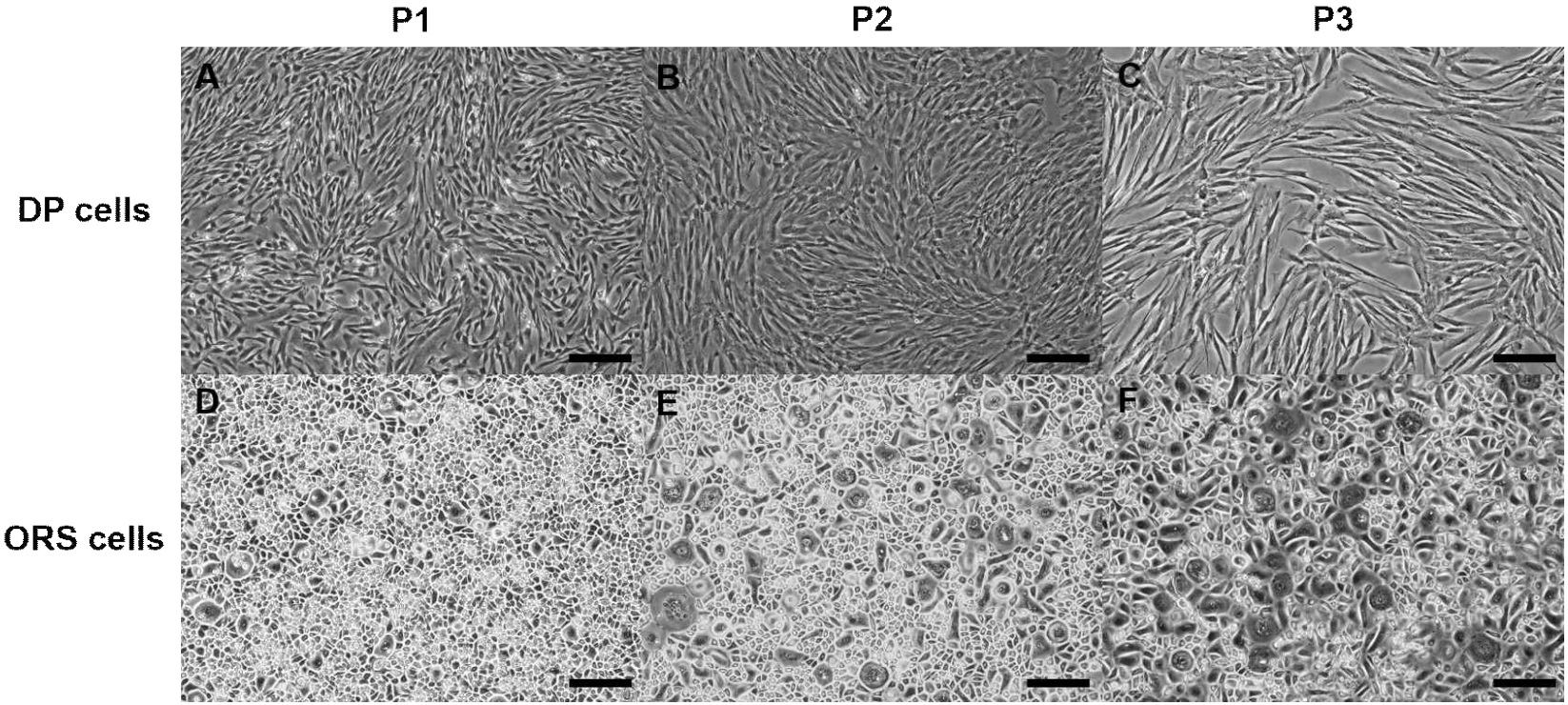

Figure 5. Microscopic image of passage 1, 2, and 3 DP cells and ORS cells. A) Passage 1 DP cells. B) Passage 2 DP cells. C) Passage 3 DP cells. D) Passage 1 ORS cells. E) Passage 2 ORS cells F) Passage 3 ORS cells (P1, passage 1; P2, passage 2; P3, passage 3). Scale bar represents 200 ㎛.

microfollicle which is more convenient and less time-consuming. Mixture of DP and ORS cells (DP cells + ORS cells), DP cells only, and ORS cells only were compared every day until day 5 in HD culture (Fig 7). DP cells + ORS cells transformed its morphology day after day (Fig 7A), whereas DP cells only maintained spheroids (Fig 7B), and ORS cells only did not aggregate until day 5 (Fig 7C). DP cells + ORS cells aggregate with each other to form spheroid, and develop into unidirectional structure similar to human hair follicle.

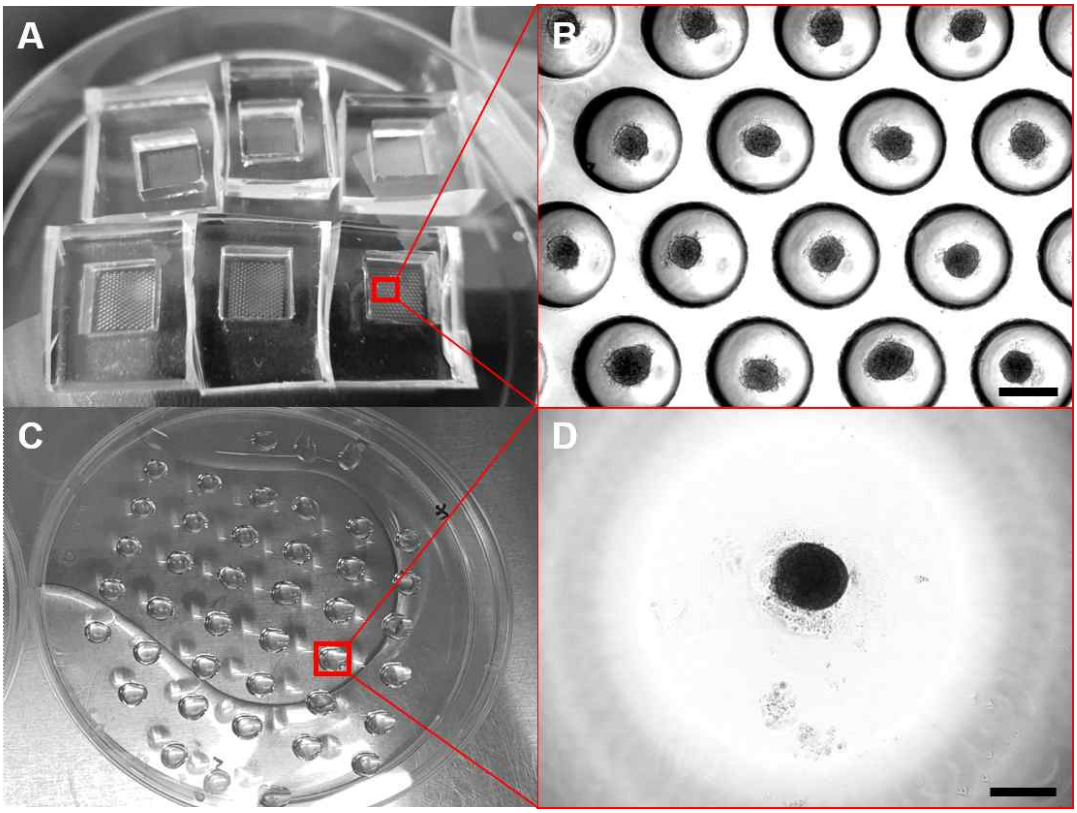

Figure 6. Photographs of concave well 3D culture and hanging drop culture. A) A photograph of 6 concave well B) DP spheroids formed by concave well cultivation. The scale bar indicates 300 ㎛. C) A photograph of hanging drop culture. D) DP spheroid was formed in each hanged medium with cells. The scale bar indicates 200 ㎛.

Interesting fact is that *de novo* hHMF was developed from mixture of the single DP cells and ORS cells. Our result suggests that the hHMF have developed in DP cells + ORS cells in HD culture on day 5 whereas, DP cells only and ORS cells did not.

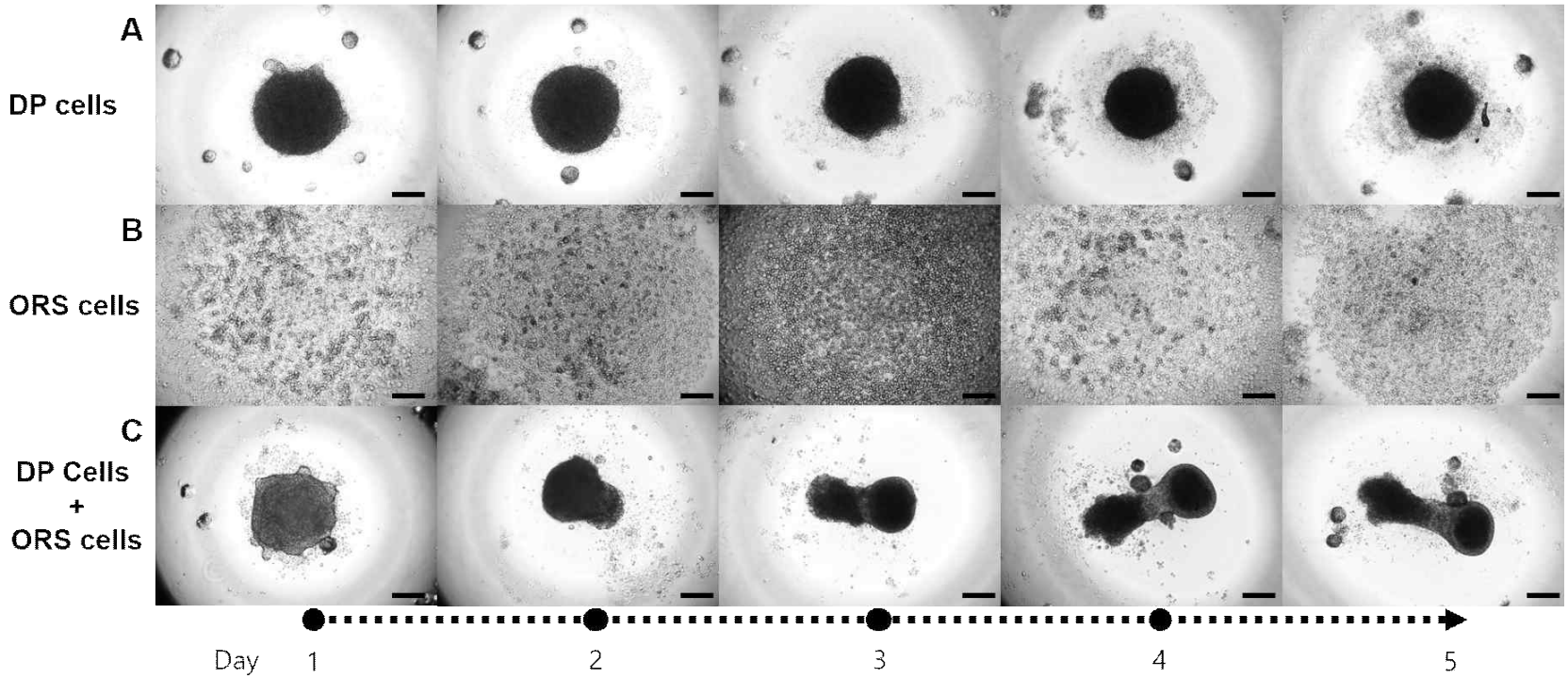

Figure 7. Time-dependent microscopic image of DP cells, ORS cells and DP cells + ORS cells in hanging drop culture. A) DP cells were seeded 1×10^4 cells/well. B) ORS cells were seeded 1×10^4 cells/well. C) DP cells and ORS cells were mixed and seeded 2×10^4 cells/well and images were taken everyday until day 5 (Scale bar = 200 ㎛).

3.4 Comparison of hair microfollicle development using different passage and cell type.

With the method we established, different passage of DP cells and ORS cells were compared. Figure 8 demonstrates various type of hHMFs when cultured with different passage of cells on HD culture day 5. hHMF were developed when cultured with passage 1 DP cells + ORS cells (Fig 8A). Interaction between two cells created an ORS-like structure (ORSS) surrounding DPS. It is also similar to the morphology of HF in that the DPS resides in the bulb region of the hHMF. P1 DP cells + P2 ORS cells (Fig 8B) and P2 DP cells + P1 ORS cells (Fig 8C) were used to develop hHMF. Unfortunately, hHMF with P2 ORS cells failed to develop the ORSS and just DPS were formed. However, DPS and ORSS have developed in P2 DP cells + P1 ORS cells. Moreover, freeze-thawed DP and ORS cells failed to develop hHMF (Fig 8D). It can be hypothesized that since the number of senescent cells increases as cells are subcultured, cells have failed to form hHMF. Our result indicates HD culture with higher passage cells resulted in failure development and the condition of ORS cells determines the development of hHMF. In addition, development of hHMF were compared with different type of cells. Hs27 (human fibroblast cell line) + ORS cell (Fig 9B) and DP cell

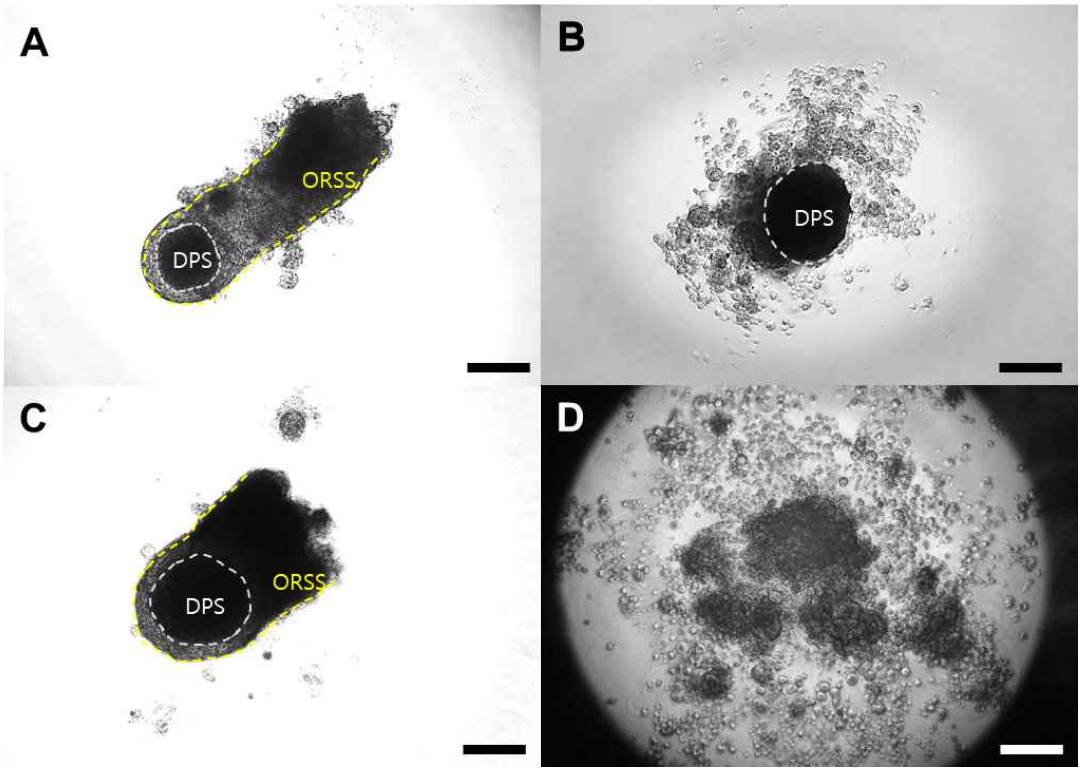

Figure 8. Comparison of hHMF morphology with different passaged cells. A) hHMF development with P1 DP cells and P1 ORS cells. B) Failure development of hHMF with P1 DP cells and P2 ORS cells. DP cells aggregated, but ORS cells scattered. C) hHMF development with P2 DP cells and P1 ORS cells. D) Failure of spheroid formation with freeze-thawed DP cells and ORS cells. DPS: Dermal Papilla cells Spheroid; ORSS: Outer Root Sheath-like Structure (Scale bar = 200 ㎛).

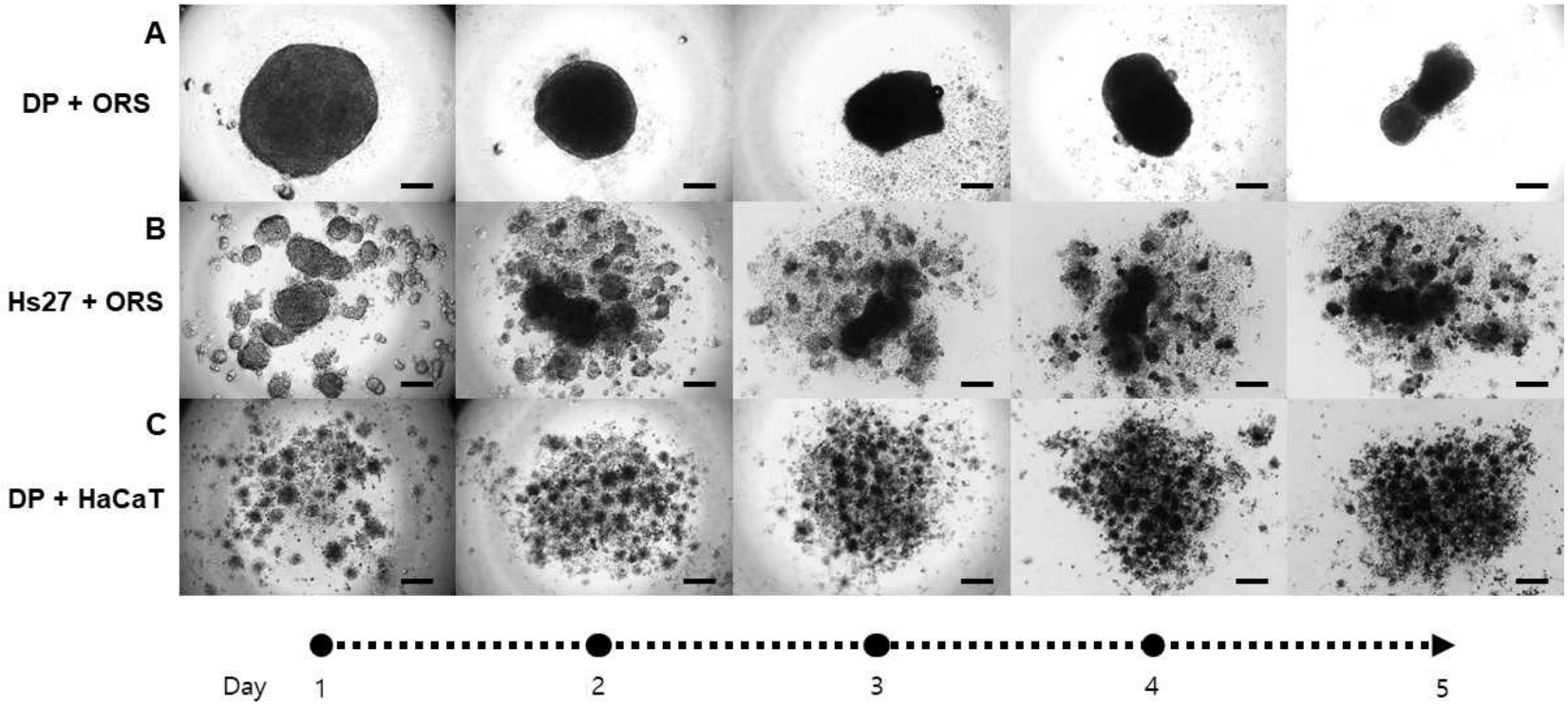

Figure 9. Time-dependent microscopic image of, Hs27s + ORS cells and DP cells + HaCaTs in hanging drop culture. A) DP cells + ORS cells were seeded 2×10^4 cells/well. B) Hs27 + ORS cells were seeded 2×10^4 cells/well. C) DP cells and HaCaTs were mixed and seeded 2×10^4 cells/well and images were taken everyday until day 5 (Scale bar = 200 ㎛).

+ HaCaT (immortalized human keratinocyte) (Fig 9C) were compared with DP cells + ORS cells (Fig 9A). Interestingly, after 5 days of incubation, only DP cells + ORS cells showed hHMF formation which indicates that DP cells and ORS cells have specific signals between these cells. This result suggests that the signal between two cells have triggered the cells to differentiate into hHMF. Further study was conducted to demonstrate allogeneic hHMF development.

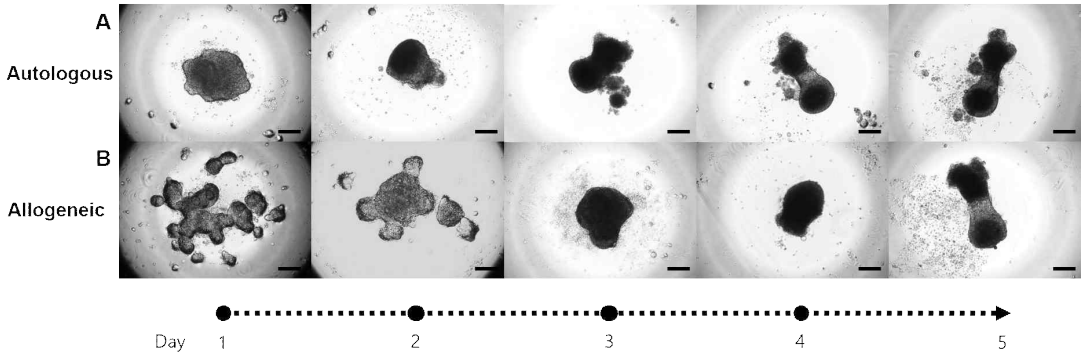

Figure 10. Comparison of autologous and allogeneic hHMF development. A) DP cells and ORS cells from same HF of patient were HD cultured for 5 days. B) DP cells and ORS cells from different patient were HD cultured for 5 days (Scale bar = 200 ㎛).

To observe allogeneic development of hHMF, DP and ORS cells from different subjects were mixed in HD culture. Time dependent development of autologous and allogeneic hHMFs are shown in Fig 10. Interestingly, allogeneic hHMF also developed from single cells. However, different morphology of autologous and allogeneic hHMF were observed on day 1 and 2. Spheroid formation has not been made in allogeneic hHMF until day 3. Our result indicates the hHMF successfully developed with allogeneic cells. To sum up, hHMF

Table I. Summary of hHMF forming efficiency

Cell type	% hHMF forming efficiency
DP P1 + ORS P1 (Autogenic)	98%
DP P1 + ORS P1 (Allogeneic)	91%
DP P1 + ORS P2	44%
DP P2 + ORS P1	93%
Hs27 + ORS P1	0
DP P1 + HaCaT	0

forming efficiency were assessed quantitatively (Table 1). Among various cell type mixtures, autologous passage 1 DP cells and ORS cells showed the highest forming capacity, likewise allogeneic cell mixture showed high percents of efficiency. When different passage cell mixtures were compared, aforesaid, hHMF with passage 2 ORS cells have develop less than 50 percent on the other hand hHMF with passage 2 DP cells showed over 90 percent of forming efficiency. Nonetheless, hHMF formation with Hs27 and HaCaT failed to develop in every single drop in HD culture.

3.5 Immunofluorescence analysis of microfollicle

De novo hHMF were analyzed with immunofluorescence staining. As shown in figure 3, expression of specific markers of DP and ORS cells were selected to identify the formation of hHMF. Expression of specific DP cell and ORS cell markers clearly demarcates the DPS and ORSS (Fig 11). Nestin and versican showed expression on DPS region and keratin 5, vimentin, and β-catenin were stained as sheath like structure. Interestingly, sheath like structure were observed around

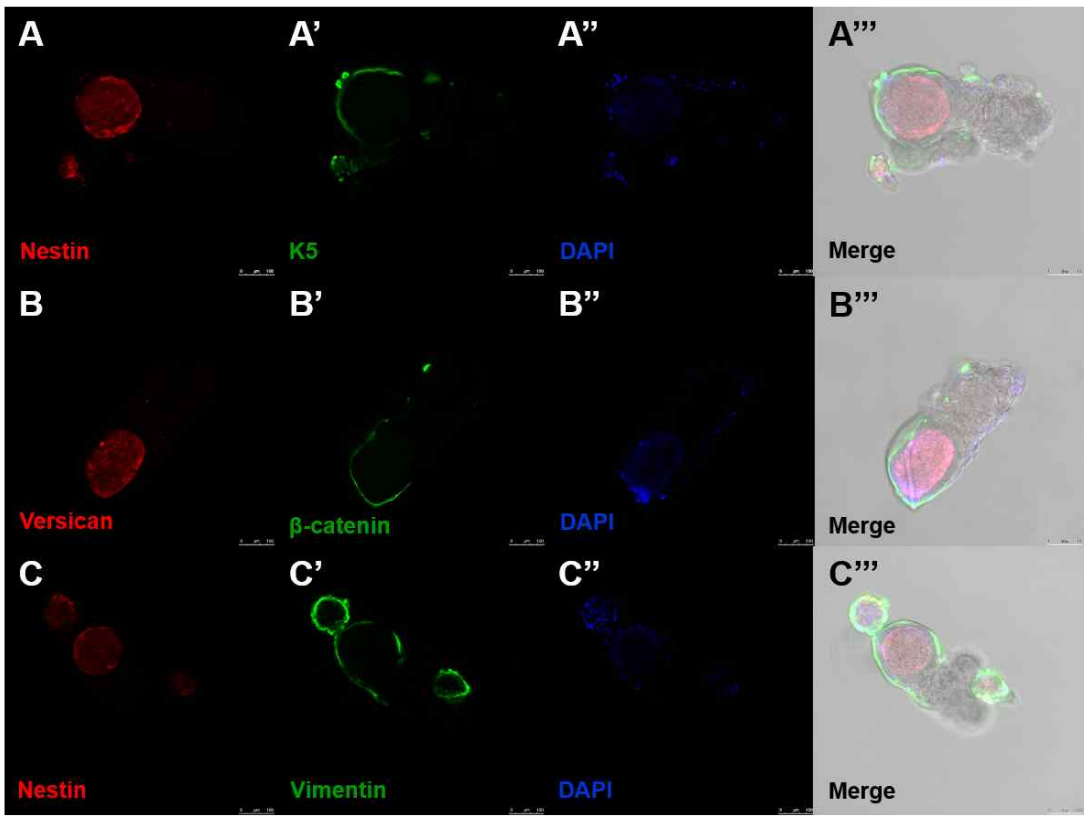

Figure 11. Characterization of hHMF by immunofluorescence staining. A) Nestin, A') Keratin 5, A") DAPI, A'") Merged. B) Versican, B') Beta-catenin, B") DAPI, B'") Merged. C) Nestin, C') Vimentin, C") DAPI, C'") Merged. Scale bars represent 100 ㎛.

every DPS including small sized DPS (Fig 11A and 11C). These results indicate that the structure of hHMF resembles the HF in that ORSS envelops the DPS and develops into a unidirectional structure. To elucidate the image more clearly, 15 confocal images were stacked together to reconstruct 3D image. ALP showed expression on the DPS region and AE13 (human hair cortex marker) showed positive expression in the ORSS area (Fig 12A). Likewise, versican and nestin expression were observed to be surrounded by K15 and beta-catenin expression respectively (Fig 12B and 12C). Together, immunofluorescence images demonstrate that DPS is surrounded by an ORSS which resembles the morphology of HF.

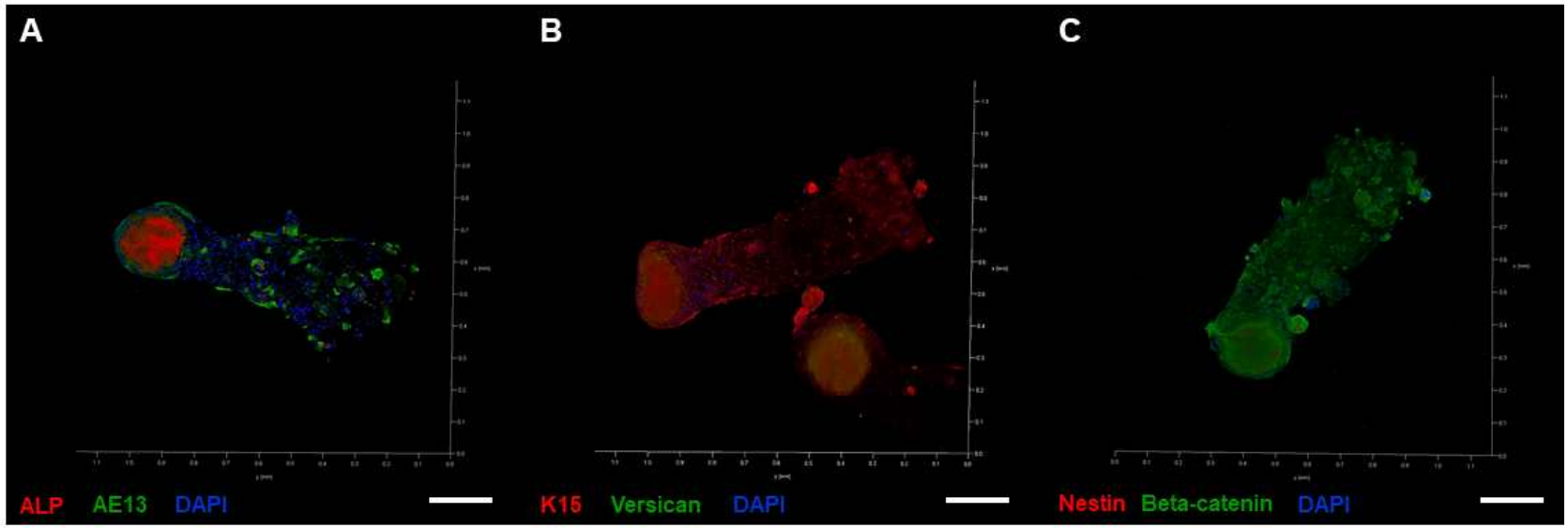

Figure 12. 3D reconstruction of hHMF confocal image. Fifteen confocal images were stacked to reconstruct 3D image. A) Alkaline phosphatase (red) and AE13 (green) were labeled on hHMF. B) Keratin15 (red) and versican (green) showed in hHMF. C) Nestin (red) positive were observed to be enveloped by beta-catenin (green) labeled cells. Scale bars represent 200 ㎛.

3.6 Establishment methodology of *de novo* hair microfollicle development

To sum up, a brief schematic overview of the development of hHMF method is shown in Figure 13. DP and ORS cells were cultured simultaneously at the same date point. It is critical to manipulate the equivalent growth rate of the two types of cells at first subculture on day 15. This is because if one of the cells grow faster than the other, it is infeasible to seed the two types of cells in HD culture on the same day. When cells were introduced to HDP, the cells developed into hHMF until day 5. With the hHMF development methodology, we have developed a number of hHMFs (Fig 14). hHMFs were able to visualize with bare eyes (Fig 14A) and under microscopy (Fig 14B). Morphology of hHMFs showed uniformity in that it has unidirectional structure; DPS were enveloped with ORSS, and resembles HF. Our results suggest that the method we established has high reproductivity showing that the robust production of hHMF is possible.

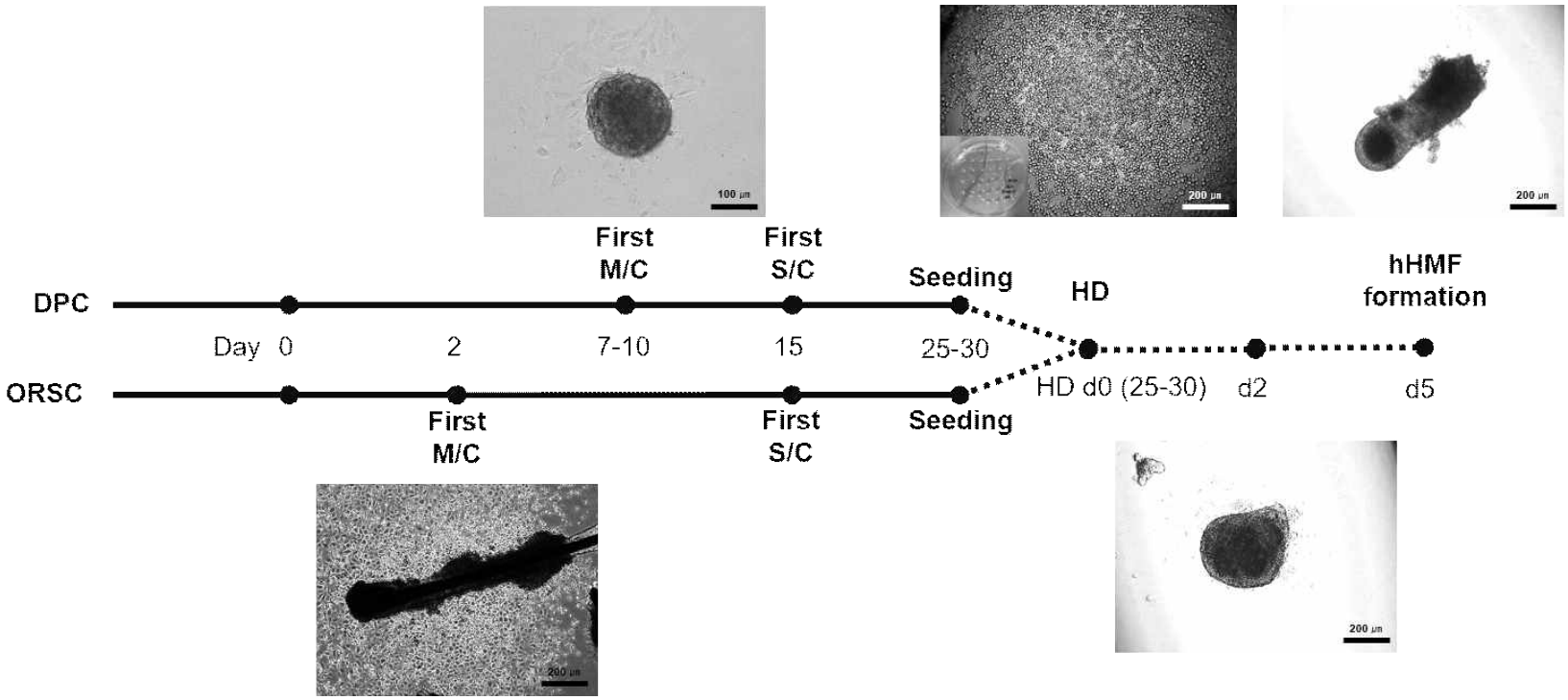

Figure 13. Schematic overview of hHMF development. DP cells and ORS cells were cultured individually at different timeline until introduction to HD culture plate. Media change of DP cells must be done after attachment of DP on the plate. On day 25-30, 1×10^4 cells of each cells were inserted into each well of HD plate. hHMF were developed after 5 days of 3D culture (DPC: Dermal Papilla cells; ORSC: Outer Root Sheath cells; M/C: Media Change; S/C: Subculture; HD: Hanging Drop culture; hHMF: human Hair Microfollicle).

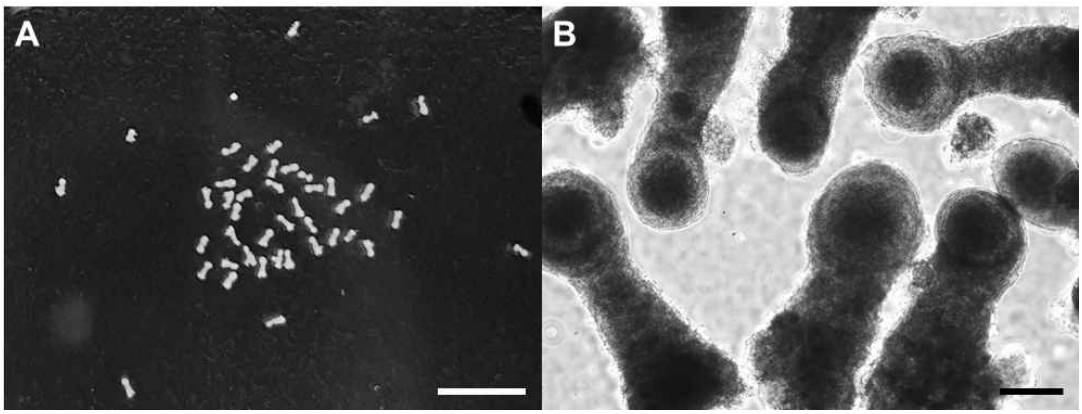

Figure 14. hHMF mass production demonstration. A) Photograph of hHMF which can be observed with bare eye (Scale bar = 1 cm). B) Morphology of hHMF under light microscopy (Scale bar = 500 ㎛).

4. Discussion

In this study, we demonstrate spontaneous reconstruction of hHMF with efficient HD culture method using primary cultured DP cells and ORS cells. Self-assembly of the DP cells and ORS cells suspension has formed into unidirectional glabrous structure which resembles the morphology of human HF.

At the initial stage of HF morphogenesis, mesenchymal cells condensate under control of epithelial-mesenchymal interactions. Then, epithelial progenitor cells surround the condensed mesenchymal cells and diverse molecular signals (e.g. BMP, wnt, notch and other genes) exchange to form a HF (Paus and Cotsarelis, 1999; Sennett and Rendl., 2012). Previous studies have been attempted to reconstruct HF using rodent mesenchymal cells and epithelial cells isolated from embryonic mice in utero (Qiao et al, 2008; Kang et al, 2012; and

Asakawa et al, 2012). However, with the ethical issues regarding the use of human embryo, it is inconceivable to attain human mesenchymal cells and epithelial cells in embryonic stage. There is another way to reconstruct HF by using induced pluripotent stem (iPS) cells to differentiate into HF cells, but it requires advanced techniques of handling iPS cells. Thus, to regenerate human HF in vitro, use of primary cultured cells isolated from human HF would be the easiest and less laborious method. Aforesaid, many preliminary studies reported the effective methods of HF cell isolation (Messenger, 1984; Warren et al., 1992; Li et al., 2005; Inoue and Yoshimura, 2013; Gledhill et al., 2013; Chan et al., 2015). Since 1984, isolation of DP from human hair follicle has been studied. We used traditional microdissection method to isolate DP without any enzyme treatment. It is efficient to cut the scalp along with the hair shaft direction for the isolation of HF. To extract ORS cells from HF, we have selected dispase II to isolate ORS cells effectively.

Unfortunately, we have not yet proved the phenomenon of hHMF development clearly. However, we were able to hypothesize the mechanisms of hHMF development through previous studies. It is well known theory that ORS cells residing in the bulge area possess epidermal stem cell properties and migrate along with the ORS at the anagen phase (Reynolds and Jahoda, 1991). Condition of ORS cells were considered to be more critical in development of hHMF.

Senescent ORS cells exponentially increased after each sub-culture. When relatively high passaged ORS cells were applied in HD culture, it is more likely to show failure development of hHMF. Also, previous studies have reported that DP cells lose their hair inductivity as they were subcultured (Oyama et al, 2012; Higgins et al, 2013). Together, we concluded that the progenitor cells associated with hHMF development are mostly distributed in ORS cells, and DP cells and ORS cells should be subcultured as minimal to form a hHMF successfully. Moreover, it was reported that DP is known as a reservoir of multipotent stem cells which activates stem cells in the hair germ at the onset of anagen stage (Driskell et al., 2011). Furthermore, DP cells gain hair inductivity when recombined with bulge-derived epithelial cells and showed chemotactic effect when co-cultured together (Fujie et al, 2001; Toyoshima et al., 2012; Hill et al., 2013). Other studies reported that DP secrete a chemotactic factor which increase migration such as insulin-like growth factor I (IGF-I) and hepatocyte growth factor (HGF) (Arase et al., 1990, 1994; Fujie et al., 2001). Therefore, we speculated that recombination of DP cells and ORS cells in HD culture may have altered DP cells to secrete GFs for ORS cells and triggered small portion of mesenchymal and epithelial progenitor cells to differentiate into HF-like structure which were demonstrated in our study.

Hair regenerative studies using murine mesenchymal cells and

epithelial cells from embryonic mouse skin have successfully reconstituted de novo HF (Qiao et al, 2008; Kang et al, 2012; and Asakawa et al, 2012). Most hair regenerative studies re-transplanted the HF germ into in vivo environment such as sub-renal capsule or under epidermis of nude mouse. Our ultimate goal is to develop a HF in vitro without using animals. The limitation of mimicking in vivo environment in in vitro is still unsolved obstacles in regenerative medicine. Although morphology of hair shaft was not clearly observed in hHMF, there are possibilities of hair shaft differentiation when transplanted in vivo or by manipulating the environment of 3D culture with additional factors (e.g. medium, GFs, ECM). Establishment of microenvironment using the factors will be required for the long-term culture of hHMF after day 5. In fact, DP cells sustained its hair inductivity with GFs such as bone morphogenetic protein (BMP), Wnt-10b, (Shimizu et al., 2004; Rendl et al., 2008; Ouji et al., 2016) and promoted hair growth ability with treatment of conditioned medium of adipose derived stem cells (ADSC) and endothelial cells (EC) (Won et al., 2010; Bassino et al., 2015). Moreover, DP cells enhanced their cell adhesivity and motility when cultured in ECM protein coated plates such as matrigel, collagen I, collagen IV and laminin (Young et al, 2009; Miao et al; 2014). Thus, with treatment of additional GFs and ECM to enhance or maintain hair-inducing properties, we suggest that hHMF could be developed as a fully

functional HF when applied in our methodology, and screening appropriate GFs and ECM will be our following study.

5. Conclusion

In conclusion, we developed an hHMF which mimics HF with spontaneous localization of DP and ORS cells using HD culture. We have demonstrated that the hHMF develops after 5 days of HD culture with DP and ORS cells mixed together. For successful development, low passaged and healthy cells are required before seeding into HD plate. Morphology of hHMF resembles the HF that ORS surrounding DP which were observed under light microscopy. Moreover, we proved that the hHMF formed with autologous cells as well as with allogeneic cells. With immunofluorescence analysis, we confirmed that the hHMF express specific characteristics of DP and ORS cells in the same location in accordance with HF. We expect that establishment of efficient hHMF development methodology will bring novel impact on hair regenerative studies and tissue engineering. With more advanced study of hHMF, we anticipate the reconstitution of HF *in vitro*. Further study is needed to investigate the specific mechanism of spontaneous localization of the cells in hHMF development. Besides, to reduce the laborious loads and long-term culture, establishment of robust culture system using microfluidics with chip-based culture would be our next task.

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국문 초록

배양된 인간의 모유두세포와 외모근초세포를 이용한 새로운 마이크로 모낭의 개발

박 지 호
협동과정 줄기세포생물학 전공
서울대학교 대학원

인간 모낭의 발달은 배아 단계에서 mesenchymal-epithelial 사이의 복잡한 상호 작용의 결과이다. 최근 mesenchymal-epithelial interaction 메커니즘을 이용한 연구가 조직 공학 및 재생 의학 분야에 많이 응용되고 있다. 모낭의 재건 또한 mesenchymal-epithelial mechanism을 이용한 많은 연구가 시도되고 있지만 in vitro상에서 완전한 모낭의 재생은 여전히 어려움을 겪고 있다. 본 연구에서 우리는 모유두세포(DP)와 외모근초세포(ORS) 인간의 두피에서 추출하였고 이를 이용하여 인간 미세 모낭

(hHMF)를 개발하였다. 배양된 DP 세포를 mesenchymal 세포로 사용하였고 ORS 세포를 epithelial 세포로 사용하여 mesenchymal-epithelial 상호작용을 유도하였고 3D hanging drop system(HD)에서 5일 동안 배양시켜 새로운 hHMF로 발달시켰다. 우리는 젊은 환자의 두피에서 간단하고 효과적인 방법으로 DP 및 ORS 세포를 추출하였고 면역 형광 염색을 통해 특이적 항체를 붙여 세포를 특정하였다. DP 세포와 ORS 세포는 우리가 확립한 특정 타임 라인에 따라 배양되었다. HD 배양에서 5일 간 배양한 후 hHMF의 발달이 관찰되었다. hHMF의 형태가 단일 방향 구조를 가지고 DP 구면이 모구 모양의 영역에 존재한다는 점에서 모낭과 유사했고 DP와 ORS 단일 세포들이 HD 배양을 통해 자발적으로 hHMF로 형성되는 것을 관찰 할 수 있었다. hHMF 또한 면역 형광 염색 분석을 통해 구조를 분석하였는데 특정 부위에 DP와 ORS 세포 마커의 발현을 관찰 할 수 있었다. 이 간단한 hHMF 발달 기술은 in vitro내에서 모낭 형성의 메커니즘을 입증할 잠재력을 제공할 것으로 기대된다. 또한, 우리가 설립한 방법에 새로운 성장인자 혹은 세포외기질을 추가하여 in vitro 상에서 완벽한 모낭이 형성 될 것이라 기대한다. 최종적으로, 진보된 배양 시스템을 통해 탈모 환자의 모발 관련 약물을 스크리닝 하거나 hHMF를 이용한 모발 이식이 가능해질 것이다. 우리 연구 결과는 조직 공학 및 모발 재생 연구에 새로운 통찰력을 가져올 것으로 기대된다.

주요어 : 모낭, 모유두세포, 외모근초세포, 3D 배양, 조직공학, 재생의학
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