

TGF- β_2 is specifically expressed in human dermal papilla cells and modulates hair folliculogenesis

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Abstract

Dermal papilla cells (DPCs) in the mammalian hair follicle have been shown to develop hair follicles through epithelial–mesenchymal interactions. A cell therapy to regenerate human hair is theoretically possible by expanding autologous human DPCs (hDPCs) and transplanting them into bald skin, though much remains to be overcome before clinical success. In this study, we compared gene signatures of hDPCs at different passages and human dermal fibroblasts, and found transforming growth factor (TGF)- β_2 to be highly expressed in cultured hDPCs. Keratinocyte conditioned medium, which is known to help preserve the hair-inducing capacity of hDPCs, up-regulated TGF- β_2 expression of hDPCs and also enhanced their alkaline phosphatase (ALP) activity, a known index for hair-inductive capacity. Through screening of components secreted from keratinocytes, the vitamin D₃ analogue was found to promote TGF- β_2 expression and ALP activity of hDPCs. In animal hair folliculogenesis models using rat epidermis and expanded hDPCs, inhibition of TGF- β_2 signalling at the ligand or receptor level significantly impaired hair folliculogenesis and maturation. These results suggest an important role for TGF- β_2 in hair follicle morphogenesis and provide insights into the establishment of future cell therapies for hair regrowth by transplanting expanded DPCs.

Keywords: TGF- β_2 • dermal papilla cell • hair regrowth • hair regeneration • tissue engineering • cell therapy • vitamin D₃

Introduction

The mammalian hair follicle is a complex mini organ that consists of different lineages of cells, including epithelial, mesenchymal and pigmented cells. The dermal papilla, considered as the most important mesenchymal component, plays versatile roles in hair follicle morphogenesis and hair cycling *via* epithelial–mesenchymal interactions [1–4]. Because cultured dermal papilla cells (DPCs) as well as organ dermal papilla were found to have hair-inductive capacity [5–7], many attempts have been made to regenerate hair follicles by transplanting expanded DPCs, sometimes together with epithelial stem cells. However, challenges in developing regeneration strategies have arisen, as the hair-inductive ability of DPCs is lost upon

culture and the molecules and mechanisms responsible for the hair-inducing capacity are not yet fully elucidated [8].

There are six major morphogenetic molecular family systems in hair follicle development and cycling: fibroblast growth factor (FGF), transforming growth factor (TGF)- β , sonic hedgehog, Wingless or Wnt pathway, neurotrophins and homeobox gene families [4, 9, 10]. In each morphogenetic stage, all function as responsible molecules for the reciprocal signalling between hair follicle epithelial and dermal components. In the context of hair follicle ‘neogenesis’, however, it is unclear which signalling molecule(s) among these pathways function in hair induction in transplanted DPCs. Thus far, specific signalling molecules, such as bone morphogenetic protein (BMP)-6 [11], have been shown to enhance mouse hair folliculogenesis. Wnt3a signalling from epithelial component is also required to maintain the inductive capacity of DPCs and to generate hair follicles [12]. These factors have been determined as candidates for hair-inducing activity by employing sophisticated transgenic approaches such as specific knockout or overexpression *in vivo*. In human beings, however,

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the difficulty in applying transgenic approaches has hampered such studies for specific *in vivo* gene function [13]. Therefore, although various biomarkers specifically expressed in human DPCs (hDPCs) have been reported [14, 15], their functions remain to be clarified.

Conditioned media obtained from epidermal keratinocyte culture (keratinocyte conditioned media, or KCM) are known to maintain DPC capacity to proliferate and induce hair follicles for a longer period than control media [16], suggesting that cultured keratinocytes release key factors for DPCs to maintain hair-inducing capacity. Keratinocytes produce a vast variety of soluble factors including growth factors, hormones and chemokines [17, 18]. Screening of biologically active components in KCM may identify the substances that stimulate DPCs to maintain their hair-inducing capability and provide an efficient method for *in vitro* expansion of hair-inductive DPCs.

We suggested that specific genes relating to hair-inducing capacity are up-regulated in hDPCs and that expression is promoted by particular components contained in human KCM. In this study, the global gene signatures of hDPCs at early and later passages and human dermal fibroblasts (hDFs) with no hair-inducing capacity were compared by microarray analysis. Our results showed that the TGF- β_2 gene was specifically expressed in hDPCs and its expression was up-regulated by KCM. We further investigated potential roles of TGF- β_2 in hair induction by hDPCs and sought to identify keratinocyte-derived components that can affect the hair-inducing capacity of hDPCs.

Materials and methods

Human DPC and DF culture

Scalp and facial skin with hair were obtained from facelift operations performed at two institutions; informed consent was obtained using protocols approved by institutional review boards from each individual institution. Dermal papillae were isolated from the hair follicles under a microscope, and placed onto a culture dish containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (10% DMEM). After 2 weeks of explant culture, expanded hDPCs were subcultured with the same medium. Human DFs were obtained from the explant culture of facial skin dermis of the same individuals and cultured in 10% DMEM.

Human epidermal keratinocyte culture and preparation of the conditioned culture media

Human facial skin was cut into 3 × 3 mm pieces and incubated in 10% DMEM supplemented with 1000 U/ml DispaseTM II (Sankyo, Tokyo, Japan) at 4°C for 15–18 hrs. The epidermis was carefully peeled off from the dermis and incubated in phosphate buffered saline (PBS) supplemented with 0.25% trypsin and ethylenediaminetetraacetic acid (EDTA) mixture at 37°C for 20 min. to obtain fresh keratinocyte cell suspension. Keratinocytes were cultured in serum free media, DKFSMTM (Gibco), for 7–10 days up to

60–80% confluence; afterwards, the culture medium was switched to 10% DMEM. The culture supernatant was collected after 1 week, centrifuged at 3000 × *g* for 30 min., and filtrated through a 0.22 μ m membrane filter (Micropore, Madison, NJ, USA). The supernatant was mixed with fresh 10% DMEM at a 1:1 ratio to make KCM for hDPC culture.

Reagents

Reagents supplemented to hDPC culture were as follows: acidic FGF (Peprotech, Rocky Hill, NJ, USA); basic FGF (Peprotech); BMP-2 (Wako, Osaka, Japan); interleukin (IL)-1 β (Endogen, Rockford, IL, USA); IL-6 (Peprotech); IL-8 (Wako); vascular endothelial growth factor (VEGF) (Wako); platelet-derived growth factor (PDGF)-BB (Wako); nerve growth factor (Sigma-Aldrich, Louis, MO, USA); heparin-binding EGF-like growth factor (Peprotech); macrophage inflammatory protein (MIP)-3 α (R&D systems, Minneapolis, MN, USA); monocyte chemotactic protein (MCP)-1 (R&D systems); insulin-like growth factor (IGF)-1 (Sigma-Aldrich); epithelial cell-derived neutrophil-activating peptide (ENA)-78 (Wako); growth-related oncogene (GRO)- α (Wako); 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (LKT laboratories, St. Paul, MN, USA); cholesterol sulphate (Sigma-Aldrich); all-trans retinoic acid (Biomol, Hamburg, Germany); 17 β -estradiol (Cayman Chemical, Ann Arbor, MI, USA) and dihydrotestosterone (Biomol). All reagents were diluted in PBS or ethanol to 1000-fold of the final working concentrations indicated in manufacturer's instructions and stored in aliquots at -20°C.

Real-time RT-PCR

RNA was isolated from cultured hDPCs or hDFs using an RNeasyTM Mini Kit (Qiagen, Valencia, CA, USA), followed by reverse transcription. PCR amplification of cDNA was performed in a 50 μ l reaction consisting of 1 × TaqManTM Universal Master Mix (Applied Biosystems, Foster City, CA, USA) with the ABI 7700 sequence detection system. Gene expression of various hair follicle-related genes was quantified based on measurement of the cycle threshold using the following TaqManTM pre-designed primers and probes (Applied Biosystems): TGF- β_2 (Hs00236092_m1); TGF- β_1 (Hs00998129_m1); BMP-2 (Hs00154192_m1); syndecan1 (Hs00896423_m1); integrin- β_1 (Hs00559595_m1); keratinocyte growth factor (KGF) (Hs00940253_m1); VEGF (Hs00900054_m1); IGF-1 (Hs01547656_m1); hepatocyte growth factor (HGF; Hs00300159_m1); PDGF (Hs00234042_m1); steroid 5 α -reductase II (Hs01399057_m1); versican (Hs00171642_m1); ephrin-A3 (Hs00191913_m1) and androgen receptor (AR; Hs00171172_m1). We used GAPDH (Hs99999905_m1) as an endogenous reference gene.

Microarray generation and analysis

To identify genes differentially expressed in hDPCs responding to the substances secreted from keratinocytes, gene expressions of hDPCs and hDFs of the same individual were compared. tRNA of hDPCs cultured in KCM (passages 2 and 8) and hDFs (passage 2) hDFs in 10% DMEM were isolated using an RNeasyTM Mini Kit. The quality of each sample was assessed by rRNA 28S/18S ratio and RNA integrity number using Agilent 2100 BioanalyzerTM (Agilent Technology, Palo Alto, CA, USA). cDNA was obtained from 5 μ g of tRNA by one-cycle of reverse transcription. The biotin-labelled cRNAs were purified, fragmented, and hybridized to the GeneChipTM Human Genome U133 Plus 2.0 Array (Affymetrix, Santa

Clara, CA), which was then scanned by the GeneChip™ 3000 Scanner (Affymetrix) following the manufacturer's protocol. The numerical data of the signal intensity was analysed using GeneChip™ Command Console™ Software (Affymetrix) and Microsoft Excel™.

Cytokine array analysis

The supernatant of human keratinocyte culture was collected at 1 or 2 weeks after the medium switch from DKSM to 10% DMEM by the methods described above. Expression levels of multiple cytokines were assayed in each sample using the Human Cytokine Array VI (Ray Biotech, Norcross, GA, USA) following the manufacturer's instruction. The image of each array was captured by a digital camera (Nikon, Tokyo, Japan) and converted to the binary image format. The signal intensity was calculated using the image processing software, Scion™ Image (Scion Corp., Frederick, MD, USA).

ELISA for TGF-β₂

The influence of KCM and other keratinocyte-derived factors on TGF-β₂ protein production in hDPCs was assessed by sandwich ELISA, Quantikine™ human ELISA for TGF-β₂ (R&D systems). Reagents supplemented to the hDPC culture were as described above. The supernatant of hDPC culture at passage 2 was collected after 96 hrs of incubation, and processed with ELISA following the manufacturer's instructions.

Alkaline phosphatase (ALP) activity assay

The influence of KCM and other keratinocyte-derived factors on hDPC ALP activity was assessed by fluorescent based ELISA, Sensolyte™ FDP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA, USA). Reagents supplemented to the hDPCs culture were as described above. hDPCs were seeded on a 24-well plate and cell lysates were collected after 48 hrs of incubation. The fluorescence intensity was measured using a microplate fluorescence reader, BTX-880 (Beckman-Coulter, Brea, CA, USA). The fluorescence reading was normalized with the cell number at time of harvest.

MTT proliferation assay

The influence of KCM and other keratinocyte-derived factors on hDPC proliferation was assessed by a MTT cell proliferation assay kit (Roche, Basel, Switzerland). hDPCs were seeded on a 96-well plate and cell lysates were collected after 96 hrs of incubation. The fluorescence intensity was measured using a microplate fluorescence reader, BTX-880 (Beckman-Coulter) and cell number was calculated following the manufacturer's instruction.

Animal assays for hair folliculogenesis

We generated rat-human chimeric hair follicles in nude mice using a previously described sandwich method [16, 19, 20] with some modifications. Briefly, hDPCs were cultured as described above, and the hDPC sheet was scraped off, cut into 1 × 1 mm pieces and used as transplanted constructs. The follicular foot pad skin of 8-week-old F344 rats was cut into

3-mm² pieces and incubated in 10% DMEM and 1000 U/ml Dispase™ II at 37°C for 20 min. to separate the epidermis and dermis. The DPC construct was placed between the epidermis and dermis of the foot pad and transplanted to the subcutis of a 6-week-old Balb/c nude mouse (Fig. S1). The transplants were harvested 4 weeks later and processed for histological evaluation of the number and maturation stage of generated hair follicles. The maturation stage was categorized into eight stages (S1 to S8) according to a previously described method [21] (Fig. S2).

A chamber grafting method was also employed according to previously reported methods [11, 20, 22, 23] with some modifications (Fig. S1). A combination of cultured hDPCs (P3) and cultured neonatal B6 mouse keratinocytes was employed to reconstitute human-mouse chimeric hair follicles. In another experiment, a combination of freshly isolated foetal dermal cells and foetal keratinocytes isolated from BL6 mouse embryos was also utilized. For preparation of cultured mKC, the dorsal skin of newborn mice was incubated in 1000 U/ml Dispase™ II at 4°C for 15–18 hrs, and the epidermis was consequently separated from the dermis. The epidermis was digested with a 0.05% Trypsin and 0.2 mM EDTA mixture at 37°C for 20 min. to obtain single cells. The resultant cell suspension was filtrated through a 40-μm cell strainer and cultured in DKSM™ for 4 days. For preparation of mouse foetal dermal cells and keratinocytes, the dorsal skin of E17.5 BL6 embryos was digested as described above. A dome-shaped polypropylene chamber made from a PCR tube lid (Greiner Bio-One, Frickenhausen, Germany) was transplanted onto the back of a nude mouse 5 days before cell transfer. The number of mesenchymal cells (cultured hDPCs or mouse fresh foetal dermal cells) and epithelial cells (cultured mouse neonatal keratinocytes or fresh foetal keratinocytes) transplanted into each chamber was 10⁶ (2 × 10⁶ in total). Skin samples of recipient nude mice were harvested 4 weeks after cell transplantation. Each group consisted of four chambers on four mice.

TGF-β signal inhibition *in vivo*

TGF-β signal inhibitors were administered to the recipient nude mice. A selective kinase inhibitor for the TGF-β type I receptor, SB431542 (10 μM; Sigma-Aldrich) or an equivalent amount of vehicle was administered continuously (0.25 μl/hr) by Alzet™ osmotic pumps (Durect, Cupertino, CA, USA), and transplanted subcutaneously beside the foot pad construct in the sandwich model or the chamber in the chamber model. A specific antibody for human TGF-β₂ (BioVision, Mountain View, France) or TGF-β_{1/2/3} (R&D systems) was used to neutralize TGF-β₂ or all three isoforms (TGF-β_{1/2/3}) of TGF-β ligand. One microgram of neutralizing or negative control IgG (R&D systems) was administered every second day by a local injection to the subcutis. Signal inhibition in both models was performed for 4 weeks.

Immunohistochemical staining

After harvest, the foot pad transplant was embedded in the OCT compound (Sakura Finetek, Tokyo, Japan), frozen in liquid nitrogen and stored at -80°C until sectioning. Frozen sections (10 μm) were placed on slides, air dried at room temperature for 1 hr, fixed in paraformaldehyde (4% in PBS) for 1 min. and washed in PBS for 5 min. Every other slide was stained with haematoxylin and eosin by the standard protocol, and the number and maturation of generated hair follicles were evaluated. The other slides were processed by immunohistochemical staining. Briefly, the sections were incubated with 5% goat serum at room temperature for 30 min., followed by incubation with mouse anti-human TGF-β₂ (1:100, Neo Markers, Fremont, CA, USA), mouse anti-human TGF-β₁ (1:100, Lab Vision,

Table 1 Selected genes up- or down-regulated in human dermal papilla cells (hDPCs). Signal intensities of gene expression in hDPCs were compared with those of human dermal fibroblasts of the same individual, and the fold changes were expressed as log ratio values in the right two columns (P2 and P8). Among the up-regulated 34 genes in Table S1, 11 genes showed a fold change of at least 2 (log ratio), with a decrease of the fold change from P2 to P8 ('Up-regulated', upper panel). Among the down-regulated 44 genes in Table S2, 5 genes showed a difference of at most -2 (log ratio) ('Down-regulated', lower panel)

	Symbol	Gene name	P2	P8
Up-regulated	<i>CCL2</i>	<i>Chemokine (C-C motif) ligand 2</i>	4.93	1.98
	<i>MGC5618</i>	<i>Hypothetical protein MGC5618</i>	4.31	1.20
	<i>G0S2</i>	<i>G0/G1 switch 2</i>	3.93	2.05
	<i>TFPI2</i>	<i>Tissue factor pathway inhibitor 2</i>	2.85	1.40
	<i>HNT</i>	<i>Neurotrimin</i>	2.84	1.01
	<i>TGFB2</i>	<i>Transforming growth factor, beta 2</i>	2.47	1.49
	<i>PRG1</i>	<i>Proteoglycan 1, secretory granule</i>	2.31	2.19
	<i>HLA-C</i>	<i>Major histocompatibility complex, class I, C</i>	2.23	1.29
	<i>FGF7</i>	<i>Fibroblast growth factor 7 (keratinocyte growth factor)</i>	2.10	1.50
	<i>TNFRSF10B</i>	<i>Tumor necrosis factor receptor superfamily, member 10b</i>	2.10	1.08
	<i>PTX3</i>	<i>Pentraxin-related gene, rapidly induced by IL-1 beta</i>	2.00	1.12
Down-regulated	<i>EGR1</i>	<i>Early growth response 1</i>	-3.46	-1.62
	<i>TK1</i>	<i>Thymidine kinase 1, soluble</i>	-2.46	-2.57
	<i>SGK</i>	<i>Serum/glucocorticoid regulated kinase</i>	-2.27	-1.23
	<i>DOK5</i>	<i>Docking protein 5</i>	-2.09	-1.14
	<i>CDC20</i>	<i>CDC20 cell division cycle 20 homolog (S. cerevisiae)</i>	-2.02	-2.86

Fremont, CA, USA), rabbit anti-rat phospho-SMAD-2 (1:100, Millipore, Billerica, MA, USA), rabbit anti-rat SMAD-7 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-rat PAI (plasminogen activator inhibitor)-1 (1:100, Innovative Research, Novi, MI, USA) antibodies at room temperature for 60 min. AlexaTM546-conjugated goat anti-mouse IgG (1:100, Molecular Probes, Eugene, CA, USA) or AlexaTM 488 conjugated goat anti-rabbit IgG (1:100, Molecular Probes) was used as a secondary antibody to detect the primary antibodies. Counter staining was performed with Hoechst33342 (Dojindo, Kumamoto, Japan).

Statistical analysis

Data were presented as mean \pm S.E. To test the significance of quantitative data, the unpaired Student's t-test was applied.

Results

TGF- β_2 gene is specifically detected in hDPCs by microarray analysis

We performed comparative microarray analysis of the molecular gene signatures of cultured hDPCs and hDFs. We suggested that gene(s) related to hair-inducing pathways are contained in DPs, but not in DFs, and that expression of the gene(s) decreases upon

passage. We did not use the same culture conditions for hDPCs and hDFs in this experiment; we used KCM for culturing hDPCs to maximize their hair-inducing capacity but used DMEM (basal medium of KCM) for hDFs not to provide this support. In each comparison, out of 54,613 genes, we first identified genes with signal intensity of at least 100. When we compared cultured hDPCs with hDFs of the same individual at the earlier passage (P2), we found 567 up-regulated and 498 down-regulated genes ('early-DPC genes') in hDPCs, of which the fold difference from hDFs was at least 1 or at most -1 in log ratio. At passage 8, the number of up-regulated and down-regulated genes with the same features decreased to 143 and 174 genes, respectively ('late-DPC genes'). We found 34 overlapping up-regulated genes (Table S1) and 48 overlapping down-regulated genes (Table S2) in both early-DPC and late-DPC genes. When limited to genes whose expression changed significantly from passage 2 to passage 8, only 11 up-regulated and 5 down-regulated genes were listed (Table 1). TGF- β_2 was included among the 11 up-regulated genes, suggesting its putative function in hair-inducing capacity.

TGF- β_2 gene is preferentially expressed in cultured hDPCs

Because cultured DPCs have been shown to contain hair-inducing capacity but lose it upon culture [6, 22], the gene expression

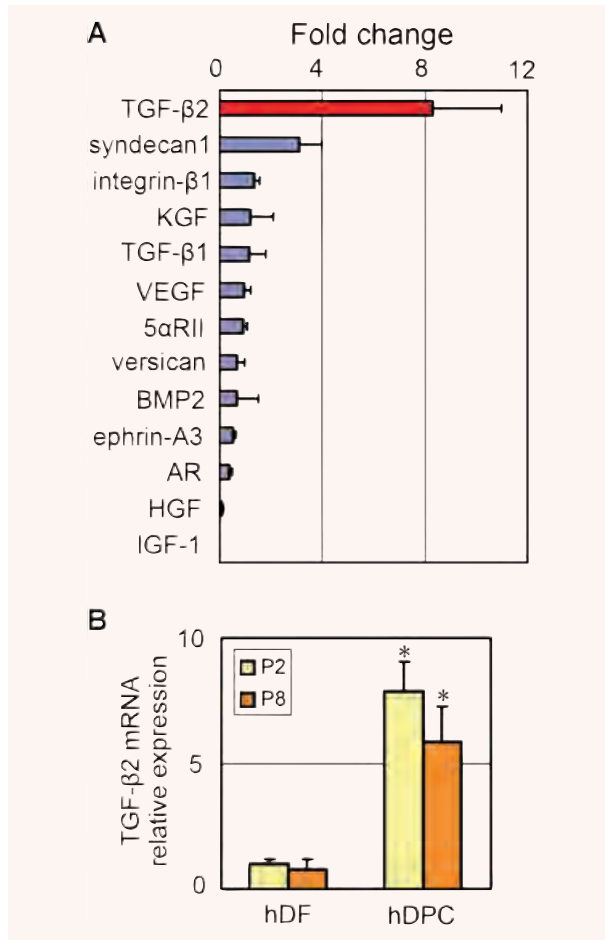


Fig. 1 TGF- β 2 mRNA is preferentially expressed in cultured human dermal papilla cells (hDPCs). **(A)** Relative mRNA expression of hair follicle-related genes in cultured hDPCs. mRNA was isolated from cultured hDPCs and hDFs of the same individual (passage 2: P2) and expression levels were quantified by real-time PCR. The expression levels were normalized to GAPDH mRNA expression and shown as fold changes (hDPC/hDF). $n = 3$. **(B)** TGF- β 2 mRNA relative expression in cultured hDPCs and hDFs of the same individual at passage 2 (P2) or 8 (P8). mRNA expression levels were normalized to GAPDH mRNA expression and fold changes to hDF (P2) were shown. $n = 3$. *Significant difference from hDFs ($P < 0.01$).

profile of cells at early passages of culture was determined. We selected a set of genes previously reported to be related to DPC function [14, 24–36], and examined which genes were up-regulated in hDPC cultured in 10% DMEM at passage 2 in comparison with expression in hDFs cultured in 10% DMEM; the set of genes included TGF- β 1, TGF- β 2, BMP-2, syndecan-1, integrin- β 1, KGF, VEGF, HGF, PDGF, 5 α -reductase II (5 α RII), versican, ephrin-A3 and AR. Quantitative real-time PCR revealed that TGF- β 2 was significantly up-regulated in cultured hDPCs compared to hDFs (Fig. 1A). We also examined hDPCs at later passages. At passage 8, hDPCs were viable enough to keep proliferating and showed no sign of apoptosis or growth arrest. TGF- β 2 gene expression was still up-

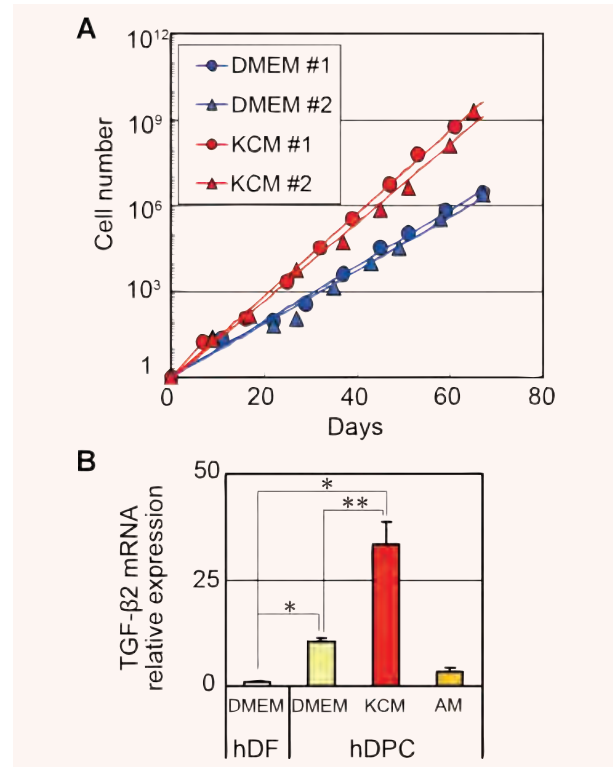


Fig. 2 Effects of keratinocyte conditioned medium (KCM) on proliferation and TGF- β 2 gene expression in human dermal papilla cells (hDPCs). **(A)** Integral cell numbers of hDPCs cultured in 10% DMEM or KCM. hDPCs and DFs from two different individuals (#1 and #2) were cultured in 10% DMEM or KCM up to passage 8. Each plot indicates the integral cell number (shown as fold increase from initial seeding) at the time of each subculture. **(B)** TGF- β 2 mRNA relative expression in hDPCs and hDFs (passage 2) cultured in 10% DMEM, KCM or Amniomax™ II (AM). mRNA expression levels were normalized to GAPDH mRNA expression and fold changes to hDF were shown. $n = 4$. *Significant difference from hDFs ($P < 0.01$). **Significant difference from hDPCs cultured in 10% DMEM ($P < 0.05$).

regulated in hDPCs at passage 8 compared to hDFs, and was slightly lower compared to hDPCs at passage 2 (Fig. 1B).

TGF- β 2 gene expression in cultured hDPCs is enhanced by epidermal KCM

It is well documented that rodent DPCs obtained from the vibrissa hair follicles maintain their proliferative and hair-inducing capacities when they are cultured in KCM [16]. To assess whether hDPCs would exhibit similar properties, we first tested the proliferative effect of human KCM on cultured hDPCs. KCM showed a marked effect on promoting hDPC proliferation to the extent of an approximate 1000-fold increase in cell number within eight passages (60–70 days) (Fig. 2A). Although KCM did not cause apparent alterations in the morphology of hDPCs, TGF- β 2 mRNA expression

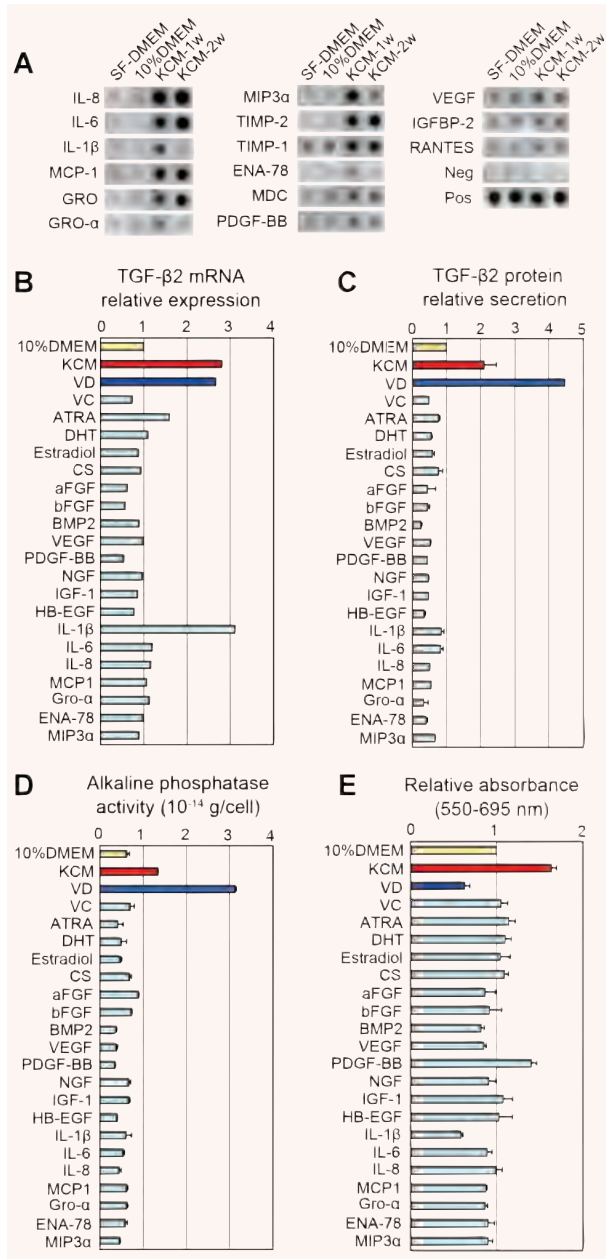


Fig. 3 Influences of keratinocyte conditioned media (KCM) components and other factors on human dermal papilla cells (hDPCs) *in vitro*. **(A)** Cytokine expression in human KCM. Each panel shows expression of each indicated cytokine released by hDPCs cultured in serum-free DMEM (SF-DMEM), DMEM with 10% foetal bovine serum (10% DMEM), and KCM harvested 1 or 2 weeks after the medium switch (KCM-1w or KCM-2w). **(B)** TGF- β_2 mRNA expression of hDPCs 48 hrs after supplementation of each factor. The data are shown as fold change compared to control media (10% DMEM). **(C)** TGF- β_2 protein in hDPCs 48 hrs after supplementation of each factor. The data are shown as fold change compared to control media (10% DMEM). **(D)** Alkaline phosphatase activity of cultured hDPCs 48 hrs after supplementation of each factor. The data are shown in 10^{-14} g/cell equivalent. **(E)** Relative cell number of hDPCs measured by the MTT assay. The data are shown as fold change compared to control media (10% DMEM). *n* = 3. Supplements and concentrations used in **(B)**, **(C)**, **(D)** and **(E)** were as follows: acidic FGF (aFGF, 100 ng/ml), basic FGF (bFGF, 100 ng/ml), BMP-2 (100 ng/ml), IL-1 β (100 ng/ml), IL-6 (100 ng/ml), IL-8 (100 ng/ml), vascular endothelial growth factor (100 ng/ml), PDGF-BB (100 ng/ml), nerve growth factor (100 ng/ml), heparin-binding EGF-like growth factor (100 ng/ml), MIP-3 α (100 ng/ml), MCP-1 (100 ng/ml), IGF-1 (100 ng/ml), ENA-78 (100 ng/ml), GRO- α (100 ng/ml), 1,25(OH) $_2$ D $_3$ (VD, 100 nM), ascorbic acid (VC, 100 μ M), cholesterol sulphate (CS, 100 μ M), all-trans retinoic acid (ATRA, 10 nM), 17 β -estradiol (10 nM) and dihydrotestosterone (DHT, 10 nM).

was significantly up-regulated in hDPCs cultured in KCM compared to control media or other kind of commercially available growth media (AmniomaxTM II) (Fig. 2B).

hDPCs respond to soluble factors from epidermal keratinocytes

We next sought to identify possible KCM components that enhance TGF- β_2 expression in hDPCs. We first examined the cytokine expression profiles of human KCM using a cytokine anti-

body array. Among 79 cytokines examined, IL-8, IL-6, IL-1 β , MCP-1, Gro, MIP-3 α , tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, ENA-78, macrophage-derived chemokine, PDGF-BB, VEGF, insulin-like growth factor binding protein (IGFBP)-2 and regulated upon activation, normal T-cell expressed and secreted (RANTES) were highly expressed in KCM compared with control media (Fig. 3A). In addition to these cytokines, soluble factors considered to have biological activity on the hair follicles [17, 18, 37–46] were tested for induction of TGF- β_2 expression in hDPCs cultured in 10% DMEM. Among 21 factors tested, a biologically active metabolite of vitamin D $_3$, 1,25(OH) $_2$ D $_3$, and IL-1 β , as well as KCM, showed a marked effect on promoting TGF- β_2 mRNA expression in hDPCs (Fig. 3B). The promotion of TGF- β_2 mRNA expression by 1,25(OH) $_2$ D $_3$ or IL-1 β was independent of serum supplementation (Fig. S3). ELISA for TGF- β_2 protein revealed that TGF- β_2 secretion from hDPCs was highly elevated upon supplementation with 1,25(OH) $_2$ D $_3$ or KCM, while no effect was observed with the other factors (Fig. 3C). Furthermore, ALP activity, a well-established marker for DPCs and hair-inducing property in hDPCs [11, 47], was significantly higher in the presence of 1,25(OH) $_2$ D $_3$ as well as KCM (Fig. 3D). In contrast, MTT assay revealed that hDPC proliferation was impaired by 1,25(OH) $_2$ D $_3$, while enhanced by KCM (Fig. 3E).

Active form of vitamin D promotes TGF- β_2 gene expression of hDPCs

Real-time RT-PCR revealed that supplementation of 10–1000 nM of 1,25(OH) $_2$ D $_3$ significantly up-regulated TGF- β_2 mRNA expression

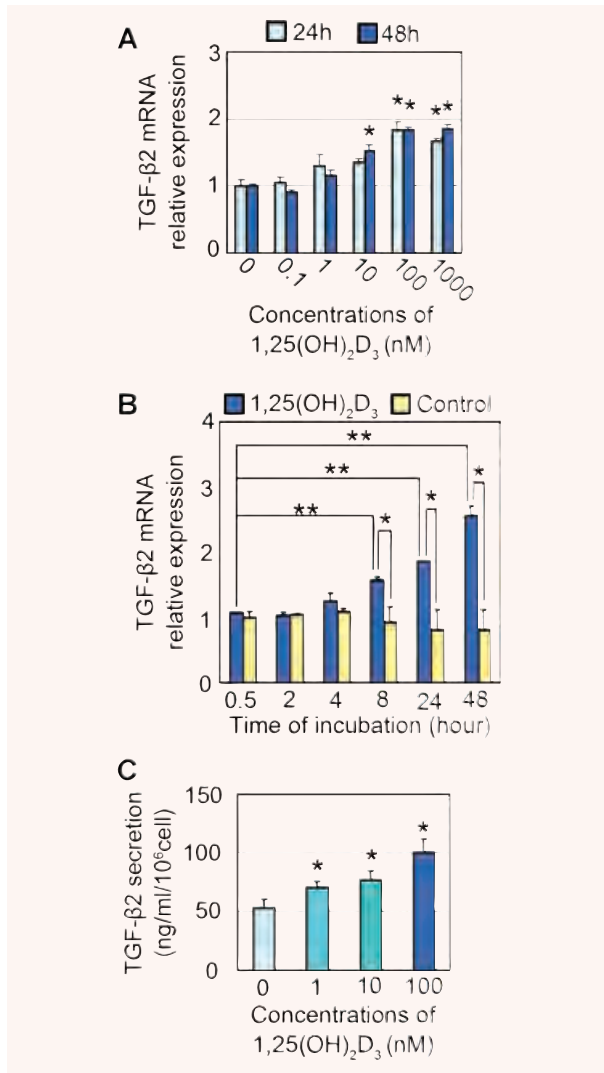


Fig. 4 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) up-regulates TGF-β₂ expression in human dermal papilla cells (hDPCs). **(A)** Quantitative real-time PCR detection of TGF-β₂ mRNA expression in hDPCs (passage 2) cultured for 24 or 48 hrs in the presence of various concentrations (0, 0.1, 1, 10, 100, 1000 nM) of 1,25(OH)₂D₃. The data are shown as fold changes to the baseline expression (at 0 nM). *n* = 5. *Significant difference from baseline expression (*P* < 0.05). **(B)** Quantitative real-time PCR detection of TGF-β₂ mRNA expression in hDPCs (passage 2) at various times of incubation (0.5, 2, 4, 8, 24, 48) in the presence of 100 nM of 1,25(OH)₂D₃. The data are shown as the fold changes to the baseline expression (at 0 hr). *n* = 3. *Significant difference between each pair (*P* < 0.05). **Significant difference from baseline (*P* < 0.05). **(C)** Quantitative detection by ELISA of TGF-β₂ protein secretion from cultured hDPCs (passage 2, normalized to 10⁶ cells equivalent, 3 days incubation) in the presence of various concentrations of 1,25(OH)₂D₃ (0, 1, 10, 100 nM). *Significant difference from 0 nM (*P* < 0.05).

in hDPCs after 24 or 48 hrs of incubation (Fig. 4A), and this up-regulation of TGF-β₂ expression was seen as early as 8 hrs (Fig. 4B). ELISA analysis of secreted TGF-β₂ protein showed that 1,25(OH)₂D₃ significantly promoted TGF-β₂ secretion from hDPCs in a dose-dependent manner (Fig. 4C).

Inhibition of TGF-β₂ signalling at either the receptor or ligand level suppresses hair folliculogenesis *in vivo*

The functional capacity of TGF-β₂ in contributing to hair induction was assessed in an animal model for hair folliculogenesis. Rat-human chimeric hair follicles were generated in nude mice using the sandwich method described above. In control mice, the chimeric hair follicles were generated in 3–4 weeks after grafting of cultured hDPCs and showed histological features at a variety of developmental stages (Fig. S2), mimicking those of foetal hair follicle morphogenesis as described previously [20, 48]. Immunohistochemistry confirmed that TGF-β₂, but not TGF-β₁, was expressed in the dermal sheath of newly developed hair follicles (Fig. 5A).

To evaluate the dependency of TGF-β signalling in hair folliculogenesis in the above animal models, signal transduction was inhibited by a continuous infiltration of SB431542, a highly selective kinase inhibitor for TGF-β type I receptor [49] *via* osmotic pump. Histological evaluation revealed that SB431542 suppressed both the frequency and maturation of hair follicle development. In control mice, well-matured hair follicles were observed that could be categorized into high maturation stages, with structures such as the hair shaft and the sebaceous gland (Fig. 5B). On the other hand, in the SB431542-administered mice, signs of impaired maturation, such as pseudo-keratosis of the inner root sheath, were seen (Fig. 5B). The total number of generated hair follicles was significantly decreased in the SB431542-administered mice (Fig. 5C). Furthermore, a morphometric categorization of maturation stages of generated hair follicles demonstrated the suppressive effects of SB431542 on folliculogenesis (Fig. 5D); follicles of control mice were observed to be in well-matured stages (stage 5 to 7), while follicles of SB431542-administered mice were classified as poorly matured stages (stages 2 to 4).

Two neutralizing antibodies were employed for the TGF-β ligand-neutralizing approach and administered *via* local injections: a neutralizing antibody specific for TGF-β₂ with no effect on TGF-β₁ or TGF-β₃ and a pan TGF-β neutralizing antibody that inhibits TGF-β₁, TGF-β₂ and TGF-β₃ activity. Although histological analysis did not reveal any significant differences in maturation stages of generated hair follicles among the groups, the number of induced hair follicles was significantly decreased in the antibody-administered mice compared with the nonspecific IgG-administered mice (Fig. 6A–C).

In addition, a hair reconstitution assay with or without signal inhibition by anti-TGF-β₂ antibody was also performed with the chamber method. The number of generated hair follicles was significantly smaller in mice treated with anti-TGF-β₂ antibody

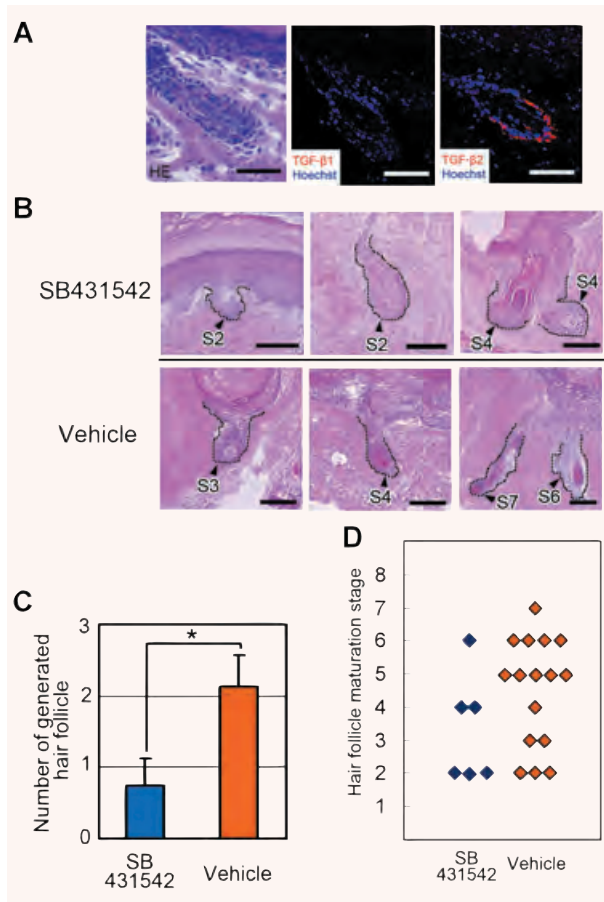


Fig. 5 Histological analysis of generated chimeric hair follicles in sandwich models with or without TGF- β signal inhibition. Cell sheets of human dermal papilla cells (hDPCs) were inserted between the dermis and epidermis of rat foot pad skin. The sandwiched construct was transplanted into the subcutis of nude mice (see Fig. 1), and harvested 4 weeks after transplantation. **(A)** The bulb region of a generated hair follicle. *Left:* Haematoxylin and eosin staining, *middle and right:* immunohistochemical stainings for TGF- β_1 and TGF- β_2 . Nuclear staining was performed with Hoechst33342. Bars = 20 μm . **(B)** Histology of generated hair follicles with or without SB431542-mediated inhibition of TGF- β signal. Generated hair follicles and boundaries are indicated with black arrowheads and dashed lines. The maturation stage is indicated beside each follicle, e.g. 'S2', 'S4' or 'S7', and was evaluated according to the classification proposed by Paus *et al.* [21] after modification. (See Table S3 and Fig. S2 for details). Haematoxylin and eosin staining. Bars = 50 μm . **(C)** Number of generated hair follicles per transplant. Hair follicle morphogenesis was significantly smaller in number in the SB431542-administered mice compared to control mice. Four recipient mice were used for each group; three hDPC sheet fragments were sandwiched for each mouse. *Significant difference from the control mice ($P < 0.05$). **(D)** Maturation stage data of generated hair follicles with or without SB431542-mediated inhibition of TGF- β signal. Note the decreased distribution of well-matured hair follicles (S5 to S7) in the SB431542-administered mice.

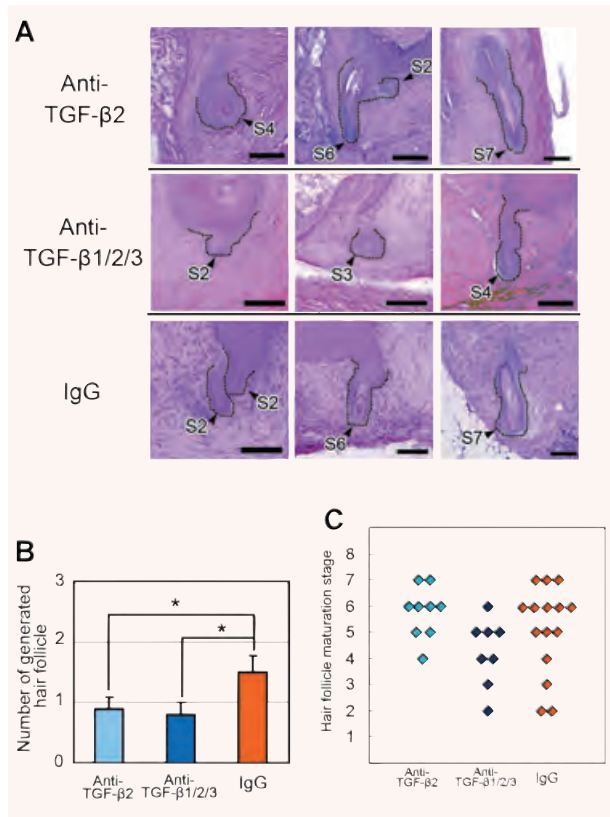


Fig. 6 Neutralizing antibodies against TGF- β s impair hair folliculogenesis in sandwich models. **(A)** Histology of generated hair follicles with or without inhibition of TGF- β signal by neutralizing antibody against TGF- β_2 or TGF- $\beta_{1/2/3}$. Generated hair follicles and their boundaries are indicated with black arrowheads and dashed lines. The maturation stage is indicated beside each follicle, e.g. 'S2', 'S4' or 'S7', and was evaluated according to the classification proposed by Paus *et al.* [21] after modification. (See Table S3 and Fig. S2 for details). Haematoxylin and eosin staining. Bars = 50 μm . **(B)** Number of generated hair follicles per transplant. Hair follicle morphogenesis was significantly smaller in number in mice treated with anti-TGF- β_2 antibody or anti-TGF- $\beta_{1/2/3}$ antibody than in those treated with control IgG. Five recipient mice were used for each group; three hDPC sheet fragments were sandwiched for each mouse. *Significant difference from the control mice ($P < 0.05$). **(C)** Maturation stage data of hair follicles generated in mice with administration of anti-TGF- β_2 or anti-TGF- $\beta_{1/2/3}$ neutralizing antibody or nonspecific control IgG.

compared to control mice (Fig. 7A and B). In chamber models using mouse DPCs (foetal dermal cells), SB431542 and anti-TGF- β_2 antibody substantially decreased the average number of generated hair follicles, though the differences did not reach statistical significance (Fig. S4).

To further assess effects of augmentation of TGF- β_2 signal on hair follicle induction, TGF- β_2 -releasing gelatin hydrogel microsphere beads (GMBs) were incorporated into the sandwich models; no positive effect of TGF- β_2 signal augmentation was observed with this approach (Fig. S5).

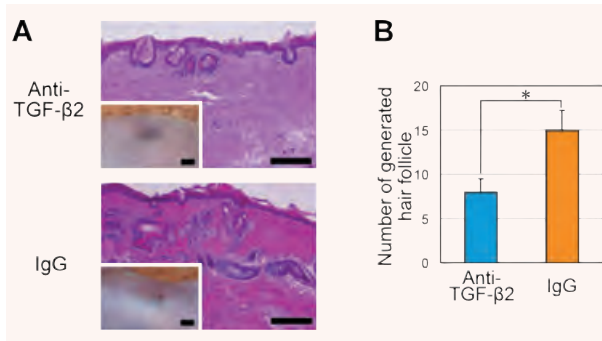


Fig. 7 Histological analysis of hair folliculogenesis in chamber models with or without TGF- β_2 signal inhibition. Cultured human dermal papilla cells (hDPCs) and cultured keratinocytes derived from newborn BL6 mice (mKC) were mixed as a cell suspension and implanted into the chamber on the back of nude mice. Skin samples were harvested 4 weeks after cell transfer; hair folliculogenesis was evaluated with histological sections. Four chambers were prepared on four mice per group. **(A)** Histology (haematoxylin and eosin staining) and macroscopic views (*inset*). Recipient nude mice were treated with a neutralizing antibody against TGF- β_2 (*top*) or a negative control IgG (*bottom*). Bars = 200 μ m (haematoxylin and eosin staining) or 2 μ m (*inset*). **(B)** Average number of generated hair follicles per section ($n = 4$). Hair follicle morphogenesis was significantly smaller in number in mice treated with anti-TGF- β_2 antibody than those treated with control IgG. *Significant difference from control mice ($P < 0.05$).

Immunohistological analysis of TGF- β signal transduction in animal models

To analyse TGF- β_2 signal transduction in hair folliculogenesis in the sandwich models, phosphorylation of SMAD-2 and expression of two SMAD-2 target genes (SMAD-7 and PAI-1) [50] were evaluated by immunohistology (Fig. 8). In control mice, phosphorylated SMAD-2 (pSMAD-2) translocation to nuclei, and positive signals of SMAD-7 and PAI-1 were observed in the epithelium of generated hair follicles. In regions in which no hair follicle development was observed despite the presence of Dil-labelled hDPCs beneath the basal lamina, signs of TGF- β_2 signalling activation were not observed in either SB431542- or anti-TGF- β_2 antibody-treated mice. In SB431542- and anti-TGF- β_2 antibody-treated mice, generated hair follicles expressed pSMAD-2, SMAD-7 or PAI-1 in the epithelium, especially when the follicles were well matured, though expression levels were not as strong compared to the control.

Discussion

Attempts over the last several decades to regenerate hair follicles by transplanting expanded hDPCs have been hampered by the lack of knowledge of the signal and mechanism in hDPCs to induce

hair folliculogenesis. How the hair-inducing capacity of hDPCs can be maintained upon expansion culture also remains unclear. Here we sought to identify a gene(s) in cultured hDPCs responsible for or contributing to hair-inductive capability. In addition, we tried to optimize the cultured method to help preserving hair-inducing capacity of hDPCs. By comparison of the gene expression profiles of hDPCs and hDFs, along with additional gene analysis of DPC biomarkers, TGF- β_2 was identified as a factor specifically expressed by cultured hDPCs. Our results showed that TGF- β_2 expression slightly decreased over culture time, as was the hair-inductive property of hDPCs reported in the literature [8, 12, 16].

For developing an expansion method of hDPCs while maintaining the hair-inductive activity, one piece of evidence may provide some insight: a previous observation that KCM showed a positive effect in maintaining the proliferative and hair-inductive ability of rodent DPCs [16]. Our results demonstrated that TGF- β_2 gene expression was up-regulated in KCM-treated hDPCs compared to non-treated hDPCs. ELISA further revealed that KCM promoted TGF- β_2 protein secretion from hDPCs. In addition, a concomitant elevation of ALP activity in KCM-treated hDPCs suggested KCM-mediated effects on the hair-inductive ability of hDPCs.

These effects of KCM indicated the possibility that KCM contains a key component to maintain hair-inductive property of hDPCs, and the key component may also stimulate cultured hDPCs to express TGF- β_2 . Because hair follicle epithelium and dermal papilla are in contact with each other and send reciprocal signals to induce hair folliculogenesis and maintain hair cycles [1–3], it is not surprising that KCM contains a key component in this process. Cytokine array analysis of KCM detected inflammatory cytokines such as IL-1 β , IL-6, IL-8, MCP-1, RANTES, ENA-78 and Gro, which are known to be secreted from keratinocytes as an acute or late-phase response to inflammation [17, 18]. Our results also revealed that KCM contained known mitotic growth factors for DPCs, such as PDGF-BB [40] or VEGF [39, 43], suggesting that the proliferative effect of KCM is attributed to such growth factors.

Screening analysis of components in KCM detected an unexpected function of vitamin D $_3$: promoting effects on TGF- β_2 expression and ALP activity of hDPCs. Cultured hDPCs express vitamin D receptor (VDR) (data not shown), and thus 1,25(OH) $_2$ D $_3$ -induced TGF- β_2 mRNA up-regulation may be mediated *via* VDR. However, TGF- β_2 mRNA expression increases over time up to 48 hrs and remains high for 5 to 7 days (data not shown), which may suggest the involvement of other signal pathways. A similar observation was made in hDFs: 1,25(OH) $_2$ D $_3$ specifically induces TGF- β_2 mRNA expression in hDFs in the early-phase of signal transduction, followed by induction of all TGF- β isoforms (TGF- β_1 , β_2 and β_3 mRNA) in an autocrine manner [51]. Thus, TGF- β_2 mRNA induction by 1,25(OH) $_2$ D $_3$ shows a monophasic increase with time [51]. Other studies showed that TGF- β signal positively regulates the vitamin D signalling pathway by formation of the Smad3-VDR complex [52], and vitamin D $_3$ induces strong activation of Smad2/Smad3 within 24 hrs in HL-60 cells [53]. These data indicate a direct interplay between TGF- β and vitamin D signalling pathways mediated by VDR and Smad proteins. Taken together, there appears to be a prolonged activation of

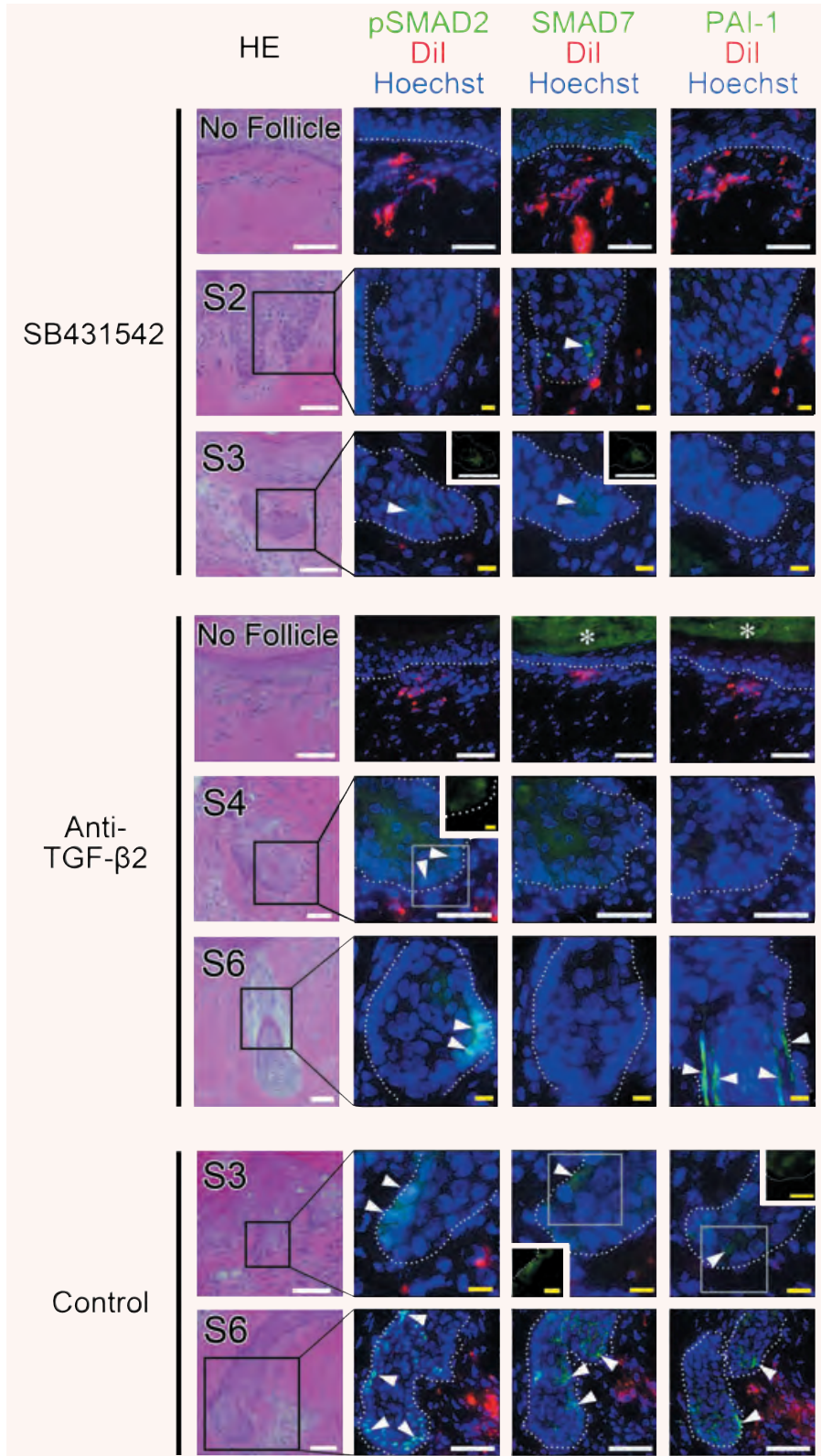


Fig. 8 Immunohistochemical staining for TGF-β signal transduction related factors in sandwich models. Cell sheet fragments of human dermal papilla cells (hDPCs) were placed between the dermis and epidermis of rat foot pad skin; the sandwiched transplant was then inserted into the subcutis of a nude mouse and harvested 4 weeks after transplantation. SB431542, anti-TGF-β₂ neutralizing antibody, or vehicle was administered to the nude mice during the 4 weeks. Each sample was serially sectioned and stained for haematoxylin and eosin or immunostained against phosphorylated SMAD2 (pSMAD-2) and two SMAD-2 target genes (SMAD-7 and PAI-1). Hair follicle maturation stages were indicated in the haematoxylin and eosin images as 'S2' or 'S6'. Representative images are also shown in which hDPCs were present but folliculogenesis was absent (shown as 'No follicle'). Immunostaining for pSMAD-2, SMAD-7 and PAI-1 were visualized in green fluorescence, red colour indicates Dil-labelled hDPCs, and Hoechst33342 was used for nuclear staining. In vehicle-administered control mice, positive pSMAD-2 signal was detected predominantly in the nuclei and positive SMAD-7 signals were located in the nuclei and/or cytoplasm of epithelial cells (arrowheads). PAI-1 signal was detected in the cytoplasm of epithelial cells and/or interstitial spaces in generated hair follicles (arrowheads). Similar findings were also observed in well-matured generated follicles, but no signals for pSMAD-2, SMAD-7 and PAI-1 were found in the 'No follicle' area. White dotted lines indicate the boundary of hair follicles and asterisks (*) indicate non-specific fluorescence in the stratum corneum. Insets are single-immunostained images for pSMAD-2, SMAD-7 or PAI-1. White bar = 50 μm, yellow bar = 10 μm.

both TGF- β and vitamin D signalling pathways in cultured hDPCs as the result of a close interplay and positive feedback loop, although further investigation should be performed.

Ablation of VDR in mice [54, 55] and mutations of VDR in human beings result in the development of alopecia [56, 57]. VDR is expressed in the two major cell components that make up hair follicles: the mesenchymal component, dermal papilla, and the epithelial component, outer root sheath keratinocytes [58]. Recently, VDR expression in follicular keratinocytes was shown to be essential in maintaining hair follicle homeostasis [59, 60]. However, less is known of the effects of vitamin D₃ signal on dermal papilla thus far. The biologically active metabolite of vitamin D₃, 1,25(OH)₂D₃, is mainly produced in kidneys [61], but is also produced and secreted by keratinocytes in the presence of endogenous 1 α -hydroxylase [37, 38, 62]. Thus, it is suggested that 1,25(OH)₂D₃ secreted by keratinocytes likely works as a signalling molecule to stimulate DPCs to secrete TGF- β ₂ and initiate the vitamin D₃ and TGF- β signalling loop.

In most types of cells, the fundamental functions of TGF- β isoforms are growth inhibition and deposition of extracellular matrix [63]. Especially during foetal development, TGF- β s are found in a broad range of organs, such as epithelium, myocardium, cartilage and bone of extremities, and in the nervous system, suggesting its critical functions in organogenesis. In hair follicle physiology, TGF- β s have been shown to exert unique multidirectional effects [4], *i.e.* both positive and suppressive effects on hair growth. TGF- β ₁ blocks anagen and induces catagen [64, 65] and inhibits hair growth [66]. TGF- β ₁ and TGF- β ₂ stimulate proliferation of outer root sheath keratinocytes [67, 68]. TGF- β ₂ induces premature hair follicle regression in adult hair cycling [35, 69], while TGF- β ₂ was also shown to be required for hair folliculogenesis [31, 70].

In the context of adult hair cycle, TGF- β ₂ is synthesized in the dermal papilla by the stimulation of dihydrotestosterone at the initiation of catagen, triggering the intrinsic caspase network and subsequent apoptotic cell death of hair follicle epithelial cells [35, 69]. In contrast, during hair development, TGF- β ₂ receptors are focally expressed initially in the placode and subsequently in the outer root sheath [71], and TGF- β ₂ exerts its morphogenetic function [31] through transient induction of the transcriptional factor Snail in the hair bud [70]. These highly elaborate spatio-temporal manners of expression suggest critical roles of the TGF- β ligand-receptor system in hair folliculogenesis. In our study, phosphorylation of SMAD-2 and expression of SMAD-2 targeted gene products (SMAD-7 and PAI-1) were seen in the epithelium of generated follicles but not in epithelium in which folliculogenesis was not induced despite the adjacent localization of transplanted hDPCs; together this suggests that the SMAD-2-mediated signal may be required for generated hair follicle maturation.

The hair folliculogenesis in our animal models depends on the epithelial-mesenchymal interaction, mimicking foetal hair follicle morphogenesis, not transition to anagen in the adult hair cycle. Suppression of hair folliculogenesis in this study by inhibition of TGF- β ₂ signal transduction both at the receptor and ligand level may reflect TGF- β signalling function observed in foetal hair follicle morphogenesis [31]. Although careful considerations should be

given to differences between our animal models and normal physiological conditions [72], our findings may draw attention to the underestimated TGF- β ₂ function in hair folliculogenesis and provide insights into clinical hair regeneration with expanded hDPCs.

In conclusion, TGF- β ₂ was specifically expressed in hDPCs at higher levels compared to hDFs, and inhibition of TGF- β ₂ signal at either the ligand or receptor level impaired hair folliculogenesis in an hDPC transplantation animal model. The vitamin D₃ analogue promoted TGF- β ₂ expression and ALP activity in hDPCs and may be a critical functional factor in KCM in the enhancement and preservation of the hair-inducing capacity of cultured hDPCs, suggesting its potential use for treatment of alopecia with expanded hDPC transplantation. The results of this study suggest a critical role for TGF- β ₂ and vitamin D₃ signalling pathways in hair folliculogenesis.

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Conflict of interests

The authors declare that they have no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Materials and methods

TGF- β ₂ releasing gelatin hydrogel microsphere beads

Gelatin hydrogel microsphere beads (GMBs) containing 1 μ g TGF- β ₂ in 40 μ l of PBS or PBS alone were prepared according to the previously reported method [73]. hDPC sheet fragments were inserted between the epidermis and dermis of rat foot pad with 2 μ l of GMB solution on top of a hDPC sheet. The sandwich transplants were transferred to the subcutis of nude mice, harvested 4 weeks after transplantation and processed for histological examination of hair folliculogenesis.

Fig. S1 Animal assays for hair folliculogenesis. **(A)** Sandwich model. Human dermal papilla cells (hDPCs) cultured at full confluence were scraped off and cut into pieces. The follicular foot pad skin of a F344 rat was cut and digested with a medium supplemented with Dispase™ II to separate the epidermis from the dermis. After placement of the hDPC sheet between the epidermis and dermis, the sandwiched transplant was inserted into the subcutis of a Balb/c nude mouse. **(B)** Chamber model. The chamber was transplanted on the back of a Balb/c nude mouse prior to cell transfer. hDPCs and keratinocytes derived from newborn BL6 mice (mKC) were cultured, combined in a cell suspension and transplanted into the chamber.

Fig. S2 Representative examples of generated hair follicles at various stages. Maturation of generated hair follicles was classified according to the scale (eight stages from S1 to S8) proposed by Paus *et al.* [21] after modification, as listed in Table S3. Haematoxylin and eosin staining. Bars = 50 μ m. DP: dermal papilla; HS: hair shaft; IRS: inner root sheath; SG: sebaceous gland.

Fig. S3 Influence of serum supplementation on TGF- β_2 mRNA expression in cultured human dermal papilla cells (hDPCs) mRNA was isolated from hDPCs cultured for 48 hrs in the presence of 1,25-dihydroxyvitamin D3 (1,25(OH) $_2$ D $_3$, 100 nM) or IL-1 β (100 ng/ml) with or without 10% serum (foetal bovine serum). TGF- β_2 mRNA expression was assessed by real-time PCR and relative expression levels normalized by GAPDH expression are shown. Serum supplementation did not influence TGF- β_2 mRNA up-regulation by 1,25(OH) $_2$ D $_3$ or IL-1 β . $n = 4$. * $P < 0.05$, ** $P < 0.01$.

Fig. S4 Histological analysis of generated murine hair follicles in chamber models with or without TGF- β signal inhibition. Fresh dermal cells and fresh keratinocytes isolated from foetal BL6 mice were mixed as a cell suspension and implanted into the chamber on the back of a nude mouse. Skin samples were harvested 4 weeks after cell transfer, and hair folliculogenesis was evaluated with histological sections. Four chambers were prepared on four mice per group. **(A)** Histology (haematoxylin and eosin staining) and macroscopic views (*inset*) of samples treated with SB431542 (*top*) or vehicle (*bottom*). Bars = 200 μ m (haematoxylin and eosin staining) or 2 mm (*inset*). **(B)** Average number of generated hair follicles per section. $n = 4$. SB431542 substantially decreased the average number of regenerated follicles, though the difference between mice treated with SB431542 and the control mice was not significant ($P = 0.075$). **(C)** Histology (haematoxylin and eosin staining) and macroscopic views (*inset*) of samples treated with a neutralizing antibody against TGF- β_2 (*top*) or a negative control IgG (*bottom*). **(D)** Average number of generated hair follicles per section. $n = 4$. TGF- β_2 decreased the average number of regenerated follicles, though the difference between mice treated with TGF- β_2 and the control mice was not significant ($P = 0.117$). Bars = 200 μ m (haematoxylin and eosin staining) or 2 mm (*inset*).

Fig. S5 Effects of augmented TGF- β_2 signal on hair folliculogenesis. TGF- β_2 signal was augmented by implanting TGF- β_2 releasing gelatin hydrogel microsphere beads (GMBs) together with sheets of Dil-labelled cultured human dermal papilla cells (hDPCs) in our sandwich models; after placement of the hDPC sheet between the epidermis and dermis of rat foot pad skin, the sandwiched transplant was inserted into the subcutis of a Balb/c nude mouse and harvested 4 weeks after transplantation. GMBs with PBS alone were used as a negative control. **(A)** Histology of sandwich model samples with or without TGF- β signal augmentation. Serial sections were stained for haematoxylin and eosin or Hoechst33342. GMBs and Dil-labelled hDPCs were observed in yellow (due to non-specific fluorescence) and red under a fluorescent microscope, respectively. Asterisks (*) indicate GMBs. Bar = 50 μ m. **(B, C)** Maturation stage **(B)** and number per sample **(C)** of generated hair follicles (TGF- β_2 -GMBs: $n = 9$, PBS-GMBs, $n = 10$). No significant difference in maturation or number of generated hair follicles between the two groups ($P = 0.283$).

Table S1 Genes up-regulated in human dermal papilla cells (hDPCs) both at passage 2 (P2) and passage 8 (P8) in comparison with human dermal fibroblasts (hDFs). Signal intensities of gene expression in hDPCs were compared with those in hDFs of the same individual, and the fold changes were expressed as log ratio values in the right two columns of P2 and P8. There were 567 up-regulated genes in hDPCs at P2 with a difference of at least 1 (log ratio), and 143 genes in hDPCs at P8. Thirty-four genes in P2 and P8 overlapped and are shown in order of the fold change at P2.

Table S2 Genes down-regulated in human dermal papilla cells (hDPCs) both at passage 2 (P2) and passage 8 (P8) in comparison with human dermal fibroblasts (hDFs). Signal intensities of gene expression in hDPCs were compared with those in hDFs of the same individual, and the fold changes were expressed as log ratio values in the right two columns of P2 and P8. There were 498 down-regulated genes in hDPCs at P2 with a difference of at most -1 (log ratio), and 174 genes in hDPCs at P8. Forty-four genes in P2 and P8 overlapped and are shown in order of the fold change at P2.

Table S3 Classification scale of generated hair follicles. The classification was originally proposed by Paus *et al.* [21]. Maturation stage is classified into eight categories (S1–S8) according to histological features. See Fig. S1 for representative histological views.

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