Preliminary evaluation of human hair follicle-derived dermal papilla cell characteristics

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ABSTRACT

Dermal Papilla Cells (DPCs) are mesenchymal cells residing at the base of the hair follicles. They play an important role in the hair cycle remodeling and hair follicle regeneration. In this study, DPCs were isolated, and cultured from human hair follicles and evaluated the expression of α-SMA, versican by immunocytochemistry, and alkaline phosphatase activity. The human fibroblasts were used as a control to confirm the non-fibroblast DPCs. The results showed that the cells grew from the hair bulb and had fibroblast morphology. These bulb cells strongly expressed alkaline phosphatase activity α-SMA, and versican while the human fibroblast lacked ALP activity and less expression of α-SMA and versican. In conclusion, the protocol is established to isolate and culture DPCs from hair follicle bulbs, and ALP activity is suggested as the major marker for distinguishing DPCs and human fibroblasts.

1. Introduction

The hair follicle is a dynamic little organ made up of a variety of mesenchymal and epithelial cells. The dermal papilla cell from the bulb is one of the hair follicle's well-known mesenchymal cells (Gan et al., 2022). The cells were roles as control centers for the growth and cycles of the hair via asymmetric divisions and differentiations to self-renewal and generate progenies to restore tissues such as hair shaft and inner root sheath (Legue & Nicolas, 2005). They release growth factors, including epidermal growth factor, transforming growth factor-b, and keratinocyte growth factor to promote the proliferation, and differentiation of follicle epithelium, and regulate mesenchymal-epithelial interaction (Lichti et al., 1993). Other studies showed that DPCs were considered as a decided factor in hair cycles, and morphology (Chi, Wu, & Morgan, 2013). Specified markers of DPCs were found such as mesenchymal morphology, expression of alkaline phosphatase (ALP), α-smooth muscle actin (αSMA), versican, and CD133 (Taghiabadi, Nilforoushzadeh, & Aghdami, 2020). ALP activity was strong in the early anagen and declined since the decrease of the hair inductivity in the dermal papilla and dermal sheath (Rendl, Polak, & Fuchs, 2008). αSMA was known as an *in vitro* marker of DPCs more than an *in vivo* marker (Jahoda, Reynolds, Chaponnier, Forester, & Gabbiani, 1991). Versican was proteoglycan and played an important role in maintaining the anagen stage and expressed during this stage (Kim, Cha, Kim, Kim, & Sung, 2006; Soma, Tajima, & Kishimoto, 2005). Previous studies showed that

the loss of versican expression was found in the alopecia patients (Yang et al., 2012). CD133 was a stem cell marker, expressed in the early anagen, and inducted hair follicle neogenesis (Ito et al., 2007). Besides, DPCs are a potential source, that contains adult stem cells of the skin, and participate in the de novo formation of hair follicle structure, reconstitution, and wound healing (Biernaskie et al., 2009; Jahoda, Horne, & Oliver, 1984). Thus, DPCs were used as a therapy to treat alopecia (Driskell, Clavel, Rendl, & Watt, 2011).

Human fibroblast-derived skin (hFs) are mesenchymal cells and located in the dermal layer of the skin (Rittie & Fisher, 2005). They have specified features, including spindle morphology, and expression of mesenchymal markers (Goodpaster et al., 2008). Markers such as fibronectin, vimentin, and CD90 were found in dermal fibroblast (Gabbiani, 2003; Goodpaster et al., 2008). hFs produced proteins of the extracellular matrix that type I, and II collagen were the main components to interact with epidermal cells and fortify the skin compartment (Kisiel & Klar, 2019). Other findings demonstrated that these cells strongly expressed αSMA, differentiated into myofibroblast, and played key roles in wounding healing and fibrosis (Darby & Hewitson, 2007; Gabbiani, 2003).

Some features of DPCs are similar to hFs because of the same mesenchymal origin. Therefore, distinguishing these cell lines is essential for isolation. In our study, we isolated DPCs and found differences between DPCs and hFs to service post-studies of hair follicle engineering.

2. Material and method

2.1. Human hair follicle isolation

Human hair follicles (HFs) were collected following a process approved by the 7A Military Hospital's Ethical Committee for Biomedical Research (protocol code 04/QY7A-HĐĐĐ, approval on May 22nd, 2020). The volunteers received informed written consent, which was then explained to them and signed. The hair follicles were collected from non-bald scalps by Follicular Unit Extraction (FUE) technique and then were treated with the mixture solution of collagenase (0.14 mg/ml) and dispase (4 mg/ml) for 15 minutes at 37^{o}C. The surrounding tissues were removed and the HFs were obtained. The anagen HFs were determined according to previous guidance (Kloepper et al., 2010), and then the bulbs were isolated from the HFs using a binocular microscope.

2.2. Culture of dermal papilla cells

Minor adjustments were made to the described methodology in order to separate the papilla bulbs from the hair follicles (Mali et al., 2018). The hair bulbs were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with supplements of 20% Fetal Bovine Serum (FBS), 1% antibiotic-antimycotic solution, 50 μg/ml hydrocortisone, 10 ng/ml insulin, and 10 ng/ml Fibroblast Growth Factor (FGF). The hair bulbs were grown on type I collagen-treated plates. For the first four days, the plates were incubated at 37°C with 5% CO_2; after that, the media was changed every three days. The sub-culture was carried out after the cell growth reached 90% confluency. 0.25% Trypsin/EDTA was used to detach the cells, and they were then re-suspended in a culture flask at a ratio of 1:3 (based on surface area). The flasks were supplemented with the fresh medium and cultured at 37^{o}C, 5% CO_2.

2.3. Culture of human fibroblasts

Human adult fibroblasts were purchased from ATCC (American Type Culture Collection). The cells were thawed and cultured following the manufacturer's instructions. The human

fibroblasts were cultured in a medium containing DMEM/F12, 10% fetal bovine serum, and 1% antibiotics and sub-cultured three passages.

2.4. Immunocytochemistry

The cultured cells were fixed in 4% paraformaldehyde. For intracellular marker staining, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS overnight at 4°C. Then, they were blocked by normal goat serum (Abcam) and incubated with anti-versican antibody (1:200, Abcam), and anti-alpha SMA antibody (1:200, Abcam) in PBST with 1% BSA overnight at 4°C. Further, the cells were incubated with Alexa Fluo® 488-labeled anti-mouse secondary antibodies (1:500, Abcam) or Alexa Fluo® 488-labeled anti-rabbit secondary antibodies (1:500, Abcam) in the dark for 1 hour at room temperature. The cell nuclei were stained with DAPI solution (1:200, Abcam). The images were captured using Cytell™ Cell Imaging System (GE Healthcare Life Sciences).

2.5. Alkaline phosphatase assay

Using the alkaline phosphatase test kit (Abcam, USA) following the manufacturer's instructions to determine alkaline phosphatase activity in the cells. Trypsin was used to detach the cultivated cells, and a hemocytometer was used to count. A volume of 50 microliters of test buffer received 10^5 cells, which were then added and homogenized. The cell lysate solution was centrifuged at 10,000rpm for 15 minutes at 4°C to get the supernatant (sample). The reaction was set up following the manual protocol. After 60 minutes of room temperature incubation, the output was measured at OD 405mn.

2.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, Incorporated company). Results were calculated as the mean of triplicate readings. The data were analyzed for statistical significance with a one-way ANOVA, where $p < 0.05$ was considered statistically significant.

3. Result

3.1. Isolation of hair follicle bulbs and primary culture of human hair follicle dermal papilla cells

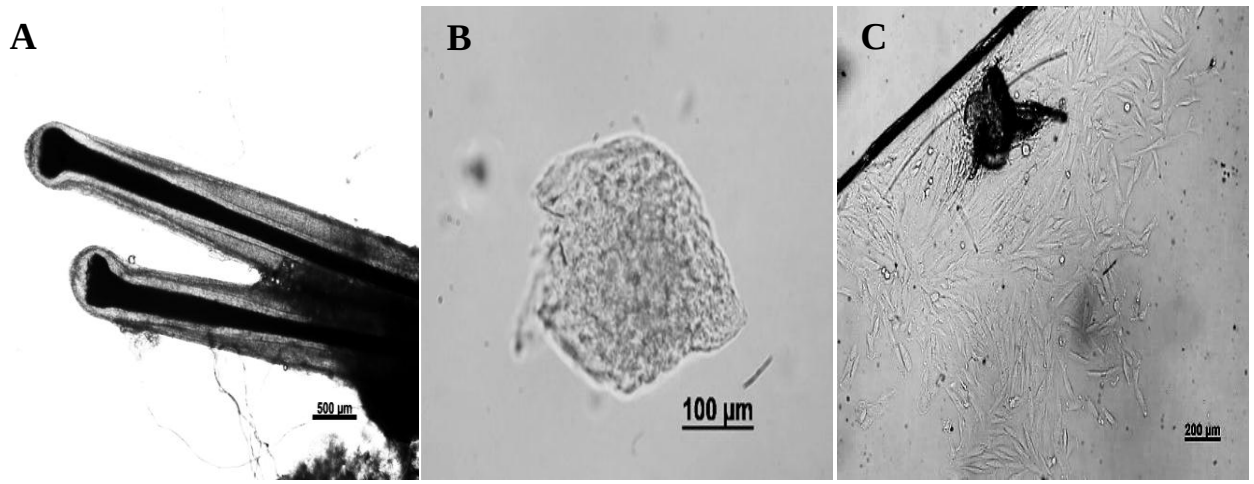


Figure 1. Collected human hair follicles (A) and isolated hair bulbs (B);
and the culture of human hair follicle dermal papilla cells: the fibroblast-shape cells adhered and expanded from the hair bulb (C)

Hair follicles were harvested from donors (Figure 1A) and the HFs at the anagen phase were used to isolate the bulbs (Figure 1B). In this 2D culture, the bulbs adhered to the plastic surface after 24 hours of culture. Dermal papilla cells grew from these bulbs, after culturing for 07 days (Figure 1C). The attached cells exhibited a spindle shape similar to fibroblasts. After 14 days of culture, the cells covered more than 90% of the culture surface and were sub-cultured. The cell population was homogenous at the first passage and maintained the morphology of mesenchymal cells after three passages.

3.2. Culture and passaging of the human fibroblasts and hair follicle dermal papilla cells

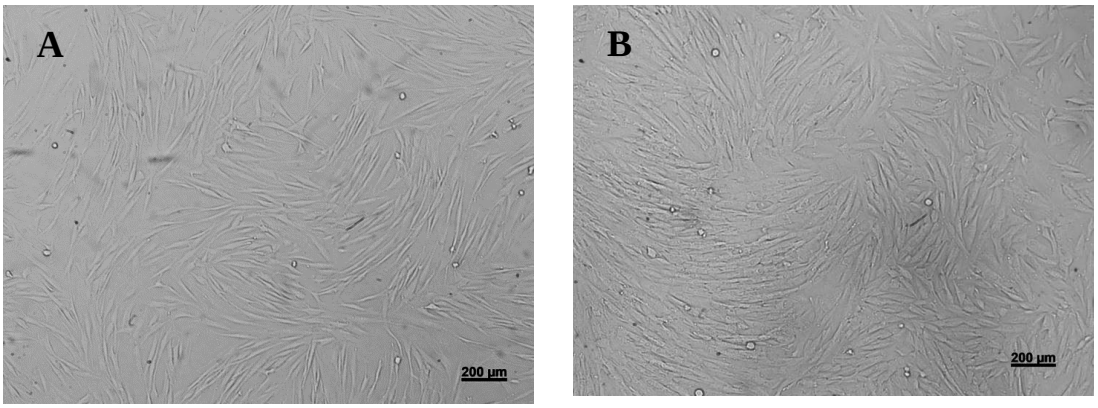
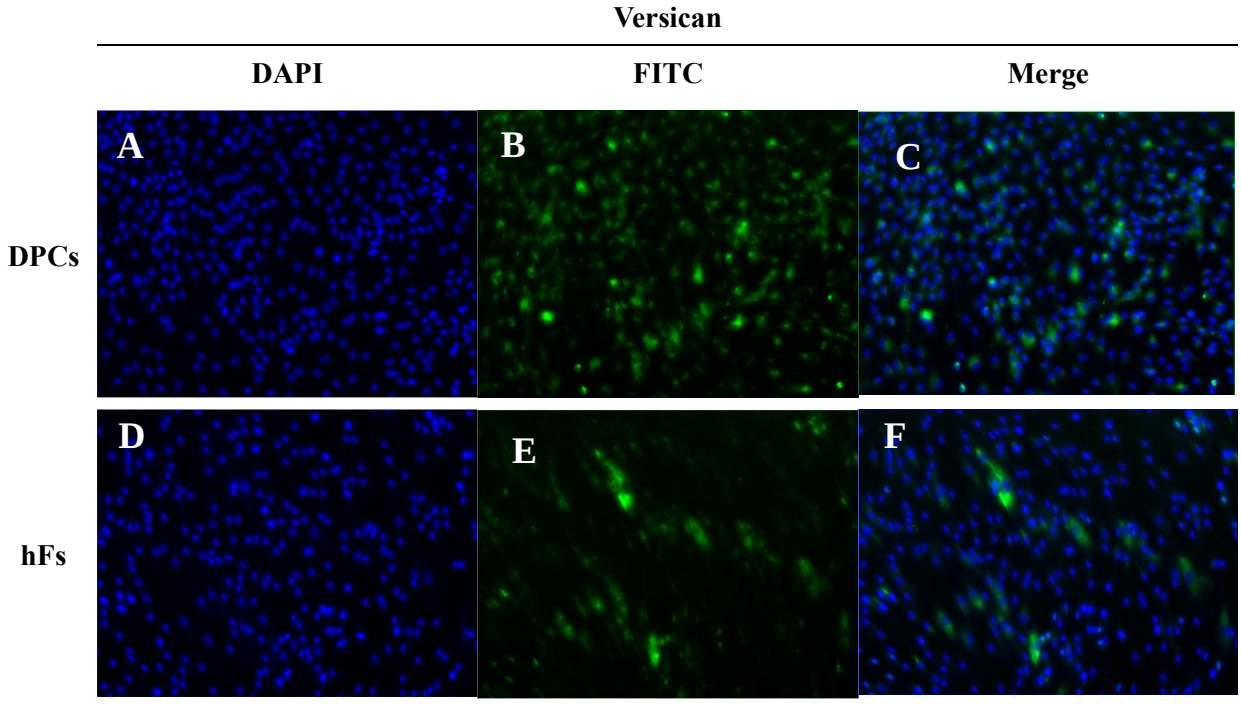


Figure 2. The morphological images of human fibroblasts (A) and human hair follicle dermal papilla cells (B)

Both human fibroblasts and human DPCs exhibited the spindle shape although there is a minor difference between them. The human fibroblasts had a long-spindle appearance whereas the DPCs were shorter and thicker (Figure 2). The DPCs at passage 3 and the human fibroblasts after sub-culture 03 times were used to analyze the characteristics of the human DPCs.

3.3. Comparative results of characterization between human dermal papilla cells and human fibroblasts



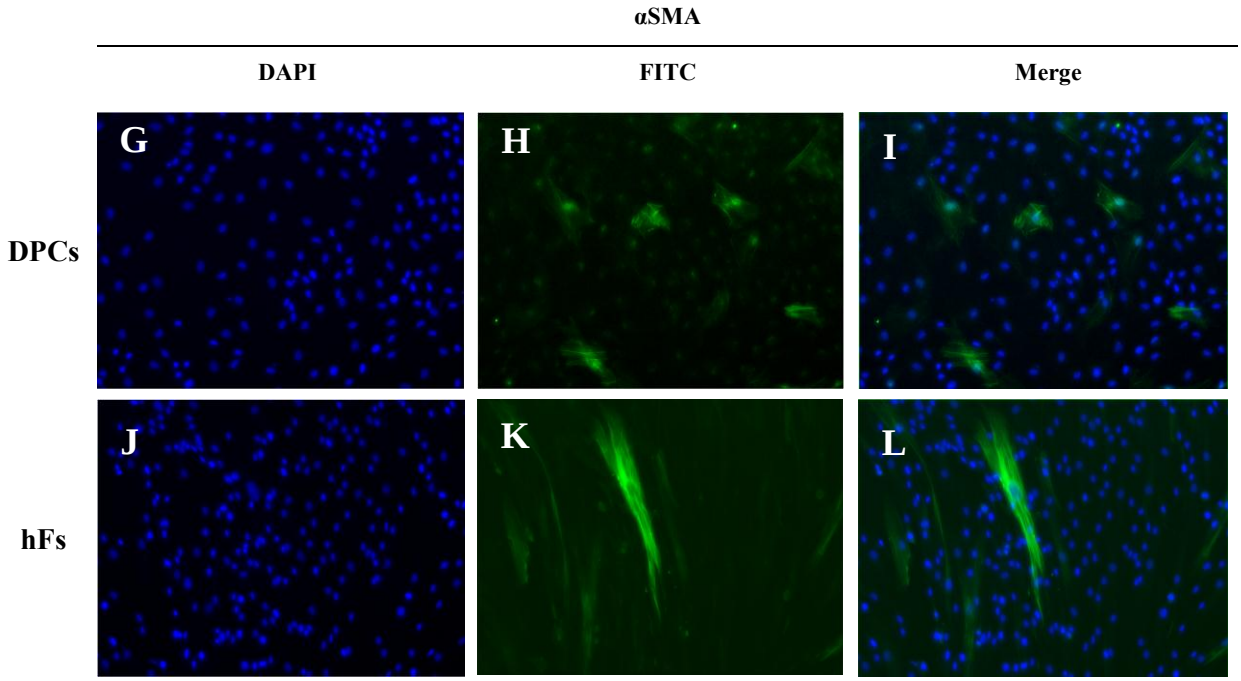


Figure 3. The expression of versican and α-SMA in human hair follicle dermal papilla cells and human adult fibroblasts: the nuclei staining with DAPI (A, D, G, J); the green fluorescence stained with versican in DPCs (B) and human fibroblast (E); the staining with α-SMA in DPCs (H) and human fibroblast (K); and the merged images of stained nuclei and makers in DPCs (C, I) and human fibroblast (F, L)

Versican and α-SMA describe its specific expression in human dermal papilla cells (Taghiabadi et al., 2020). In this study, the result of immunostaining with versican showed that this marker was strongly expressed in the cultured DPCs while its expression was lower in human fibroblasts (Figure 3A-F). The result of the versican expression was consistent with the α-SMA expression result. The expression of α-SMA was more obvious in DPCs than in its dispersed expression in human fibroblasts (Figure 3G-L). Thus, the expression of versican and α-SMA was detected in both human fibroblasts and DPCs although there is a small difference between them. We performed the alkaline phosphatase (ALP) activity assay to evaluate human fibroblasts and DPCs. The result revealed that ALP activity was preferentially expressed in DPCs than in human fibroblasts (Figure 4). This data reinforces that ALP activity is the most specific characteristic of the human dermal papilla cells.

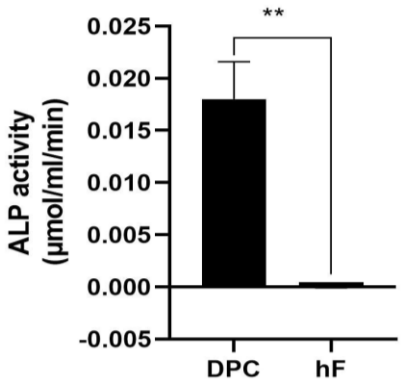


Figure 4. The evaluation of alkaline phosphatase activity in human hair follicle Dermal Papilla Cell (DPC) and human fibroblast (hF) ($p < 0.05$)

Note: ** represents the statistical significance

4. Discussion

Dermal Papilla Cells (DPCs) are mesenchymal cells and play a key role in hair follicle morphogenesis and regeneration. DPCs regulate the hair cycle which includes three major phases: anagen (growth), catagen (degeneration), and telogen (resting). The DPC signaling and secretome are responsible for many events occurring in the hair cycle (Bejaoui, Taarji, Saito, Nakajima, & Isoda, 2021; Nilforoushzadeh, Aghdami, & Taghiabadi, 2020). DPCs reside at the base of hair follicles and could be *in vitro* cultured in 2D and 3D manner (Mali et al., 2018; Ohyama, Kobayashi, Sasaki, Shimizu, & Amagai, 2012; Topouzi, Logan, Williams, & Higgins, 2017). In 2D culture, the DPCs adhere to the plastic surface and have fibroblast morphology. There are several important notes that could influence the efficacy of DPCs isolation: that is, (i) the right determination of the anagen phase; (ii) the right excision of the HF bulb regions; (iii) the right culture medium; and (iv) the characterization of the DPCs. In this study, we harvested the Hair Follicles (HFs) by using the Follicular Unit Extraction (FUE) technique, and the HFs were then examined during the anagen period following the previously described publication (Kloepper et al., 2010). The bulbs were isolated from the anagen HFs by micro-dissection technique. The cells explanted from the bulb tissue after 07 days of culture and exhibited the typical shape of mesenchymal cells. That is easy to mistake dermal papilla cells for fibroblasts and other mesenchymal cells if only based on their morphologies. Thus, we suggest evaluating several typical characteristics of DPCs to find the differences between the isolated DPCs and standard fibroblasts supplied by ATCC. The typical characteristic prominently expressed in DPCs is alkaline phosphatase (ALP) activity (Gentile & Garcovich, 2019; Nilforoushzadeh et al., 2020). ALP activity also plays a useful role in determining the position and function of the DPCs (hair-inductive capacity) (Kwack et al., 2019). In our study, the expression of ALP activity was much more dominant in DPCs than in human fibroblasts. Their results demonstrated that DPCs cultured in 2D and 3D expressed strong ALP (Betriu, Jarrosson-Moral, & Semino, 2020). Other research showed that ALP gene expression of DPCs higher than fibroblast (Ohyama et al., 2012). These results suggested that ALP activity is a useful marker for distinguishing DPCs and human fibroblasts. The other specific markers of DPCs are versican and α-SMA (Nilforoushzadeh et al., 2020). Versican is a proteoglycan preferentially expressed in DPCs than other HF cells (Taghiabadi et al., 2020). Versican and its gene, VCAN, express strongly in DPCs at the anagen phase (Taghiabadi et al., 2020) and down regulation in DPCs from aging (Jo et al., 2016) or androgenetic alopecia humans (Taghiabadi et al., 2020). α-Smooth Muscle Actin (α-SMA) is another *in vitro* marker to identify DPCs. α-SMA is expressed in both DPCs and the mesenchymal cells from a dermal sheath of the hair follicle. Therefore, α-SMA might not be a unique and high-confidence marker to identify DPCs. Indeed, in our study, the expression of α-SMA and versican were obviously found in cultured DPCs and less in human fibroblasts. These data suggest that α-SMA and versican could be auxiliary markers for identifying the DPCs from the bulb but not appropriate to distinguish DPCs and human fibroblasts.

5. Conclusion

In summary, we established the condition for 2D culturing human dermal papilla cells from hair follicle bulbs. The cultured dermal papilla cells possessed the fibroblast morphology and expressed the typical characteristics including the expression of alkaline phosphatase activity and proteoglycan versican, and the α-SMA. In addition, the alkaline phosphatase activity was proved to be a useful marker to distinguish DPCs and human adult fibroblasts.

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