Journal of Cell Science
Research article

Title: Restoration of the intrinsic properties of human dermal papilla in vitro

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Running title: Restoration of dermal papilla

Key words: Human, dermal papilla, molecular signature, culture, restoration

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Word count: 6820 (excluding references)
Abstract word count: 247
Figures: 6
Tables: 2, with 5 supplemental tables

Conflict of interest: none declared
Summary

The dermal papilla (DP) plays pivotal roles in hair follicle morphogenesis and cycling. However, characterization and/or propagation of human DP have been unsatisfactory because of the lack of efficient isolation methods and the loss of innate characteristics in vitro. We hypothesized that culture conditions sustaining the intrinsic molecular signature of human DP could facilitate expansion of functional DP cells. To test this, we first characterized the global gene expression profile of microdissected, non-cultured human DPs. We performed a “two-step” microarray analysis to exclude the influence of unwanted contaminants in isolated DPs and successfully identified 118 human DP signature genes, including 38 genes listed in the mouse DP signature. The bioinformatics analysis of the DP gene list revealed that WNT, BMP and FGF signaling pathways were upregulated in intact DP and addition of 6-bromoindirubin-3'-oxime, recombinant BMP2 and basic FGF to stimulate these respective signaling pathways resulted in maintained expression of in situ DP signature genes in primarily cultured human DP cells. More importantly, the exposure to these stimulants restored normally reduced DP biomarker expression in conventionally cultured DP cells. Cell growth was moderate in the newly developed culture condition. However, rapid DP cell expansion by conventional culture followed by the restoration by defined activators provided a sufficient number of DP cells, which demonstrated characteristic DP activities in functional assays. The present study revealed previously unreported molecular mechanisms contributing to human DP properties and describes a useful technique for the investigation of human DP biology and hair follicle bioengineering.
Introduction

The dermal papilla (DP) is a highly specialized mesenchymal component located at the base of hair follicles (Paus and Cotsarelis, 1999; Stenn and Paus, 2001; Driskell et al., 2011). Previous studies, including hair reconstitution experiments, have demonstrated the indispensable roles of DP in epithelial–mesenchymal interactions that enable hair follicle morphogenesis and cycling (for reviews see Oliver, 1991; Jahoda and Reynolds, 1996; Millar, 2002; Botchkarev and Kishimoto, 2003; Ohyama et al., 2010; Yang and Cotsarelis, 2010; Driskell et al., 2011). In mice, elegant transgenic approaches (Kishimoto et al., 1999; Rendl et al., 2005; Driskell et al., 2009) and newly identified cell surface markers such as CD133 (Ito et al., 2007) and α9-integrin (Rendl et al., 2008) enabled fluorescent labeling and targeted isolation of living DP cells. Sufficient numbers of mouse DP cells are now available for downstream applications such as microarray analysis and tissue regeneration assays (Rendl et al., 2005; Rendl et al., 2008; Driskell et al., 2009). However, isolation of human DP cells mostly depends on manual microdissection, which provides a limited number of DP cells (Messenger, 1984; Magerl et al., 2002; Ohyama et al., 2010). Accordingly, previous studies of human DP predominantly utilized cultured DP cells (Inui et al., 2003; Pflieger et al., 2006; Iino et al., 2007; Park et al., 2007; Shin et al., 2010). A potential disadvantage of this approach is the loss of some intrinsic properties of DP cells during culture (Ohyama et al., 2010; Yang and Cotsarelis, 2010). Loss of biological characteristics, especially hair inductive capacity, has been reported in rodent (Jahoda et al., 1984; Horne et al., 1986; Lichti et al., 1993; Weinberg et al., 1993) and canine (Kobayashi et al., 2011) DP cells, and is likely to occur in human DP cells. Thus, a clear demand exists for an approach that allows in vitro expansion of human DP cells without affecting their in situ characteristics.

Previous studies reported various approaches to maintaining in vivo characteristics in cultured DP cells, including co-culture with keratinocytes (Reynolds and Jahoda, 1996; Inamatsu et al., 1998; Kobayashi et al., 2011), cultivation on extracellular matrices, and sphere formation (Osada et al., 2007; Young et al., 2009; Higgins et al., 2010; Kobayashi et al., 2011). The hair inductive capacity of rodent and canine DP cells was maintained in cultures treated with keratinocyte-derived factors (Inamatsu et al.,
1998; Kobayashi et al., 2011) and ligands in signaling pathways involved in hair follicle morphogenesis, including Wnt3a and Bmp6 (Kishimoto et al., 2000; Shimizu and Morgan, 2004; Rendl et al., 2008). However, whether a similar approach is feasible with human DP cells has not been fully assessed.

In the present study, we first elucidated the molecular signature of intact, non-cultured human DP. Subsequent bioinformatics analysis identified biological pathways that are activated in DP. By administering an optimized combination of activators of those pathways, a culture condition that maintained or even restored intrinsic DP characteristics was successfully developed. The current study uncovered previously unreported molecular characteristics of in vivo human DP and described an efficient methodology with which to propagate DP cells while maintaining intrinsic properties.
Results

Microarray analysis of total RNA isolated from freshly microdissected human DP

In our attempt to elucidate the global gene expression profile of intact human DP (Fig. 1A), 15 DPs were microdissected from anagen hair follicles obtained from four volunteers (Fig. 1A). As controls, human DP cells and fibroblasts primarily cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (10% FBS-DMEM) (de Almeida et al., 2005; Wu et al., 2005; Iino et al., 2007) were prepared from the same individuals (Fig. 1A). Real-time PCR analysis of total RNA isolated from freshly isolated human DP (fDP), cultured DP (cDP) cells, and fibroblasts (Fibros) detected upregulation of genes that have been used to confirm successful DP cell isolation (Botchkarev and Kishimoto, 2003; Rendl et al., 2005; Ito et al., 2007; Osada et al., 2007; Driskell et al., 2009; Ohyama et al., 2010), such as alkaline phosphatase (ALP), noggin (NOG), WNT inhibitory factor 1 (WIF1), and versican (VCAN), in fDP as compared with cDP cells and Fibros, demonstrating successful RNA recovery (Fig. 1B). The same total RNA samples were analyzed using four sets of Affymetrix HG-U133A 2.0 GeneChip microarrays. Despite limitations in the starting materials, the GeneChip data were of high quality (Khanna et al., 2004) and comparable with one another (Ohyama et al., 2006), with 55.5 ± 3.14% of transcripts found to be expressed and an average correlation coefficient of 0.95 ± 0.03 for the microarrays representing each population. The raw GeneChip data files are accessible at NCBI’s Gene Expression Omnibus through GEO Series accession number GSE31324 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31324).

Elucidation of gene expression profiles that distinguish intact human DP from conventionally cultured human DP cells and fibroblasts

Global gene expression profiles for fDP, cDP, and Fibro GeneChips were subsequently compared to identify genes that are overrepresented in fDP. It should be noted that fDP samples contained microvasculature and possibly minor contaminants such as matrix cells and melanocytes (Magerl et al., 2002; Kobayashi et al., 2011), whereas cDP and Fibro samples contained almost pure cell populations (Fig. 1A). To minimize the influence of unwanted contaminants on gene expression profiles, two respective
microarray analyses were performed. In each analysis, DP signature genes were selected by a “two-step” filtering approach comprising primary gene screening by comparing global gene expression profiles of two well-defined subsets, and subsequent selection by assessing the expression patterns of candidate genes among additional cell populations (Ohyama et al., 2006).

In the first analysis (Approach 1), cDP and Fibro GeneChip data (n = 4) were compared to identify genes upregulated in cDP. The expression levels of those genes in fDP and cDP cells were then compared to identify genes that were equally or more highly expressed in fDP (Fig. 1C). Among 105 genes detected as upregulated in cDP compared with in Fibros, 70 genes (Table S1) were removed because they were downregulated in fDP compared with in cDP. Bioinformatics analysis using Database for Annotation, Visualization and Integrated Discovery software (DAVID, ver. 6.7; http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a; Huang da et al., 2009b) revealed that those genes were involved in metabolic processes and responses to external stimulus, suggesting that their upregulation was due to in vitro expansion. No previously described DP gene (Botchkarev and Kishimoto, 2003; Rendl et al., 2005; Ito et al., 2007; Osada et al., 2007; Driskell et al., 2009; Ohyama et al., 2010) was excluded in this step. This analysis identified 35 genes upregulated in fDP (Tables 1, S2). Differential expression of the selected genes was visualized by a heat map (Fig. 1D) and validated by real-time PCR analyses of 11 randomly selected genes (representative data in Fig. 1E). However, this approach did not allow for the detection of genes whose expression was rapidly downregulated in cDP cells to levels similar to those in Fibros (Fig. 1F).

Previous studies reported that DP cell aggregation by centrifugation in low cell binding plates, culturing on synthetic membranes, or hanging drop culture recovered DP functions, including DP biomarker expression. These results suggest that genes upregulated by DP cell aggregation could contribute to the biological distinctiveness of in situ DP (Osada et al., 2007; Young et al., 2008; Higgins et al., 2010). Accordingly, in the second analysis (Approach 2), two additional sets of GeneChips were generated from aggregated cDP (agDP) cells and cDP cells used for agDP (cDP for agDP) and compared. Among the genes detected as upregulated in agDP arrays, those more highly
expressed in fDP compared with both cDP and Fibros were finally selected as DP signature genes (Fig. 1F). A total of 77 genes were identified in this analysis (Tables 2, S3). The data are displayed in a heat map (Fig. 1G). Real-time PCR analyses of 16 randomly selected genes demonstrated expression patterns consistent with microarray data, although statistical significance between agDP and cDP was not sufficient for two genes (representative data in Fig. 1H). The genes upregulated by DP cell aggregation accounted for approximately 20% of all genes detected as increased in fDP among fDP, cDP, and Fibro subsets. Accordingly, a large proportion of fDP genes (n = 321, Table S4) were filtered out in the first step. A total of 36 known hair follicle keratinocyte-related genes, 12 melanocyte-related genes, and 8 vasculature-related genes (Table S4) (Rendl et al., 2005; Huang da et al., 2009a; Huang da et al., 2009b) were found in the removed genes, whereas such genes were not included in those upregulated in agDP cells. These results suggest that the genes expressed by the contaminants in microdissected DP were indeed excluded in the two-step approach.

A total of 108 genes were found to be upregulated in intact human DP cells by two microarray analyses. When the genes detected as increased in fDP prior to microarray generation (Fig. 1B) were combined, 118 human DP signature genes were identified in this study (Fig. 2A).

Bioinformatics analysis revealing functional aspects of DP signature genes, including the signaling pathways involved

To categorize human DP signature genes, a functional classification analysis was performed using DAVID software (Huang da et al., 2009a; Huang da et al., 2009b), in which DP signature genes were divided into 14 groups according to enrichment scores (Fig. 2A). The clusters with high enrichment scores (>2.0) mainly comprised genes involved in WNT (frizzled-related protein [FRZB], Norrie disease [NDP], WIF1, wingless-related MMTV integration site 5A [WNT5A]), bone morphogenetic proteins 2 and 4 [BMP2, BMP4], NOG, and sclerostin domain–containing 1 [SOSTDC1]) and fibroblast growth factor (FGF; FGF7 and sprouty homologs 1 and 4 [SPRY1, SPRY4]) signaling pathways, suggesting the significance of these pathways in the maintenance of intrinsic DP properties (Fig. 2A).
DAVID gene ontology analysis annotated 100 of 118 signature genes as those with binding functions and 23 and 17 genes with transcription regulator and enzyme regulator activity, respectively. These results indicate that most DP signature genes were involved in intercellular or intracellular signal transduction or regulation. Previously unreported human DP genes encoding receptor binding molecules (e.g., glia maturation factor gamma \([\text{GMFG}]\), lipoprotein lipase \([\text{LPL}]\), \(\text{NDP}\), and metastasis suppressor 1 \([\text{MTSS1}]\)), activators or inhibitors of enzyme activity (e.g., apolipoprotein E \([\text{APOE}]\); G-protein signaling 2, 24 kDa \([\text{RGS2}]\); c cystatin A \([\text{CSTA}]\); and stratifin \([\text{SFN}]\)), and transcription factors (e.g., ets variant 1 \([\text{ETV1}]\), forkhead box O1 \([\text{FOXO1}]\), growth arrest–specific 7 \([\text{GAS7}]\), and myocyte enhancer factor 2C \([\text{MEF2C}]\)) were listed, which could imply novel mechanisms contributing to human DP function (Fig. 2B). The genes of membrane proteins (e.g., endothelin receptor type A \([\text{EDNRA}]\), G-protein–coupled receptor 125 \([\text{GPR125}]\), and low-density lipoprotein receptor–related protein 4 \([\text{LRP4}]\)) and extracellular matrix proteins (e.g., spondin 1 \([\text{SPON1}]\) and matrix Gla protein \([\text{MGP}]\)) were also identified (Fig. 2B).

Importantly, 38 of these genes are reportedly over-represented in mouse DP cells (Fig. 2C) (Sleeman et al., 2000; Botchkarev and Kishimoto, 2003; Panteleyev et al., 2003; O'Shaughnessy et al., 2004; Rendl et al., 2005; Driskell et al., 2009). Most of them were established DP signature genes (Botchkarev and Kishimoto, 2003; Rendl et al., 2005; Ito et al., 2007; Osada et al., 2007; Driskell et al., 2009; Ohyama et al., 2010), including those encoding key molecules that enhance (e.g., \(\text{FGF7}\), insulin-like growth factor-1 \([\text{IGF1}]\), and \(\text{WNT5A}\)) or inhibit (e.g., \(\text{FRZB}\) and \(\text{SOSTDC1}\)) interactions between DP and the hair matrix (Fig. 2B). These findings indicate that a fundamental molecular mechanism is conserved between mouse and human DP cells.

**Development of DP activation culture condition (DPAC): optimized culture condition that maintains the intrinsic properties of human DP cells**

As an application of the results obtained above, we attempted to develop a culture condition that allows human DP cell expansion while sustaining intrinsic properties, including the molecular signature. Because the bioinformatics analysis implied the involvement of WNT, BMP, and FGF signaling pathways in the maintenance of human
DP properties, we anticipated that stimulation of these pathways during culture would improve the biological characteristics of cultured DP cells. To test this hypothesis, 6-bromoindirubin-3'-oxime (BIO), recombinant BMP2 (rBMP), and basic FGF (bFGF) were individually added to conventionally used 10% FBS-DMEM to stimulate the WNT, BMP, and FGF pathways, respectively.

To assess the effect of each activator on DP properties, expression of the classic DP genes ALP, lymphoid enhancer-binding factor 1 (LEF1), and NOG, as well as of RGS2, a DP gene identified in this study, was monitored (Fig. 3A). Interestingly, the expression of these genes was not always upregulated in a dose-dependent manner (Fig. 3A). BIO markedly increased the expression of all tested genes at a concentration of 2 μM. In contrast, expression of ALP and RGS2 was somewhat suppressed by 4 μM BIO.

The ameliorative effect of rBMP on DP gene expression did not differ significantly between 200 or 400 ng/ml. bFGF dose-dependently enhanced DP cell proliferation. However, an increase in dead cells, probably due to overproliferation, was observed at 40 ng/ml (data not shown). Although downregulation of ALP was observed at a higher concentration, upregulation of other genes was maintained at 20 ng/ml. Accordingly, each activator differently maintained DP signature gene expression at an individual optimal concentration of 2 μM for BIO, 200 ng/ml for rBMP, and 20 ng/ml for bFGF (Fig. 3A).

We next prepared 10% FBS-DMEM containing optimal concentrations of two or all three factors and cultured fDP cells. The combination of rBMP and bFGF enhanced DP cell proliferation, whereas the mixture of BIO and bFGF or all three factors reduced DP cell growth to approximately 30% that of controls (Fig. 3B). In addition, DP cells hardly proliferated when exposed to the combination of BIO and rBMP (data not shown). Importantly, the combination of all three factors caused statistically significant (p < 0.05) upregulation of all tested DP signature genes compared with controls (Fig. 3C). Thus, 10% FBS-DMEM containing 2 μM BIO, 200 ng/ml rBMP, and 20 ng/ml bFGF provided the most favorable conditions for maintaining proliferative capacity and intrinsic gene expression in cultured human DP cells. We named this condition “dermal papilla activation culture condition” or DPAC.

Expression of other classic DP signature genes in DP cells primarily cultured in
DPAC (dpacDP) was comparable with or higher than that in fDP (Fig. 3D). In addition, pentraxin 3 (PTX3), a Fibro signature gene upregulated in cDP compared with fDP, was significantly underrepresented in dpacDP vs. cDP, further confirming the effect of DPAC (Fig. 3D).

Both the conventional condition (10% FBS-DMEM) and DPAC allowed DP cell expansion for more than five passages (Fig. 3E). Early-passage dpacDP cells predominantly demonstrated a small polygonal appearance distinct from the fibroblast-like morphology of cDP cells. However, numbers of spindle-shaped cells increased in higher-passage dpacDP (Fig. 3E). Consistent with this observation, ALP, NOG, and BMP4 gene expression and ALP enzyme activity, which correlates with the hair inductive capacity of DP cells (McElwee et al., 2003; Rendl et al., 2008), were maintained in early-passage dpacDP at levels comparable with those in fDP (Fig. 3F, G). Thus, the effects of DPAC persisted for several passages.

**DPAC sustained the intrinsic molecular signature of intact human DP**

Human DP cells primarily cultured in DPAC sustained the expression of previously undescribed human DP signature genes (Fig. 4). LRP4, which is reportedly a specific marker of murine DP (Lrp4/corin) (Enshell-Seijffers et al., 2008; Enshell-Seijffers et al., 2010), was almost identically expressed in fDP and dpacDP (Fig. 4). Likewise, BMP and activin membrane–bound inhibitor homolog (BAMBI) and SPRY4, antagonists of TGF-β and FGF pathways (Furthauer et al., 2001; Sekiya et al., 2004), respectively, and NDP, an activator of WNT signaling (Seitz et al., 2010), were similarly expressed in fDP and dpacDP cells (Fig. 4). Expression of those not directly related to the activated pathways, including ETV1; GPR125; guanylate cyclase 1, soluble, alpha 3 (GUCY1A3); and semaphorin-4C (SEMA4C), were also well maintained (Fig. 4). Trichorhinophalangeal syndrome I (TRPS1), a gene responsible for human congenital hair anomalies (Momeni et al., 2000), and sex-determining region Y-box 2 (SOX2), a mouse DP gene that defines a hair shaft phenotype (Driskell et al., 2009), were highly expressed in dpacDP compared with cDP cells (Fig. 4). These findings indicate that DPAC provides a useful tool to probe the links between potential DP signature genes and human DP biology.
**Restoration of impaired DP properties in conventionally cultured human DP cells by DPAC**

When DP cells initially cultured in 10% FBS-DMEM were passaged into DPAC (yielding dpacDP P1 cells; Fig. 5A), their morphology changed from spindle-shaped to a polygonal form resembling that of DP cells primarily cultured in DPAC (Fig. 5B). In line with this observation, DP signature genes such as *ALP*, *LEF1*, and *NOG* were upregulated after passaging into DPAC (Fig. 5C). In addition, ALP activity was much higher in dpacDP P1 cells compared with passage-one cDP cells (cDP P1 cells; Fig. 5A, D). Thus, DPAC ameliorated DP biomarker expression reduced in conventionally cultured DP cells.

To assess whether exposure to DPAC improved DP functions, either dpacDP P1 or cDP P1 cells were co-cultured with normal human hair follicle keratinocytes (Fig. 6A) (Inui et al., 2002). In the bulb of the hair follicle, DP cells interact with hair matrix cells, actively dividing keratinocytes that generate the hair shaft (Rendl et al., 2005, 2008; Ohyama et al., 2010). Intriguingly, dpacDP P1 cells significantly increased the expression of hair matrix signature genes *LEF1* and *msh homeobox 2 (MSX2)* (Rendl et al., 2005) in keratinocytes compared with cDP P1 cells, suggesting that exposure to DPAC ameliorated the capacity of DP cells to provoke epithelial-mesenchymal interactions in the hair follicle (Fig. 6B).

An in vivo hair induction assay was subsequently performed. DP-like cell aggregates were generated from dpacDP P1 or cDP P1 cells and placed between enzymatically separated afollicular murine sole epidermis and dermis. The “sandwich” composites were then inserted subcutaneously into immunodeficient mice (Fig. 6C). Intriguingly, developments suggestive of hair morphogenesis, including epidermal invagination or papillary mesenchymal body formation (Osada and Kobayashi, 2000; Osada et al., 2009) (Fig. 6D), were observed in 9 of 21 mice in the DPAC-treated group, but only in 2 of 18 mice in the control group (p < 0.05). These developments were surrounded by dermal cells positive for ALP and human-specific vimentin staining (Fig. 6D, E), suggesting that they were induced by transplanted human DP cells.

These findings demonstrate that DPAC restores some aspects of DP cell function in...
conventionally cultured DP cells.
Discussion

The success of microarray analysis greatly depends on the purity of the tested cell populations (Rendl et al., 2005). Microdissected DP predominantly comprised DP cells, as demonstrated by high expression of established DP signature genes. However, the vascular structure within DP could not be removed, and complete detachment of the surrounding hair matrix cells and melanocytes was hardly achieved by the microdissection technique (Ohyama et al., 2010; Kobayashi et al., 2011). Because the isolation of a pure DP cell population is currently not feasible in humans (Ohyama et al., 2010), a methodology that allows for the exclusion of non–DP-related genes from fDP upregulated genes must be developed to obtain a bona fide intact DP gene expression profile.

We previously successfully identified the markers of human hair follicle putative stem cells by “two-step” microarray analyses (Ohyama et al., 2006). We adopted this approach to filter out unwanted genes. Because no signal from possible contaminants was detected on cDP, Fibro, or agDP arrays, comparisons between cDP and Fibro GeneChips and agDP and cDP GeneChips should detect the differential expression of the genes expressed in DP cells. These preceding microarray analyses provided the criteria with which to link a gene to DP fate in each subsequent step. In Approach 1, the second step could be crucial because some DP signature genes might be detected as downregulated in fDP compared with cDP cells and discarded due to the lower occupancy of DP cells in fDP subsets. However, previously reported DP genes (Botchkarev and Kishimoto, 2003; Rendl et al., 2005; Ito et al., 2007; Osada et al., 2007; Driskell et al., 2009; Ohyama et al., 2010) were not among the genes excluded in this step, suggesting that this loss is unlikely.

Theoretically, Approach 1 could not detect any DP gene downregulated in cDP cells to a level similar to that in Fibros. In truth, cDP cells exhibited a global gene expression pattern resembling that of Fibros (Fig. 1G), indicating the need for additional analyses. Although Approach 2 identified as many as 77 DP signature genes, excluding those expressed in contaminants in microdissected DP, some DP signature genes likely remained undetected because of the limitation intrinsic to this approach that not all DP signature genes were upregulated by cell aggregation. Indeed, 12 established DP genes
(Botchkarev and Kishimoto, 2003; Rendl et al., 2005; Ito et al., 2007; Osada et al., 2007; Driskell et al., 2009; Ohyama et al., 2010) were upregulated in fDP compared with cDP cells and Fibros, but were not detected by Approach 2. Importantly, half of those genes were selected by another analysis and finally listed as DP signature genes. This result, together with the observation that only four genes were common to both analyses, suggests that the combination of individual approaches was beneficial.

The current study identified 118 human DP signature genes, whereas global gene expression profiling of FACS-sorted pure mouse DP cells returned 225 DP signature genes (Rendl et al., 2005), most of which overlapped with the genes recently identified in CD133+ mouse DP cells (Driskell et al., 2009). Thus, a considerable number of human DP signature genes were not detected in the present study. Our two-step criteria were sufficiently stringent to identify selective markers, but may have been too restrictive to fully unveil the molecular signature of tested populations. Successful genetic fluorescent labeling of living human hair follicle cells through specific promoter expression has been reported (Tiede et al., 2009). A similar approach utilizing a promoter for a DP signature gene identified in this study or the use of potential cell surface markers described below might enable the isolation of more homogenous human DP cell samples and allow for the detection of unidentified DP genes.

In addition to the elucidation of the roles of WNT, BMP, and FGF signaling in the maintenance of DP properties, the bioinformatics analysis discovered attractive gene groups implying future applications. For instance, the modulation of enzyme activators, including \textit{APOE} and \textit{RGS2}, could augment DP cell functions. Among transcription regulator genes, those involved in cell differentiation, such as \textit{FOXO1} (Takano et al., 2007), \textit{GAS7} (Moorthy et al., 2005), and \textit{MEF2C} (Lin et al., 1997), are of particular interest. The introduction of exogenous transcription factors could reprogram the biological properties of target cells (Takahashi and Yamanaka, 2006; Tsai et al., 2010; Tsai et al., 2011). Likewise, forced expression of these factors may facilitate the conversion of other dermal cells into cells with DP-like properties. Membrane proteins, represented by \textit{GPR125} and \textit{LRP4}, may provide useful cell surface markers for living human DP cell isolation. Of note, \textit{Lrp4}/corin is reportedly specific for mouse DP cells (Enshell-Seijffers et al., 2008; Enshell-Seijffers et al., 2010). The development of
antibodies against these molecules for immunohistochemical- and/or fluorescence-activated cell sorting analyses could represent an important next step. Interaction with extracellular matrix plays a key role in the maintenance of DP cell properties (Ohyama et al., 2010). Upregulation of DP biomarker was observed when DP cells were cultured on laminin (Kobayashi et al., 2011). SPON1 or MGP may provide a supportive environment for human DP cells in culture.

For the development of DPAC, we adopted BIO, rBMP, and basic FGF to stimulate biological pathways downregulated during conventional culture. BIO is a small molecule commonly used to activate canonical WNT signaling in cultured cells (Sato et al., 2004; Yamauchi and Kurosaka, 2009). Although BMP6 improved mouse DP cell properties more efficiently than BMP2/BMP4 (Rendl et al., 2008), we did not detect upregulation of BMP6 in human fDP. Based on the observations that BMP2 was markedly suppressed during conventional culture and upregulated during DP cell aggregation, we selected this molecule as a BMP signaling activator. bFGF upregulated some DP signature genes, suggesting that active FGF signaling contributes not only to cell proliferation, but also to the maintenance of DP properties. As suggested by decreased expression of NOG, SOX2, and TRPS1 in dpacDP compared with that in fDP cells, dpacDP cells probably did not perfectly reproduce the molecular signature of in situ DP. Once a pure human DP cell population is isolated, microarray comparison between intact DP and dpacDP cells must be performed to more precisely assess the ability of DPAC to sustain DP properties. The findings obtained by this analysis could enable further improvement of DPAC.

In previous studies, supplementation of exogenous Wnt or Bmp molecules maintained mouse DP cell properties in culture, but did not affect their proliferative capacities (Rendl et al., 2008). For clinical application, the use of BIO may be advantageous because Wnt is not widely available in large quantities and may exhibit unwanted effects via non-canonical pathways (Shimizu and Morgan, 2004). However, considering that DP growth suppression due to BIO (Yamauchi and Kurosaka, 2009) represents a major drawback of DPAC, BIO may be substituted by recombinant WNT3A (Kishimoto et al., 2000; Shimizu and Morgan, 2004) to maintain the intrinsic properties and proliferative capacity of dpacDP cells. Because past reports suggested
that activation of multiple biological pathways is necessary to fully maintain DP characteristics in vitro (Kishimoto et al., 2000; Shimizu and Morgan, 2004; Rendl et al., 2008), DPAC could be more potentiated by the addition of activators of other signaling pathways, including the Sonic hedgehog, Notch, and Endothelin pathways (Gao et al., 2008; Driskell et al., 2009; Hu et al., 2010). In contrast to DP cells, fibroblasts cultured in DPAC reduced ALP expression (data not shown). Thus, the conversion of other dermal cells into the DP cell lineage using DPAC is not feasible.

A unique feature of DPAC is that it restored the DP signature gene expression and functions normally reduced in conventionally cultured DP cells. The gene expression recovery seems to be imperfect by, for instance, moderate upregulation of \textit{RGS2}. For full assessment of functional restoration, additional examinations, including the evaluation of the response to androgen (Inui et al., 2002) or adenosine (Iino et al., 2007), may be performed. However, the co-culture experiment and in vivo hair induction assay demonstrated that the most characteristic DP property, communication with the epithelial component (Ohyama et al., 2010; Driskell et al., 2011), was at least partly restored by DPAC. These observations imply a promising strategy for the efficient propagation of functional human DP cells by primary expansion under conventional culture conditions, followed by functional restoration. In fact, the yield of DP cells after the second passage was more than 10 times higher than that obtained through DPAC expansion from primary cultures, and the time required was approximately 50% less, indicating substantial advantages. The phase of isolated DPs might have affected the efficiency of the restoration. In the present study, DPs were mostly microdissected from full-anagen hair follicles. The role of DP in such hair follicles is to support hair shaft elongation rather than hair induction (Oliver, 1967; Paus and Cotsarelis, 1999). Early-anagen DP cells may possess more potent biological capacities, including resilience, than late-anagen DP cells. Although the use of such DP cells is technically very challenging, it could improve the outcome of the restoration experiments.

In summary, we have elucidated the molecular signature of intact human anagen DP cells. This guided the development of DPAC for the efficient in vitro expansion of human DP cells that retain their intrinsic properties. The present study sheds new light on the biological properties of human DP cells and implies a novel strategy for
supplying sufficient functional human DP cells for the development of molecules acting on in vivo DPs or for regenerative medicine-based approaches to treat intractable hair loss disorders.
Materials and Methods

Preparation of human dermal papillae

Human hair follicles were manually isolated from intact scalp skin obtained during surgical removal of benign skin tumors. DPs were microdissected from the bulbar portion of hair follicles under a dissection microscope as previously described (Williams et al., 1994). All experimental procedures were officially approved by the Institutional Review Board of Keio University. All human donors provided written informed consent in accordance with Declaration of Helsinki guidelines.

Cell culture

Five to six DPs were placed on the bottom of a 60-mm dish and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (Roche, Basel, Switzerland) and 10% FBS. For fibroblast collection, the dermis of scalp samples was cut into small blocks, 2-3 mm square. The blocks were attached to the bottom of 60-mm culture dishes and cultured in 10% FBS-DMEM. All cell culture was performed at 37°C in 5% CO₂. When confluent, primary cultured cells were harvested and re-seeded (20,000 cells/dish). After primary culture, all cells were passaged on a weekly basis.

Dermal papilla cell aggregation

Cultured DP cells were incubated with 2% trypsin and then transferred into low-cell-binding 96-well plates and centrifuged for several seconds to spin down the DP cells, and then incubated overnight at 37°C in 5% CO₂.

RNA isolation and real time PCR analysis

Total RNA was isolated from microdissected DP, DP cell aggregates and cultured DP cells using an RNeasy Micro Kit (QIAGEN, Hilden, Germany). For real-time PCR analyses, cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Real-time PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Santa Clara, CA, USA) and a StepOne Real-Time PCR system (Applied Biosystems). All primers were designed using Primer Express® software.
(Applied Biosystems) and reactions were performed under the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primer sets used are shown in Table S5. Target mRNA expression levels normalized to the GAPDH mRNA using the ΔCt method were calculated as $2^{-\Delta C_t}$. GAPDH was used as the reference gene because of its similar expression in microdissected and cultured DP cell populations, as shown by consistent Ct values. Three independent experiments were performed in duplicate. The results are presented as the means ± standard error of the means (s.e.m.).

Microarray generation

Isolated total RNA was amplified and biotin-labeled using a T7-primer based GeneChip® Two-Cycle cDNA Synthesis Kit and an IVT Labeling Kit (Affymetrix Inc., Santa Clara, CA, USA), respectively. Then, 20 μg of each labeled cRNA sample was fragmented and hybridized to an Affymetrix HG-U133A 2.0 GeneChip microarray and scanned using GeneChip Scanner 3000 7G (Affymetrix Inc.) according to the manufacturer’s protocol.

Microarray data analysis

To identify genes that are differentially expressed in intact human DP, two data analyses individually consisting of two-step microarray comparisons were performed using Gene Chip Operating Software ver. 1.4 (Affymetrix Inc.). For all microarrays, chip files (CHIP files) were generated according to the following parameters: target signal scaling, 500; Alpha1, 0.05; Alpha2, 0.065; and Tau, 0.015. Gene expression files were generated based on the following parameters: Gamma1H, 0.0045; Gamma1L, 0.0045; Gamma2H, 0.006; Gamma2L, 0.006; and Perturbation, 1.1. Only probe sets detected as being expressed (present call) in these files were analyzed. In the first analysis, global gene expression for the cDP and Fibro chips was compared (n=4), and probe sets whose targets were upregulated ≥2-fold in cDPs were selected. Next, the expression levels of the targets for these probe sets in fDP and cDP GeneChips were compared in order to identify genes whose expression was up-regulated in fDP or equally expressed in fDP and cDPs. In the second analysis, two sets of GeneChips were
used to analyze total RNA samples from agDPs and cDPs in order to identify probe sets whose targets were >1.5 fold upregulated by DP cell aggregation. Among the probe sets upregulated in agDPs, those found to be >3-fold more highly expressed in fDP, as compared to both cDPs and Fibros, were selected. The Affymetrix IDs of these probe sets were uploaded into the gene batch viewer of DAVID (http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a; Huang da et al., 2009b) to generate a list of genes whose expression is upregulated in intact human DP.

Bioinformatics analysis

The list of DP human signature genes was uploaded in DAVID, and the gene clustering analysis was performed with the following settings: similarity term overlap, 3; similarity threshold, 0.25; initial group membership, 2; final group membership, 2; and multiple linkage threshold, 0.50. Ontological functional annotation for each gene was obtained by DAVID. In addition, gene expression profiles for Approaches 1 and 2 were analyzed by GeneSpring GX 11.5 software (Agilent Technologies, Santa Clara, CA, USA). The MAS without baseline transformation was applied for normalization. The genes were analyzed by hierarchical clustering and drawn as heat maps using the GeneSpring default setting.

Development of dermal papilla activation culture condition (DPAC)

DP cells were cultured in 10% FBS-DMEM containing BIO (1, 2, or 4 μM; Sigma, St. Louis, MO, USA) rBMP2 (100, 200, or 400 ng/ml; R&D, Minneapolis, MN, USA), or bFGF (10, 20, or 40 ng/ml; PEPROTECH, Rocky Hill, NJ, USA). The growth rates of DP cells under these different culture conditions were monitored every 4–5 days by assessing the ratio between the size of a starting DP and an outgrowing colony. Four weeks later, DP cells were collected and total RNA was extracted. Then, ALP, NOG, LEF1, and RGS2 expression levels were measured by real-time PCR as described above. Subsequently, DP cells were cultured in 10% FBS-DMEM containing two activators (2 μM BIO and 200 ng/ml rBMP, 2 μM BIO and 20 ng/ml FGF, or 200 ng/ml rBMP and 20 ng/ml FGF) or all three activators (2 μM BIO, 200 ng/ml rBMP, and 20 ng/ml FGF). DP cell growth and signature gene expression were then assessed as described above.
Alkaline phosphatase activity detection
Detection of ALP activity in DP cells was performed using an ALP detection kit (Chemicon Millipore, Temecula, CA, USA), according to the manufacturer’s protocol.

Keratinocyte and DP co-culture
DP cells primarily cultured in 10% FBS-DMEM were seeded on the upper compartment of 6 well Transwell culture dishes (Corning, Lowell, MA, USA) and cultured either in DPAC or 10% FBS-DMEM for 1 week. At the same time, human middle follicle outer root sheath keratinocytes were cultured on the collagen type I coated 6 well plates in defined keratinocyte serum free medium (Invitrogen, Carlsbad, CA, USA). At day 7, Transwell dishes with DP cells were inserted into the plates with keratinocytes and cultured for additional 2 days in DMEM without FBS. Then, total RNA was isolated from keratinocytes for gene expression analyses.

In vivo hair induction assay
Freshly isolated human DP cells were conventionally cultured in 10% FBS-DMEM and passaged either in DPAC or 10% FBS-DMEM. After 4 weeks of culture, aggregates of DPAC-treated and control DP cells were generated as described above. Afollicular footpads were surgically removed from 8-week-old ICR mice under a dissection microscope and incubated with dispase I (Godo Syusei, Tokyo, Japan) (1,500 U/ml) for 30 min at 37°C. The epidermis was separated from the dermis and cell aggregates were placed between them. The footpad skin composites were incubated on Millicell-CM culture plate inserts (Millipore, Billerica MA, USA) for 2 h at 37°C in 10% FBS-DMEM and grafted subcutaneously into BALB/c nu/nu nude mice. Skin samples were harvested 2 weeks later for histological analysis.

Human vimentin detection
Frozen sections were fixed through incubation with acetone for 20 min at -20°C and incubated overnight at 4°C with a monoclonal anti-human vimentin-Cy3 antibody (Sigma). Cell nuclei were counterstained with 4’,6-diamidino-2-phenylindole.
Statistical analysis

Statistically significant differences were identified by two-sided Student’s \( t \)-test or Mann-Whitney \( U \)-test, depending on the size of the sample population. A p-value of less than 0.05 was considered significant.
Acknowledgements

We thank the late Dr. Jonathan C. Vogel (Dermatology Branch, CCR, National Cancer Institute, NIH, Bethesda, USA) for his technical advice and discussion. This work was supported by a Grant-in-Aid for Scientific Research (KAKEN-HI) awarded to MO and MA by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a JSID Fellowship SHISEIDO Award 2008, Keio University Research Grants for Life Sciences and Medicine, and Keio Gakuji Academic Development Funds to MO.
References


Figure legends

Fig. 1 Microarray comparisons between human DP and controls identified human DP signature genes. (A) Morphology and histology of human DP in a human anagen hair follicle. For microarray generation, total RNA was isolated from freshly microdissected DP (fDP) and cultured DP (cDP) cells and fibroblasts (Fibros). Scale bars: fDP, 100 μm; cDP and Fibros, 300 μm. (B) Real-time PCR analysis detected higher expression of established DP signature genes in fDP compared with cDP cells and Fibros, confirming successful RNA isolation (*p < 0.05). (C) In Approach 1 microarray analysis, the genes upregulated in cDP versus Fibros and not downregulated in fDP versus cDP cells were selected. (D) Heat map of DP signature genes detected by Approach 1. (E) Real-time PCR confirmation of Approach 1 genes (*p < 0.05). (F) In Approach 2, the genes increased in agDP versus cDP cells and upregulated in fDP compared with cDP cells and Fibros were selected. Scale bar: agDP, 200 μm. (G) Heat map of DP signature genes detected by Approach 2. (H) Approach 2 genes confirmed by real-time PCR (*p < 0.05).

Fig. 2 Bioinformatics analyses of the molecular signature of human DP. (A) Human DP signature genes were subdivided into 14 groups by functional classification. Clusters with high enrichment scores (>2) contained genes involved in WNT, BMP, and FGF signaling. (B) Schematic illustration of the outcome of gene ontology analysis. DP signature genes were enriched for those with binding functions, including signaling molecules, enzyme activity regulators, and transcription factors. Previously unreported encoding membrane and extracellular matrix molecules were also detected. (C) Thirty-eight DP signature genes were conserved between mice and humans, suggesting that they have essential roles in DP biology.

Fig. 3 Development of dermal papilla activation culture condition (DPAC). (A) Differential effects of BIO, rBMP and bFGF on human DP signature gene expression. (B) The combination of rBMP and bFGF significantly enhanced DP cell proliferation, while the combination of BIO plus bFGF and a combination of all three activators moderately suppressed DP cell growth. Scale bar: 300 μm. (C) Combined treatment
with all three activators significantly increased the expression of all DP signature genes (*p<0.05). As a result, 10% FBS-DMEM containing 2 μM BIO, 200 ng/ml rBMP and 20 ng/ml bFGF was named “dermal papilla activation culture condition” or DPAC. (D) Human DP cells primarily cultured in DPAC (dpacDP) maintained the expression of some DP signature genes at a level comparable to that in fDP, while suppressing expression of the fibroblast gene *PTX3* (*p<0.05 for cDPs). (E) At lower passages, the morphology of DP cells in DPAC was distinct from those cultured under control conditions (10% FBS-DMEM). Numbers of cells with a fibroblast-like morphology increased as the passage number increased. Scale bar: 300 μm. (F) DPAC sustained DP signature gene expression across a number of passages. (G) Alkaline phosphatase (ALP) activity (red) was maintained for more than four passages in DPAC, while it was almost completely lost within two passages under control conditions. Scale bar: 100 μm.

**Fig. 4** DPAC maintained novel human DP signature gene expression in vitro. The expression of newly identified human signature genes was increased in cells cultured in DPAC (*p<0.05 for cDPs). Increased expression of previously unreported human DP signature genes under DPAC could further confirm their roles in DP biology.

**Fig. 5** Restoration of impaired DP properties in conventionally cultured human DP cells by DPAC. (A) Intact human DP (fDP) were cultured under conventional culture conditions (which allow rapid cell proliferation), and passaged into either DPAC or the conventional culture medium. (B) DP cells started to change their morphology when passaged into DPAC. Scale bar: 300 μm. (C) Restoration of human DP signature gene expression after culture in DPAC (*p<0.05). (D) ALP activity was recovered in cDPs cultured in DPAC, but was barely detectable in cDPs passaged into a conventional culture condition. Scale bar: 100 μm.

**Fig. 6** Functional restoration of human DP cells by DPAC. (A) Co-cultured system to assess the capacity to provoke the epithelial mesenchymal interaction in the hair follicle bulb. (B) dpacDP P1 cells upregulated hair matrix signature genes in co-cultured
hair follicle keratinocytes compared to cDP P1 cells. (C) A “Sandwich” assay was performed using dpacDP and cDP cell aggregates. The aggregates were placed between mouse sole afollicular epidermis and the dermis and composites were transplanted subcutaneously into nude mice. Scale bar: 100 μm. (D) A hair peg-like papillary mesenchymal body formed after transplantation of dpacDP aggregates. Note the ALP-positive dermal cells (arrowheads) surrounding the structure, and the loss of ALP expression in the control aggregate. Scale bar: 50 μm. (E) An anti-human vimentin antibody specifically stains human DP, but not mouse DP. This antibody detected ALP-positive cells surrounding a hair like structure (arrowheads), indicating that they were of human origin. Scale bar: mouse DP, 10 μm; human DP, 50 μm; experimental sample, 100 μm.
### Table 1
Top 15 human DP signature genes detected by approach 1

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene name</th>
<th>Gene Symbol</th>
<th>Fold change cDP vs Fibro</th>
<th>Fold change fDP vs cDP</th>
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<td><strong>3.0</strong></td>
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*For the genes supported by multiple probe sets, the probe set with the highest fold change between cDP and Fibro was indicated by bold letters.*
Table 2

Top 25 human DP signature genes detected by approach 2

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<th>Affymetrix ID</th>
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<th>Fold change fDP vs cDP</th>
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*For the genes supported by multiple probe sets, the probe set with the highest fold change between agDP and cDP was indicated by bold letters.
**Relative mRNA expression**

**Approach 1**

- cDP > Fibro
- fDP ≥ cDP

**Approach 2**

- AgDP > cDP and cDP < fDP > Fibro

**Gene Expression**

- **cDP for agDP**
- **cDP**
- **fDP**
- **Fibro**

**Gene Expression**

- **AgDP**
- **cDP**
- **fDP**
- **Fibro**

**Forced DP cell aggregation**

- cDP cells
- agDPs

**Forced D P cell aggregation**

- (freshly microdissected DP)
- (cultured DP cells)
- (fibroblast)

**Journal of Cell Science Accepted manuscript**
Relative mRNA expression

BAMBI

ETV1

GPR125

GUCY1A3

LRP4

MEF2C

NDP

RBP1

SEMA4C

SOX2

SPRY4

TRPS1
A. fDP → cDP P0 (conventional culture)

Possible restoration in DPAC

dpacDP P1 → passage → cDP P1

B. cDP P1 vs. dpacDP P1

C. Relative mRNA expression

- ALP
- BAMBI
- BMP4
- LEF1
- LRP4
- NOG
- RGS2
- SPRY4
- WNT5A

D. cDP P1 vs. dpacDP P1