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Title: TGF-β2 is specifically expressed in human dermal papilla cells and modulates hair folliculogenesis

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Abbreviation
AR: androgen receptor
BMP: bone morphogenetic protein
DMEM: Dulbecco's modified Eagle's medium
ELISA: enzyme-linked immunosorbent assay
ENA: epithelial cell-derived neutrophil-activating peptide
FGF: fibroblast growth factor
GMB: gelatin hydrogel microsphere bead
GRO: growth-related oncogene
HB-EGF: heparin-binding EGF-like growth factor

hDPC: human dermal papilla cell
hDF: human dermal fibroblast
HGF: hepatocyte growth factor
IGF: insulin-like growth factor
IGFBP: insulin-like growth factor binding protein
KCM: keratinocyte conditioned media
KGF: keratinocyte growth factor
MCP: monocyte chemotactic protein
MDC: macrophage-derived chemokine
MIP: macrophage inflammatory protein
NGF: nerve growth factor
PAI-1: plasminogen activator inhibitor 1
PDGF: platelet-derived growth factor
pSMAD2: phosorylated SMAD2
RANTES: regulated upon activation, normal T-cell expressed and secreted
RT-PCR: reverse transcription polymerase chain reaction
TIMP: tissue inhibitor of metalloproteinase
TGF: transforming growth factor
VDR: vitamin D receptor
VEGF: vascular endothelial growth factor

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 and transplanting them into bald skin, though much remains to be overcome before clinical success. In this study, we compared gene signatures of human DPCs at different passages and human dermal fibroblasts, and found TGF-β2 to be highly expressed in cultured human DPCs. Keratinocyte conditioned medium, which is known to help preserve the hair inducing capacity of hDPCs, upregulated TGF-β2 expression of human DPCs and also enhanced their alkaline phosphatase activity, a known index for hair inductive capacity. Through screening of components secreted from keratinocytes, the vitamin D_3 analogue was found to promote TGF-β2 expression and alkaline phosphatase activity of human DPCs. In animal hair folliculogenesis models using rat epidermis and expanded human DPCs, inhibition of TGF-β2 signaling at the ligand or receptor level significantly impaired hair folliculogenesis and maturation. These results suggest an important role for TGF-β2 in hair follicle morphogenesis and provide insights into the establishment of future cell therapies for hair regrowth by transplanting expanded DPCs.

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]. Since 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 signaling between hair follicle epithelial and dermal components. In the context of hair follicle "neogenesis", however, it is unclear which signaling molecule(s) among these pathways function in hair induction in transplanted DPCs. Thus far, specific signaling molecules, such as bone morphogentic protein (BMP)-6 [11], have been shown to enhance mouse hair folliculogenesis. Wnt3a signaling 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 over-expression in vivo. In humans, however, 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 have been reported [14, 15], their functions remain to be clarified.

Conditioned media obtained from epidermal keratinocyte culture (keratinocyte conditioned media, or KCM) is 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 hypothesized that specific genes relating to hair inducing capacity are upregulated in human DPCs (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-β2 gene was specifically expressed in hDPCs and its expression was upregulated by KCM. We further investigated potential roles of TGF-β2 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 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (10% DMEM). After two 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 mm square pieces and incubated in 10% DMEM supplemented with 1,000 unit/ml Dispase™ II (Sankyo, Tokyo, Japan) at 4°C for 15-18 hours. The epidermis was carefully peeled off from the dermis and incubated in phosphate buffered saline (PBS) supplemented with 0.25% trypsin and EDTA mixture at 37°C for 20 minutes to obtain fresh keratinocyte cell suspension. Keratinoctyes were cultured in serum free media, DKSFM™ (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 one week, centrifuged at 3,000 g for 30 minutes, and filtrated through a 0.22 μm membrane filter (Micropore, Madison, NJ). 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); basic FGF (Peprotech); BMP2 (Wako, Osaka, Japan); interleukin (IL)-1β (Endogen, Rockford, IL); IL-6 (Peprotech); IL-8 (Wako); vascular endothelial growth factor (VEGF) (Wako); platelet-derived growth factor (PDGF)-BB (Wako); nerve growth factor (NGF) (Sigma-Aldrich, Louis, MO); heparin-binding EGF-like growth factor (HB-EGF) (Peprotech); macrophage inflammatory protein (MIP) 3α (R&D systems, Minneapolis, MN); 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 D_3 [$1,25(OH)_2D_3$] (LKT laboratories, St. Paul, MN); cholesterol sulfate (Sigma-Aldrich); all-trans retinoic acid (Biomol, Hamburg, Germany); 17β-estradiol (Cayman Chemical, Ann Arbor, MI); and dihydrotestosterone (Biomol). All reagents were diluted in PBS or ethanol to 1,000 fold of the final working concentrations indicated in manufacturer's instructions and stored in aliquots at -20°C.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated from cultured hDPCs or hDFs using an RNeasy™ Mini Kit (Qiagen, Valencia, CA), followed by reverse transcription. PCR amplification of cDNA was performed in a 50 μl reaction consisting of 1x TaqMan™ Universal Master Mix (Applied Biosystems, Foster City, CA) 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 TaqMan™ pre-designed primers and probes (Applied Biosystems): TGF-β2 (Hs00236092_m1); TGF-β1 (Hs00998129_m1); BMP2 (Hs00154192_m1); syndecan1

(Hs00896423_m1); integrin-β1 (Hs00559595_m1); keratinocyte growth factor (KGF) (Hs00940253_m1); VEGF (Hs00900054_m1); IGF-1 (Hs01547656_m1); HGF (Hs00300159_m1); PDGF (Hs00234042_m1); steroid 5α-reductase II (Hs01399057_m1); versican (Hs00171642_m1); ephrin-A3 (Hs00191913_m1); and androgen receptor (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 (passage 2 and 8) and hDFs (passage 2) hDFs in 10% DMEM were isolated using an RNeasy™ Mini Kit. The quality of each sample was assessed by rRNA 28S/18S ratio and RIN (RNA Integrity Number) using Agilent 2100 Bioanalyzer™ (Agilent Technology, Palo Alto, CA). cDNA was obtained from 5 μg of tRNA by one-cycle of reverse transcription. The biotin-labeled cRNAs were purified, fragmented, and hybridized to the GeneChip™ 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 analyzed using GeneChip™ Command Console™ Software (Affymetrix) and Microsoft Excel™.

Cytokine array analysis

The supernatant of human keratinocyte culture was collected at one or two weeks after the medium switch from DKSFM 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) 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).

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 hours of incubation, and processed with ELISA following the manufacturer's instructions.

Alkaline phosphatase activity assay

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

were seeded on a 96-well plate and cell lysates were collected after 96 hours 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-mm square pieces, and used as transplanted constructs. The follicular foot pad skin of 8-week-old F344 rats was cut into 3-mm square pieces and incubated in 10% DMEM and 1,000 U/ml Dispase™ II at 37°C for 20 minutes 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 (Supplemental Fig. 1). 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 8 stages (S1 to S8) according to a previously described method [21] (Supplemental Fig. 2).

A chamber grafting method was also employed according to previously reported methods [11, 20, 22, 23] with some modifications (Supplemental Fig. 1). 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 fetal dermal cells and fetal keratinocytes isolated from BL6 mouse embryos was also utilized. For preparation of cultured mKC, the dorsal skin of newborn mice was incubated in 1,000 U/ml Dispase™ II at 4°C for 15-18 hours, 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 minutes to obtain single cells. The resultant cell suspension was filtrated through a 40-μm cell strainer and cultured in DKSFM™ for 4 days. For preparation of mouse fetal 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 fetal dermal cells) and epithelial cells (cultured mouse neonatal keratinocytes or fresh fetal keratinocytes) transplanted into each chamber was 10^6 (2×10^6 in total). Skin samples of recipient nude mice were harvested 4 weeks after cell transplantation. Each group consisted of 4 chambers on 4 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 administrated continuously (0.25 μl/hour) by Alzet™ osmotic pumps (Durect, Cupertino, CA), 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-β1/2/3 (R&D systems) was used to neutralize TGF-β2 or all three isoforms (TGF-β1/2/3) of TGF-β ligand. One μg 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 hour, fixed in paraformaldehyde (4% in PBS) for 1 minute, and washed in PBS for 5 minutes. Every other slide was stained with hematoxylin 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 minutes, followed by incubation with mouse anti-human TGF-β2 (1:100, Neo Markers, Fremont, CA), mouse anti-human TGF-β1 (1:100, Lab Vision, Fremont, CA), rabbit anti-rat phospho-SMAD2 (1:100, Millipore, Billerica, MA), rabbit anti-rat SMAD7 (1;100, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-rat PAI (plasminogen activator inhibitor)-1 (1:100, Innovative Research, Novi, MI) antibodies at room temperature for 60 minutes. Alexa^{TM}546-conjugated goat anti-mouse IgG (1:100, Molecular Probes, Eugene, CA) or Alexa^{TM} 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 using Hoechst33342 (Dojindo, Kumamoto, Japan).

Statistical analysis

Data were presented as mean ± standard error. To test the significance of quantitative data, the unpaired Student's t-test was applied.

RESULTS

TGF-β2 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 hypothesized 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 54613 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 upregulated and 498 downregulated 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 upregulated and downregulated genes with the same features decreased to 143 and 174 genes, respectively ("late-DPC genes"). We found 34 overlapping upregulated genes (Supplemental Table 1) and 48 overlapping downregulated genes (Supplemental Table 2) in both early-DPC and late-DPC genes. When limited to genes whose expression changed significantly from passage 2 to passage 8, only 11 upregulated and 5 downregulated genes were listed (Table 1). TGF-β2 was included among the 11 upregulated genes, suggesting its putative function in hair inducing capacity.

TGF-β2 gene is preferentially expressed in cultured hDPCs

Since cultured DPCs have been shown to contain hair inducing capacity but lose it upon culture [6, 22], the gene expression 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 upregulated 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-β1, TGF-β2, BMP2, syndecan1, integrin-β1, KGF, VEGF, HGF, PDGF, 5α-reductase II (5αRII), versican, ephrin-A3, and androgen receptor (AR). Quantitative real-time PCR revealed that TGF-β2 was significantly upregulated 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-β2 gene expression was still upregulated in hDPCs at passage 8 compared to hDFs, and was slightly lower compared to hDPCs at passage 2 (Fig. 1B).

TGF-β2 gene expression in cultured hDPCs is enhanced by epidermal keratinocyte conditioned media

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 1,000 fold increase in cell number within 8 passages (60-70 days) (Fig. 2A). Although KCM did not cause apparent alterations in the morphology of hDPCs, TGF-β2 mRNA expression was significantly upregulated in hDPCs cultured in KCM compared to control media or other kind of commercially available growth media (Amniomax^{TM} II) (Fig. 2B).

Human DPCs respond to soluble factors from epidermal keratinocytes

We next sought to identify possible KCM components that enhance TGF-β2 expression in hDPCs. We first examined the cytokine expression profiles of human KCM using a cytokine antibody array. Among 79 cytokines examined, IL-8, IL-6, IL-1β, MCP-1, Gro, MIP3α, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, ENA-78, macrophage-derived chemokine (MDC), PDGF-BB, VEGF, insulin-like growth factor binding protein (IFGBP)-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-β2 expression in hDPCs cultured in 10% DMEM. Among 21 factors tested, a biologically active metabolite of vitamin D_3, $1,25(OH)_2D_3$, and IL-1β, as well as KCM, showed a marked effect on promoting TGF-β2 mRNA expression in hDPCs (Fig. 3B). The promotion of TGF-β2 mRNA expression by $1,25(OH)_2D_3$ or IL-1β was independent of serum supplementation (Supplemental Fig. 3). ELISA for TGF-β2 protein revealed that TGF-β2 secretion from hDPCs was highly elevated upon supplementation with $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ as well as KCM (Fig. 3D). In contrast, MTT assay revealed that hDPC proliferation was impaired by $1,25(OH)_2D_3$, while enhanced by KCM (Fig. 3E).

Active form of vitamin D promotes TGF-β2 gene expression of human DPCs

Real-time RT-PCR revealed that supplementation of 10-1000 nM of $1,25(OH)_2D_3$ significantly upregulated TGF-β2 mRNA expression in hDPCs after 24 or 48 hours of incubation (Fig. 4A), and this upregulation of TGF-β2 expression was seen as early as 8 hours (Fig. 4B). ELISA analysis of secreted TGF-β2 protein showed that $1,25(OH)_2D_3$ significantly promoted TGF-β2 secretion from hDPCs in a dose-dependent manner (Fig. 4C).

Inhibition of TGF-β2 signaling 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 (Supplemental Fig. 2), mimicking those of fetal hair follicle morphogenesis as described previously [20, 48]. Immunohistchemistry 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 signaling 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-maturated 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-maturated stages (Stage 5 to 7), while follicles of SB431542-administered mice were classified as poorly-maturated 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 inducted 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 using the chamber method. The number of generated hair follicles was significantly smaller in mice treated with anti-TGF-β2 antibody compared to control mice (Fig. 7A, 7B). In chamber models using mouse DPCs (fetal dermal cells), SB431542 and anti-TGF-β2 antibody substantially decreased the average number of generated hair follicles, though the differences did not reach statistical significance (Supplemental Figure 4).

To further assess effects of augmentation of TGF-β2 signal on hair follicle induction, TGF-β2-releasing gelatin hydrogel microsphere beads (GMBs) were incorporated into the sandwich models; no positive effect of TGF-β2 signal augmentation was observed with this approach (Supplemental Fig. 5).

Immunohistological analysis of TGF-β signal transduction in animal models

To analyze TGF-β2 signal transduction in hair folliculogenesis in the sandwich models, phosphorylation of SMAD2 and expression of two SMAD2 target genes (SMAD7 and PAI-1) [50] were evaluated by immunohistology (Fig. 8). In control mice, phosphorylated SMAD2 (pSMAD2) translocation to nuclei, and positive signals of SMAD7 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 DiI-labeled hDPCs beneath the basal lamina, signs of TGF-β2 signaling activation were not observed in either SB431542- or anti-TGF-β2 antibody-treated mice. In SB431542- and anti-TGF-β2 antibody-treated mice, generated hair follicles expressed pSMAD2, SMAD7 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-β2 was identified as a factor specifically expressed by cultured hDPCs. Our results showed that TGF-β2 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-β2 gene expression was upregulated in KCM-treated hDPCs compared to non-treated hDPCs. ELISA further revealed that KCM promoted TGF-β2 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-β2. Since 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-1β, 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-β2 expression and ALP activity of hDPCs. Cultured hDPCs

express vitamin D receptor (VDR) (data not shown), and thus $1,25(OH)_2D_3$-induced TGF-β2 mRNA upregulation may be mediated via VDR. However, TGF-β2 mRNA expression increases over time up to 48 hours 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: $1,25(OH)_2D_3$ specifically induces TGF-β2 mRNA expression in hDFs in the early phase of signal transduction, followed by induction of all TGF-β isoforms (TGF-β1, β2, and β3 mRNA) in an autocrine manner [51]. Thus, TGF-β2 mRNA induction by $1,25(OH)_2D_3$ shows a monophasic increase with time [51]. Other studies showed that TGF-β signal positively regulates the vitamin D signaling pathway by formation of the Smad3-VDR complex [52], and vitamin D_3 induces strong activation of Smad2/Smad3 within 24 hours in HL-60 cells [53]. These data indicate a direct interplay between TGF-β and vitamin D signaling pathways mediated by VDR and Smad proteins. Taken together, there appears to be a prolonged activation of both TGF-β and vitamin D signaling 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 humans 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 D_3 signal on dermal papilla thus far. The biologically active metabolite of vitamin D_3, $1,25(OH)_2D_3$, is mainly produced in kidneys [61], but is also produced and secreted by keratinocytes in the presence of endogenous 1α-hydroxylase [37, 38, 62]. Thus, it is suggested that $1,25(OH)_2D_3$ secreted by keratinocytes likely works as a signaling molecule to stimulate DPCs to secret TGF-β2 and initiate the vitamin D_3 and TGF-β signaling loop.

In most types of cells, the fundamental functions of TGF-β isoforms are growth inhibition and deposition of extracellular matrix [63]. Especially during fetal development, TGF-βs 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-βs have been shown to exert unique multidirectional effects [4], i.e., both positive and suppressive effects on hair growth. TGF-β1 blocks anagen and induces catagen [64, 65] and inhibits hair growth [66]. TGF-β1 and TGF-β2 stimulate proliferation of outer root sheath keratinocytes [67, 68]. TGF-β2 induces premature hair follicle regression in adult hair cycling [35, 69], while TGF-β2 was also shown to be required for hair folliculogensis [31, 70].

In the context of adult hair cycle, TGF-β2 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-β2 receptors are focally expressed initially in the placode and subsequently in the outer root sheath [71], and TGF-β2 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 SMAD2 and expression of SMAD2 targeted gene products (SMAD7 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 SMAD2-mediated signal may be required for generated hair follicle maturation.

The hair folliculogenesis in our animal models depends on the epithelial-mesenchymal interaction, mimicking fetal hair follicle morphogenesis, not transition to anagen in the adult hair cycle. Suppression of hair folliculogenesis in this study by inhibition of TGF-β2 signal transduction both at the receptor and ligand level may reflects TGF-β signaling function observed in fetal 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-β2 function in hair folliculogenesis and provide insights into clinical hair regeneration with expanded hDPCs.

In conclusion, TGF-β2 was specifically expressed in hDPCs at higher levels compared to hDFs, and inhibition of TGF-β2 signal at either the ligand or receptor level impaired hair folliculogenesis in an hDPC transplantation animal model. The vitamin D_3 analogue promoted TGF-β2 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-β2 and vitamin D_3 signaling pathways in hair folliculogenesis.

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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 upregulated 34 genes in Supplemental Table 1, 11 genes showed a fold change of at least 2 (log ratio), with a decrease of the fold change from P2 to P8 ("Upregulated", upper panel). Among the downregulated 44 genes in Supplemental Table 2, 5 genes showed a difference of at most -2 (log ratio) ("Downregulated", lower panel).

	Symbol	Gene name	P2	P8
Upregulated	*CCL2*	*chemokine (C-C motif) ligand 2*	4.93	1.98
	MGC5618	*hypothetical protein MGC5618*	4.31	1.20
	G0S2	*G0/G1 switch 2*	3.93	2.05
	TFPI2	*tissue factor pathway inhibitor 2*	2.85	1.40
	HNT	*neurotrimin*	2.84	1.01
	TGFB2	*transforming growth factor, beta 2*	2.47	1.49
	PRG1	*proteoglycan 1, secretory granule*	2.31	2.19
	HLA-C	*major histocompatibility complex, class I, C*	2.23	1.29
	FGF7	*fibroblast growth factor 7 (keratinocyte growth factor)*	2.10	1.50
	TNFRSF10B	*tumor necrosis factor receptor superfamily, member 10b*	2.10	1.08
	PTX3	*pentraxin-related gene, rapidly induced by IL-1 beta*	2.00	1.12
Downregulated	*EGR1*	*early growth response 1*	-3.46	-1.62
	TK1	*thymidine kinase 1, soluble*	-2.46	-2.57
	SGK	*serum/glucocorticoid regulated kinase*	-2.27	-1.23
	DOK5	*docking protein 5*	-2.09	-1.14
	CDC20	*CDC20 cell division cycle 20 homolog (S. cerevisiae)*	-2.02	-2.86

Figure legends

Figure 1

TGF-β2 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-β2 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 ($p < 0.01$).

Figure 2

Effects of keratinocyte conditioned medium (KCM) on proliferation and TGF-β2 gene expression in human dermal papilla cells (hDPCs).
(A) Integral cell numbers of hDPCs cultured in 10% DMEM or KCM. Human DPCs 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-β2 mRNA relative expression in hDPCs and hDFs (passage 2) cultured in 10% DMEM, KCM or Amniomax^{TM} 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 ($p < 0.05$).

Figure 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% fetal bovine serum (10% DMEM), and KCM harvested one or two weeks after the medium switch (KCM-1w or KCM-2w). (B) TGF-β2 mRNA expression of hDPCs 48 hours after supplementation of each factor. The data is shown as fold change compared to control media (10% DMEM). (C) TGF-β2 protein in hDPCs 48 hours after supplementation of each factor. The data is shown as fold change compared to control media (10% DMEM). N = 3. (D) Alkaline phosphatase activity of cultured hDPCs 48 hours after supplementation of each factor. The data is shown in 10^{-14} g/cell equivalent. N = 3. (E) Relative cell number of hDPCs measured by the MTT assay. The data is 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). BMP2 (100 ng/ml), IL-1β (100 ng/ml), IL-6 (100 ng/ml), IL-8 (100 ng/ml), VEGF (100 ng/ml), PDGF-BB (100 ng/ml), NGF (100 ng/ml), HB-EGF (100 ng/ml), MIP3α (100 ng/ml), MCP1 (100 ng/ml), IGF-1 (100 ng/ml), ENA-78 (100 ng/ml), GRO-α (100 ng/ml), $1,25(OH)_2D_3$ (VD, 100 nM), ascorbic acid (VC, 100 μM), cholesterol sulfate (CS, 100 μM), all-trans retinoic acid (ATRA, 10 nM), 17β-estradiol (10 nM), and dihydrotestosterone (DHT, 10 nM).

Figure 4

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) upregulates 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 hours in the presence of various concentrations (0, 0.1, 1, 10, 100, 1000 nM) of $1,25(OH)_2D_3$. The data are shown as fold changes to the base-line expression (at 0 nM). N = 5. *Significant difference from base-line 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 $1,25(OH)_2D_3$. The data are shown as the fold changes to the base-line expression (at 0 hour). N = 3. *Significant difference between each pair ($p < 0.05$). **Significant difference from base-line ($p < 0.05$). (C) Quantitative detection by ELISA of TGF-β2 protein secretion from cultured hDPCs (passage 2, normalized to 10^6 cells equivalent, 3 days incubation) in the presence of various concentrations of $1,25(OH)_2D_3$ (0, 1, 10, 100 nM). *Significant difference from 0 nM ($p < 0.05$).

Figure 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*: Hematoxylin and eosin staining (HE staining), *middle and right*: immunohistochemical stainings for TGF-β1 and TGF-β2. Nuclear staining was performed using 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 Supplemental Table 3 and Supplemental Figure 2 for details). Hematoxylin 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-maturated hair follicles (S5 to S7) in the SB431542-administered mice.

Figure 6
Neutralizing antibodies against TGF-βs 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-β2 or TGF-β1/2/3. 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 Supplemental Table 3 and Supplemental Figure 2 for details). Hematoxylin 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-β2 antibody or anti-TGF-β1/2/3 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-β2 or anti-TGF-β1/2/3 neutralizing antibody or nonspecific control IgG.

Figure 7
Histological analysis of hair folliculogenesis in chamber models with or without TGF-β2 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 (HE staining) and macroscopic views (*inset*). Recipient nude mice were treated with a neutralizing antibody against TGF-β2 (*top*) or a negative control IgG (*bottom*). Bars = 200 μm (HE staining) or 2 mm (*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-β2 antibody than those treated with control IgG. *Significant difference from control mice ($p < 0.05$).

Figure 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 four weeks. Each sample was serially sectioned and stained for HE or immunostained against phosphorylated SMAD2 (pSMAD2) and two SMAD2 target genes (SMAD7 and PAI-1). Hair follicle maturation stages were indicated in the HE 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 pSMAD2, SMAD7 and PAI-1 were visualized in green fluorescence, red color indicates DiI-labeled hDPCs, and Hoechst33342 was used for nuclear staining. In vehicle-administered control mice, positive pSMAD2 signal was detected predominantly in the nuclei and positive SMAD7 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 pSMAD2, SMAD7 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 pSMAD2, SMAD7, or PAI-1. White bar = 50 μm, yellow bar = 10 μm.

Supplemental contents

Supplemental Materials and Methods

TGF-β2 releasing gelatin hydrogel microsphere beads
Gelatin hydrogel microsphere beads (GMBs) containing 1 μg TGF-β2 in 40 μl of PBS or PBS alone were prepared according to the previously reported method [Hiraoka, et al. *Tissue Eng.* 2006; 12:1475-87]. hDPC sheet fragments were inserted between the epidermis and dermis of rat foot pad with 2 μl 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.

Supplemental Figure 1
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™ 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. Human DPCs and keratinocytes derived from newborn BL6 mice (mKC) were cultured, combined in a cell suspension and transplanted into the chamber.

Supplemental Figure 2
Representative examples of generated hair follicles at various stages.
Maturation of generated hair follicles was classified according to the scale (8 stages from S1 to S8) proposed by Paus et al. [21] after modification, as listed in Supplemental Table 3. Hematoxylin and eosin staining. Bars = 50 μm.
DP: dermal papilla; HS: hair shaft; IRS: inner root sheath; SG: sebaceous gland.

Supplemental Figure 3
Influence of serum supplementation on TGF-β2 mRNA expression in cultured human dermal papilla cells (hDPCs)
mRNA was isolated from hDPCs cultured for 48 hours in the presence of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$, 100 nM) or IL-1β (100 ng/ml) with or without 10% serum (fetal bovine serum). TGF-β2 mRNA expression was assessed by real-time PCR and relative expression levels normalized by GAPDH expression are shown. Serum supplementation did not influence TGF-β2 mRNA upregulation by $1,25(OH)_2D_3$ or IL-1β. N = 4. $*p < 0.05$, $**p < 0.01$.

Supplemental Figure 4
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 fetal 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 (HE staining) and macroscopic views (*inset*) of samples treated with SB431542 (*top*) or vehicle (*bottom*).

Bars = 200 μm (HE 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 (HE staining) and macroscopic views (*inset*) of samples treated with a neutralizing antibody against TGF-β2 (*top*) or a negative control IgG (*bottom*). (D) Average number of generated hair follicles per section. N = 4. TGF-β2 decreased the average number of regenerated follicles, though the difference between mice treated with TGF-β2 and the control mice was not significant ($p = 0.117$). Bars = 200 μm (HE staining) or 2 mm (*inset*).

Supplemental Figure 5
Effects of augmented TGF-β2 signal on hair folliculogenesis.
TGF-β2 signal was augmented by implanting TGF-β2 releasing gelatin hydrogel microsphere beads (GMBs) together with sheets of DiI-labeled 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 HE (Hematoxilin and eosin) or Hoechst33342. GMBs and DiI-labeled hDPCs were observed in yellow (due to non-specific fluorescence) and red under a fluorescent microscope, respectively. Asterisks (*) indicate GMBs. Bar = 50 μm. (B, C) Maturation stage (B) and number per sample (C) of generated hair follicles (TGF-β2-GMBs: n=9, PBS-GMBs, n=10). No significant difference in maturation or number of generated hair follicles between the two groups ($p = 0.283$).

Supplemental Table 1. Genes upregulated 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 upregulated 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.

Supplemental Table 2. Genes downregulated 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 downregulated 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.

Supplemental Table 3. Classification scale of generated hair follicles.
The classification was originally proposed by Paus et al [21]. Maturation stage is classified into 8 categories (S1-S8) according to histological features. See Supplemental Figure 1 for representative histological views.

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Supplemental contents

Supplemental Table 1.

Symbol	Gene name	P2	P8
CCL2	*chemokine (C-C motif) ligand 2*	4.93	1.98
MGC5618	*hypothetical protein MGC5618*	4.31	1.20
G0S2	*G0/G1 switch 2*	3.93	2.05
TFPI2	*tissue factor pathway inhibitor 2*	2.85	1.40
HNT	*neurotrimin*	2.84	1.01
TGFB2	*transforming growth factor, beta 2*	2.47	1.49
PRG1	*proteoglycan 1, secretory granule*	2.31	2.19
HLA-C	*major histocompatibility complex, class I, C*	2.23	1.29
FGF7	*fibroblast growth factor 7 (keratinocyte growth factor)*	2.10	1.50
TNFRSF10B	*tumor necrosis factor receptor superfamily, member 10b*	2.10	1.08
PTX3	*pentraxin-related gene, rapidly induced by IL-1 beta*	2.00	1.12
CCPG1	*cell cycle progression 1*	1.97	1.40
NTN4	*netrin 4*	1.86	2.18
HLA-G	*HLA-G histocompatibility antigen, class I, G*	1.85	1.04
ANTXR2	*anthrax toxin receptor 2*	1.80	1.15
EDIL3	*EGF-like repeats and discoidin I-like domains 3*	1.66	1.58
FBN2	*fibrillin 2 (congenital contractural arachnodactyly)*	1.63	3.14
IGFBP7	*insulin-like growth factor binding protein 7*	1.62	1.16
GCLM	*glutamate-cysteine ligase, modifier subunit*	1.58	1.15
RPL37	*ribosomal protein L37*	1.58	1.13
HIST1H2BK	*histone 1, H2bk*	1.57	1.40
FLJ11259	*hypothetical protein FLJ11259*	1.56	1.71
C9orf5	*chromosome 9 open reading frame 5*	1.35	1.07
GNPTAB	*N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits*	1.32	1.35
ATP10D	*ATPase, Class V, type 10D*	1.23	1.41
SEMA3C	*sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C*	1.23	1.65
H2BFS	*H2B histone family, member S*	1.23	1.23
KLF3	*Kruppel-like factor 3 (basic)*	1.22	1.13
ADAMTS5	*ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)*	1.16	1.20
DPP4	*dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)*	1.09	1.01
LAMA2	*laminin, alpha 2 (merosin, congenital muscular dystrophy)*	1.08	1.02
RSNL2	*restin-like 2*	1.07	1.37
BACH1	*BTB and CNC homology 1, basic leucine zipper transcription factor 1*	1.03	1.05
TGM2	*transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)*	1.03	1.41

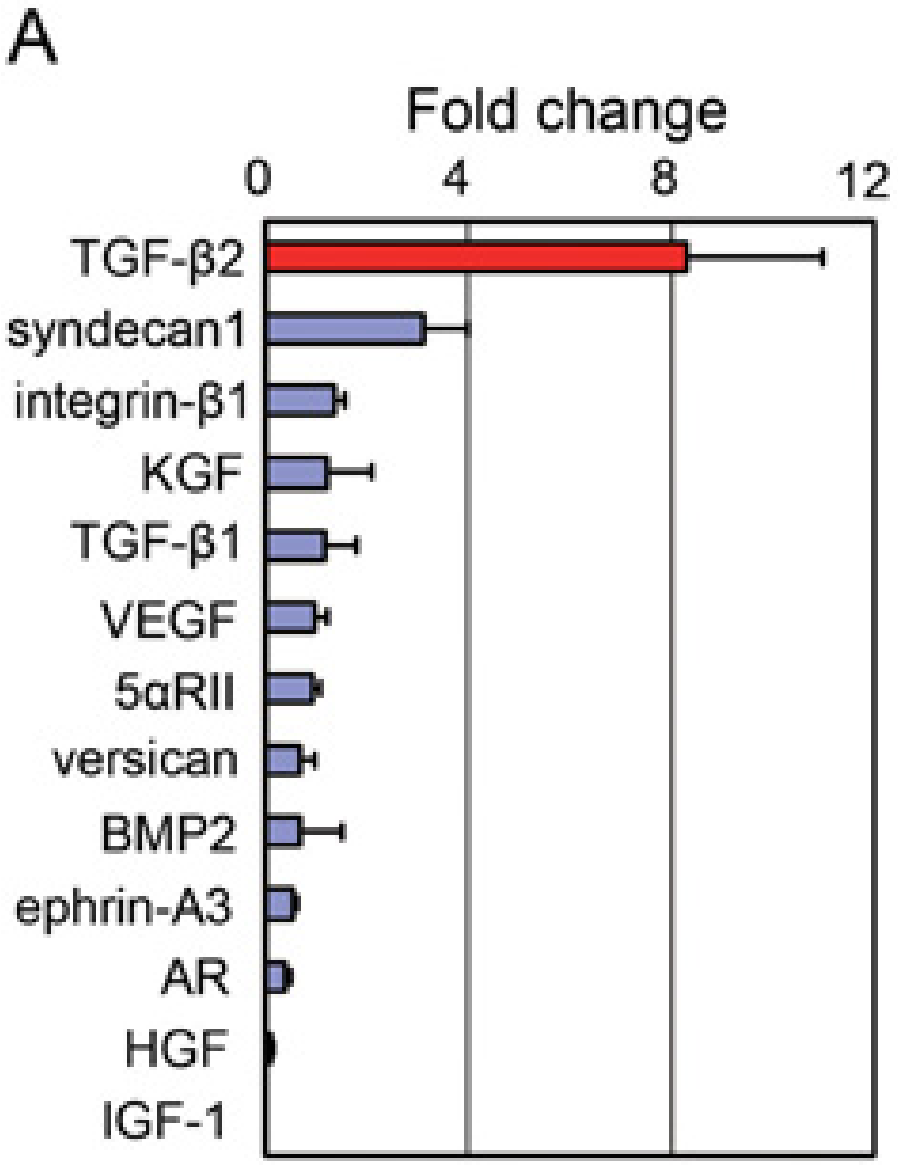
Supplemental Table 2.

Symbol	Gene name	P2	P8
EGR1	*early growth response 1*	-3.46	-1.62
TK1	*thymidine kinase 1, soluble*	-2.46	-2.57
SGK	*serum/glucocorticoid regulated kinase*	-2.27	-1.23
DOK5	*docking protein 5*	-2.09	-1.14
CDC20	*CDC20 cell division cycle 20 homolog (S. cerevisiae)*	-2.02	-2.86
LBR	*lamin B receptor*	-1.99	-1.31
CDKN3	*cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)*	-1.94	-2.18
CDCA3	*cell division cycle associated 3*	-1.76	-2.23
CCNB1	*cyclin B1*	-1.54	-2.15
MCM5	*MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)*	-1.53	-2.26
LRRC15	*leucine rich repeat containing 15*	-1.48	-2.52
CCNA2	*cyclin A2*	-1.47	-1.84
TPX2	*TPX2, microtubule-associated, homolog (Xenopus laevis)*	-1.44	-1.64
LOC283824	*hypothetical protein LOC283824*	-1.44	-1.10
GPSM2	*G-protein signalling modulator 2 (AGS3-like, C. elegans)*	-1.40	-1.52
KIF2C	*kinesin family member 2C*	-1.39	-2.21
CEP55	*centrosomal protein 55kDa*	-1.38	-2.14
BM039	*uncharacterized bone marrow protein BM039*	-1.37	-1.84
UHRF1	*ubiquitin-like, containing PHD and RING finger domains, 1*	-1.35	-2.04
ASPM	*asp (abnormal spindle)-like, microcephaly associated (Drosophila)*	-1.30	-2.24
BIRC5	*baculoviral IAP repeat-containing 5 (survivin)*	-1.30	-2.11
TOP2A	*topoisomerase (DNA) II alpha 170kDa*	-1.29	-2.51
DTYMK	*deoxythymidylate kinase (thymidylate kinase)*	-1.29	-1.54
CCNB2	*cyclin B2*	-1.27	-2.15
RRM2	*ribonucleotide reductase M2 polypeptide*	-1.27	-2.44
TYMS	*thymidylate synthetase*	-1.27	-1.97
MCM6	*MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)*	-1.27	-1.03
DDX39	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 39*	-1.23	-1.59
ANLN	*anillin, actin binding protein (scraps homolog, Drosophila)*	-1.22	-2.00
U2AF2	*U2 (RNU2) small nuclear RNA auxiliary factor 2*	-1.22	-1.11
ANP32E	*acidic (leucine-rich) nuclear phosphoprotein 32 family, member E*	-1.22	-1.50
WHSC1	*Wolf-Hirschhorn syndrome candidate 1*	-1.18	-1.53
ZWILCH	*Zwilch, kinetochore associated, homolog (Drosophila)*	-1.18	-1.22
PRC1	*protein regulator of cytokinesis 1*	-1.17	-2.07
C6orf173	*chromosome 6 open reading frame 173*	-1.17	-1.54
H2AFX	*H2A histone family, member X*	-1.16	-1.54
NUSAP1	*nucleolar and spindle associated protein 1*	-1.16	-2.16
HMGB2	*high-mobility group box 2*	-1.15	-1.61
SUV39H2	*suppressor of variegation 3-9 homolog 2 (Drosophila)*	-1.15	-1.04
KIAA0101	*KIAA0101*	-1.13	-2.08
DNM3	*dynamin 3*	-1.12	-1.06
SHCBP1	*SHC SH2-domain binding protein 1*	-1.11	-2.00

CSTF3	*cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa*	-1.09	-1.32
NOLC1	*nucleolar and coiled-body phosphoprotein 1*	-1.09	-1.00
RACGAP1	*Rac GTPase activating protein 1*	-1.07	-1.91
THOC4	*THO complex 4*	-1.03	-1.03
TMPO	*thymopoietin*	-1.03	-1.19
RPA3	*replication protein A3, 14kDa*	-1.02	-1.52

Supplemental Table 3.

Maturation stage	Histological feature
S1	Epidermal thickening and dermal condensation
S2	Downward elongation of hair-germ without dermal papilla formation
S3	Formation of hair-peg-like structure with concaved basal border surrounding rounded dermal papilla
S4	Formation of bulb in the proximal end of hair germ and formation of corn-shaped inner root sheath distal to the hair matrix
S5	Distal elongation of internal root sheath and pseudo-keratinization of inner root sheath
S6	Formation of hair shaft and differentiation of sebocytes
S7	Distal elongation of hair shaft into hair canal and formation of sebaceous grand
S8	Elongation of hair shaft through epidermis and proximal growth of hair follicle to subcutaneous layer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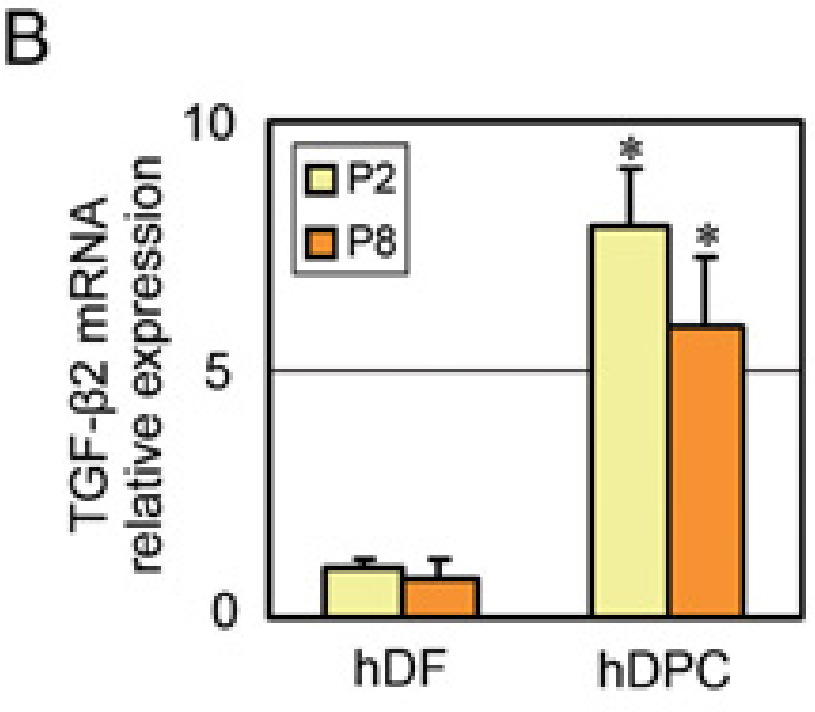
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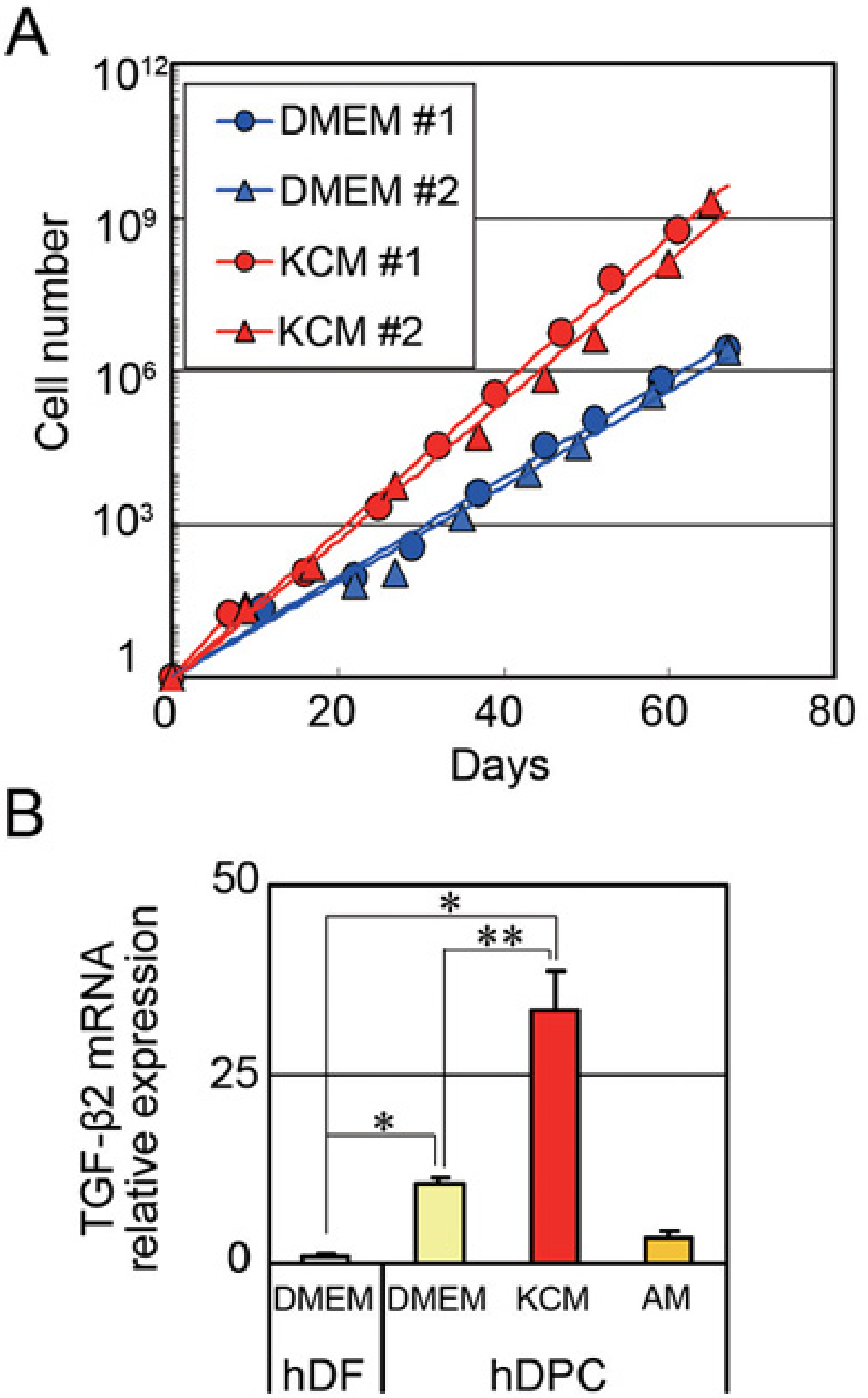
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40
60
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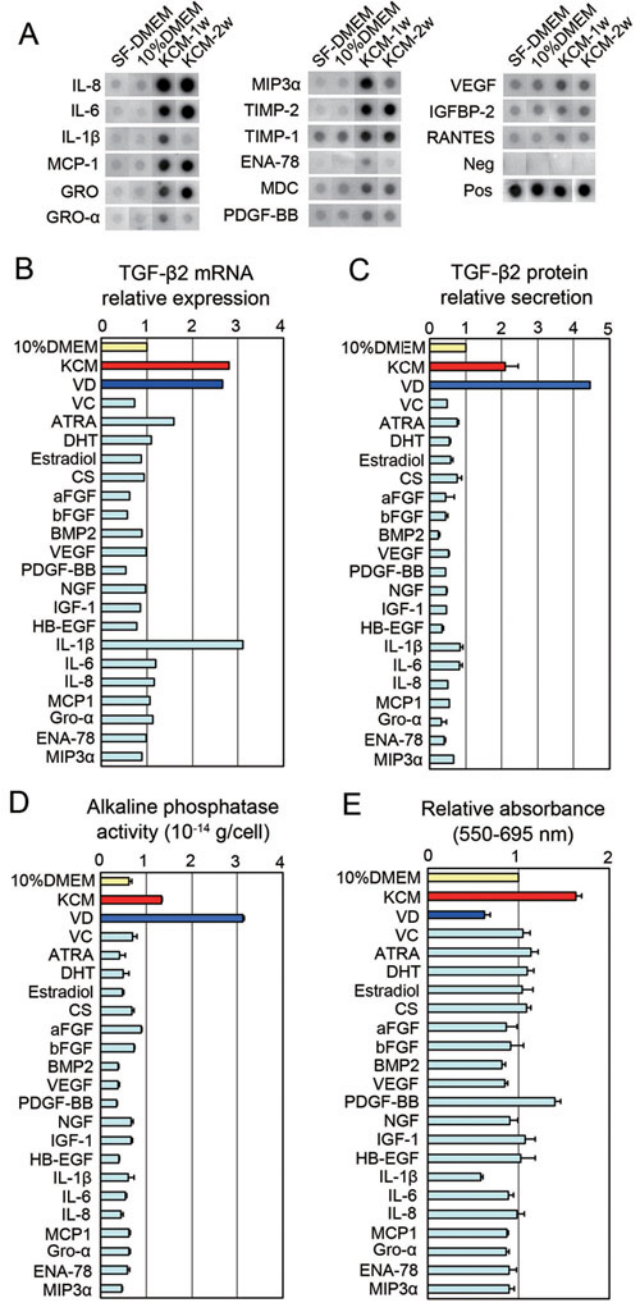
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TIMP-2
TIMP-1
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VEGF
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KCM
VD
VC
ATRA
DHT
Estradiol
CS
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bFGF
BMP2
VEGF
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NGF
IGF-1
HB-EGF
IL-1β
IL-6
IL-8
MCP1
Gro-α
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MIP3α
C
TGF-β2 protein
relative secretion
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VC
ATRA
DHT
Estradiol
CS
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bFGF
BMP2
VEGF
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NGF
IGF-1
HB-EGF
IL-1β
IL-6
IL-8
MCP1
Gro-α
ENA-78
MIP3α
D
Alkaline phosphatase
activity (10-14 g/cell)
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10%DMEM
KCM
VD
VC
ATRA
DHT
Estradiol
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VEGF
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NGF
IGF-1
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Gro-α
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MIP3α
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Relative absorbance
(550-695 nm)
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KCM
VD
VC
ATRA
DHT
Estradiol
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bFGF
BMP2
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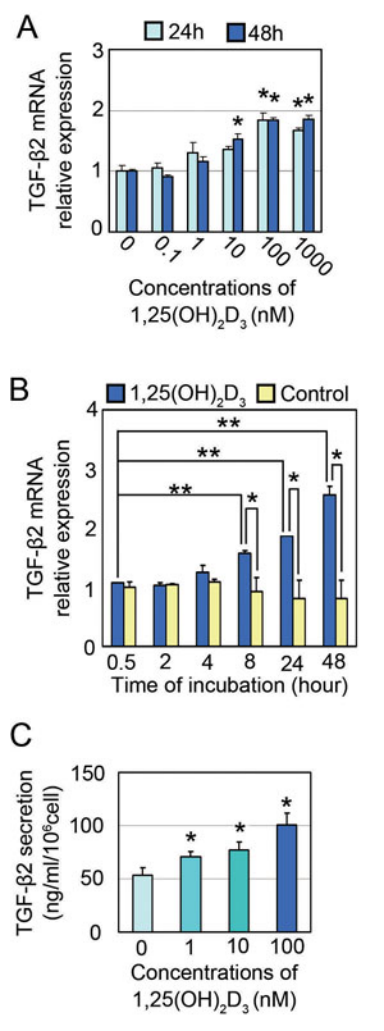
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1,25(OH)2D3
Control
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8
24
48
Time of incubation (hour)
C
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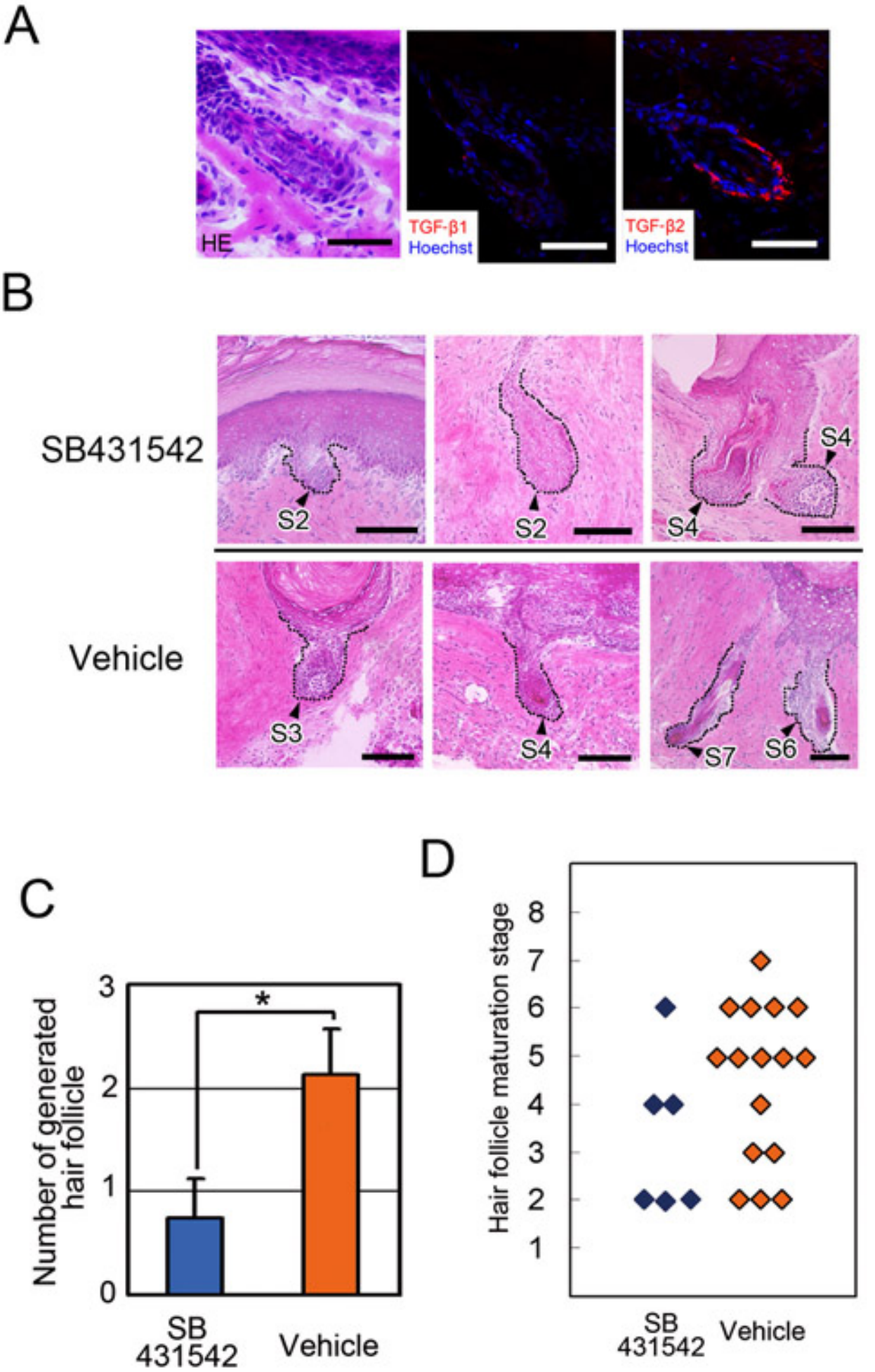
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S3
S4
S7
S6
C
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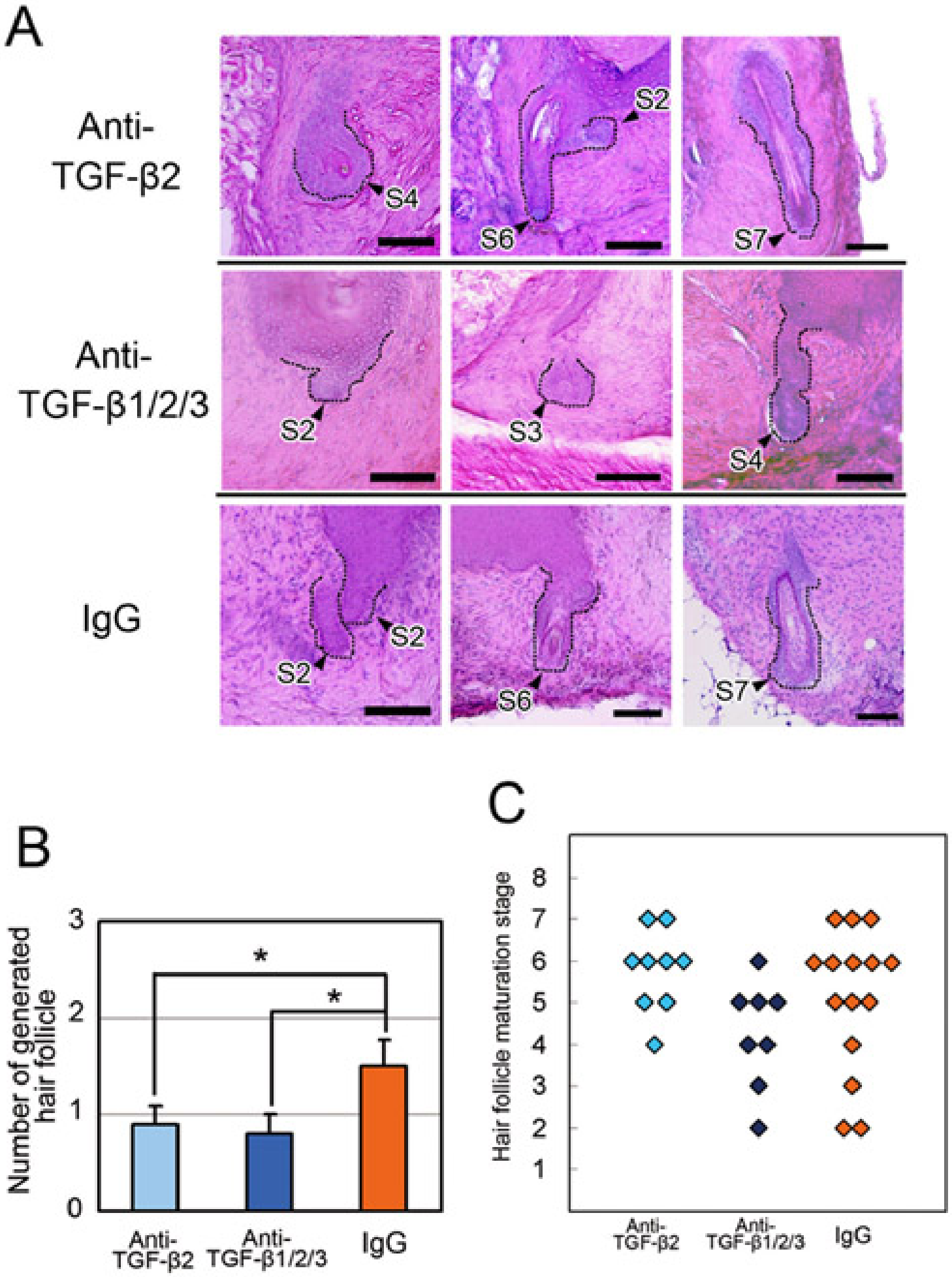
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S2
S6
S7
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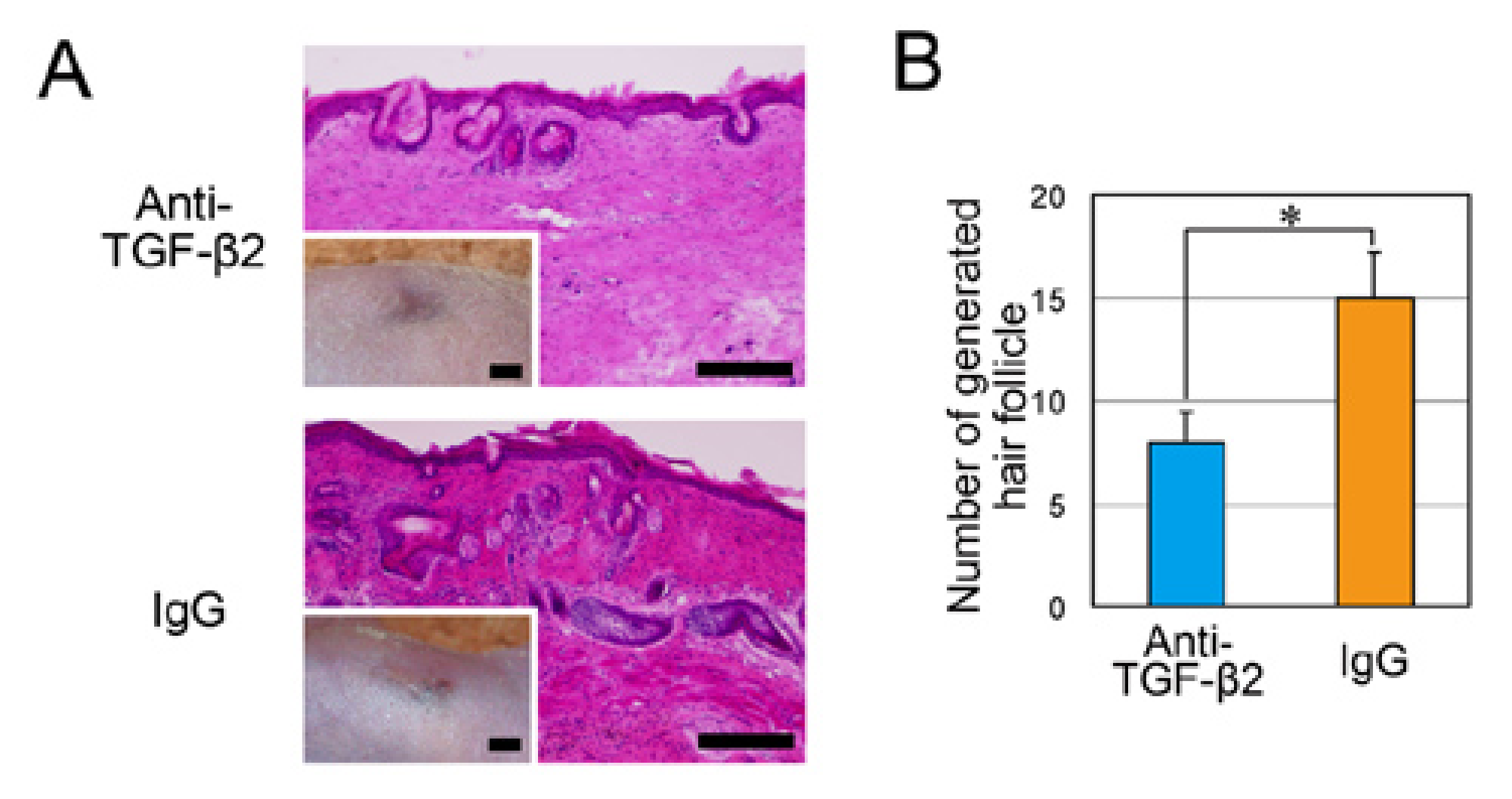
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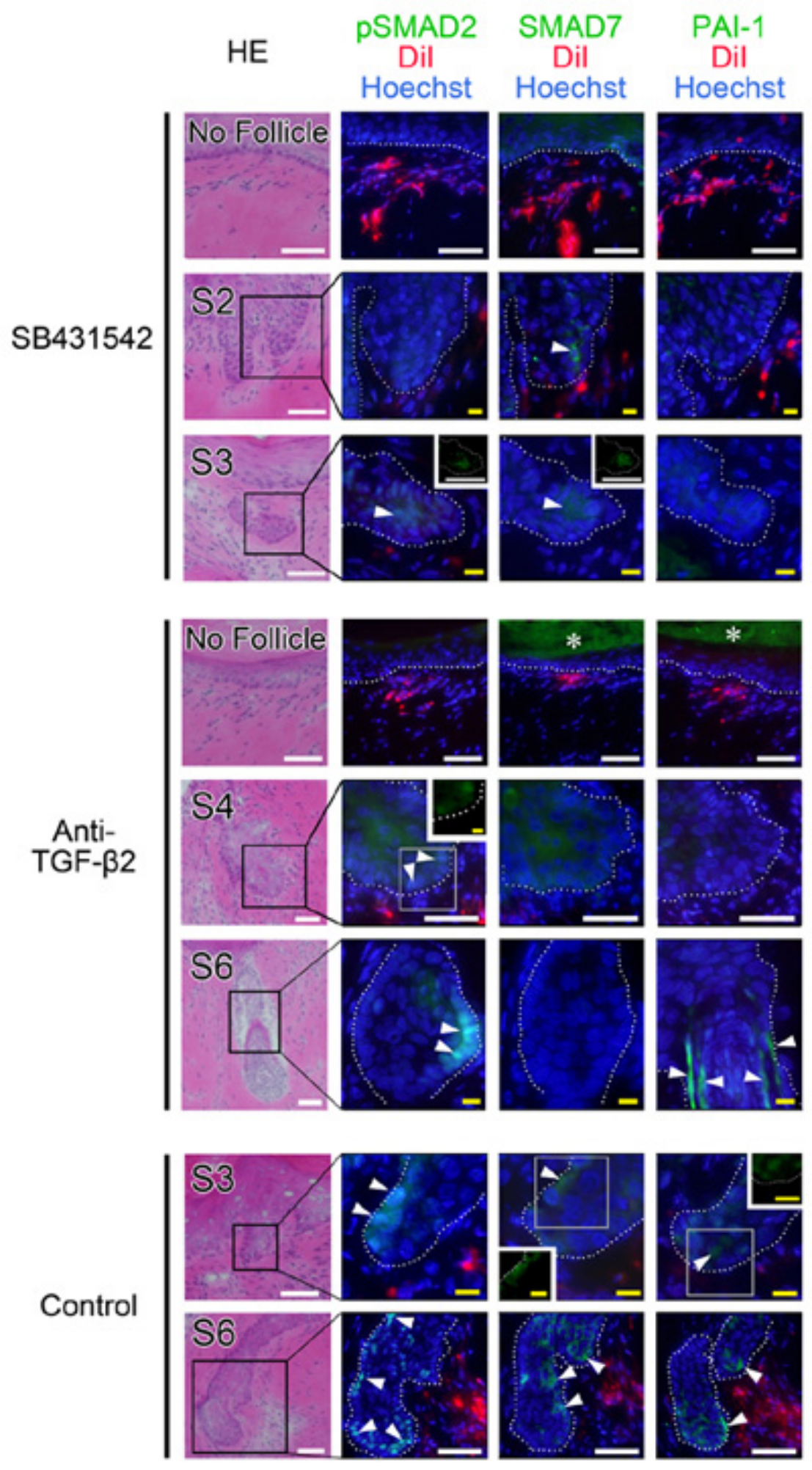
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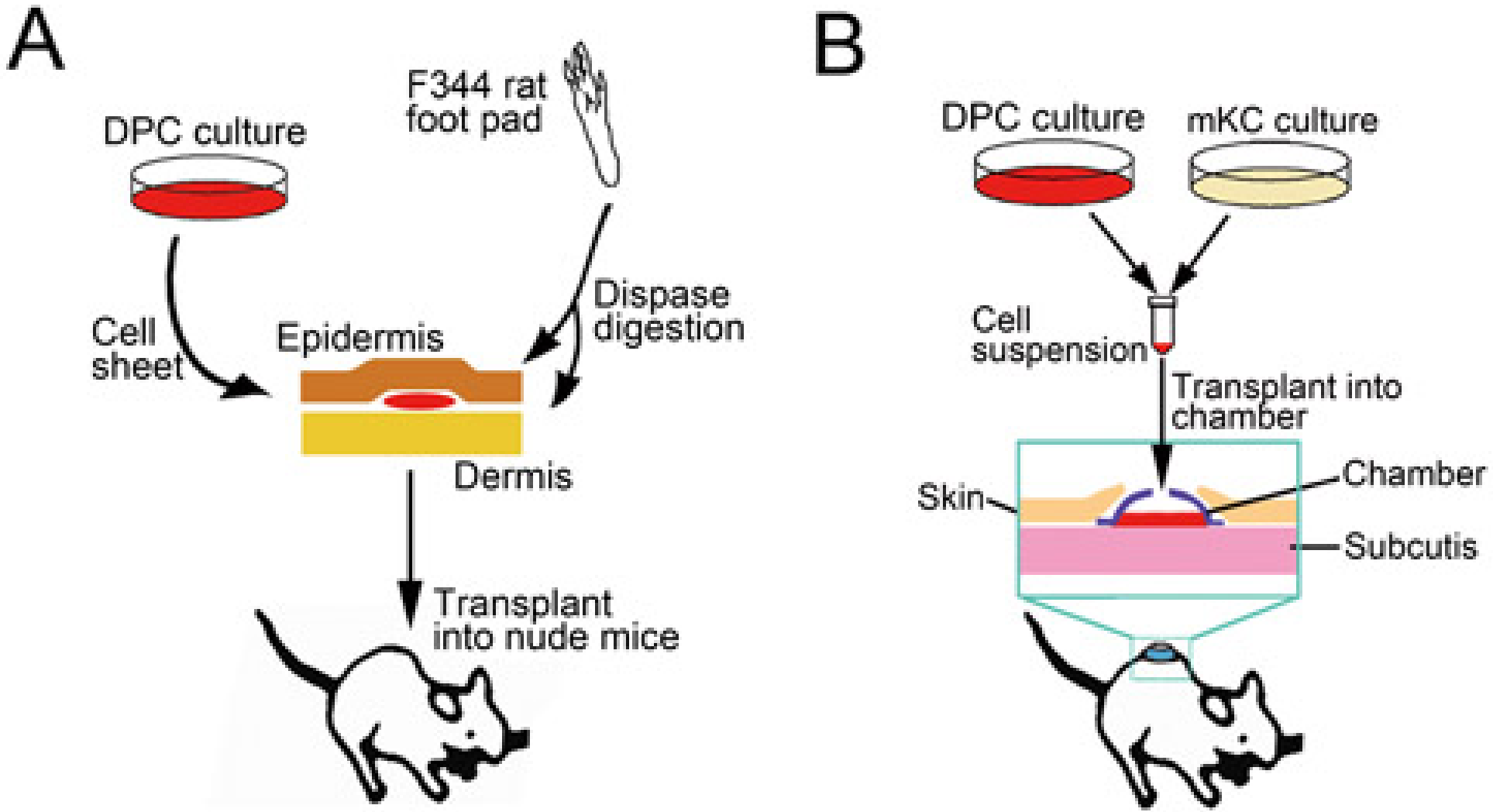
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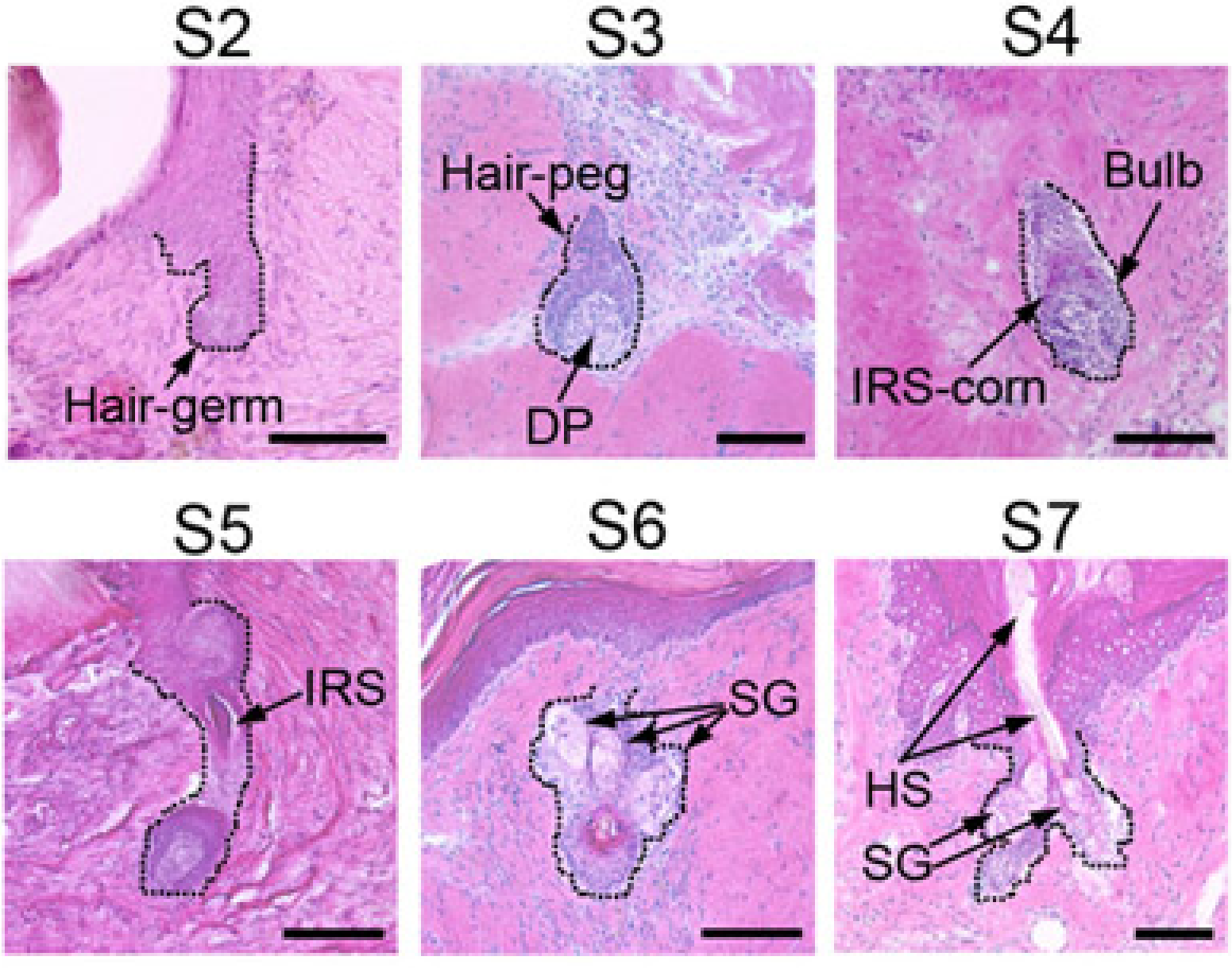
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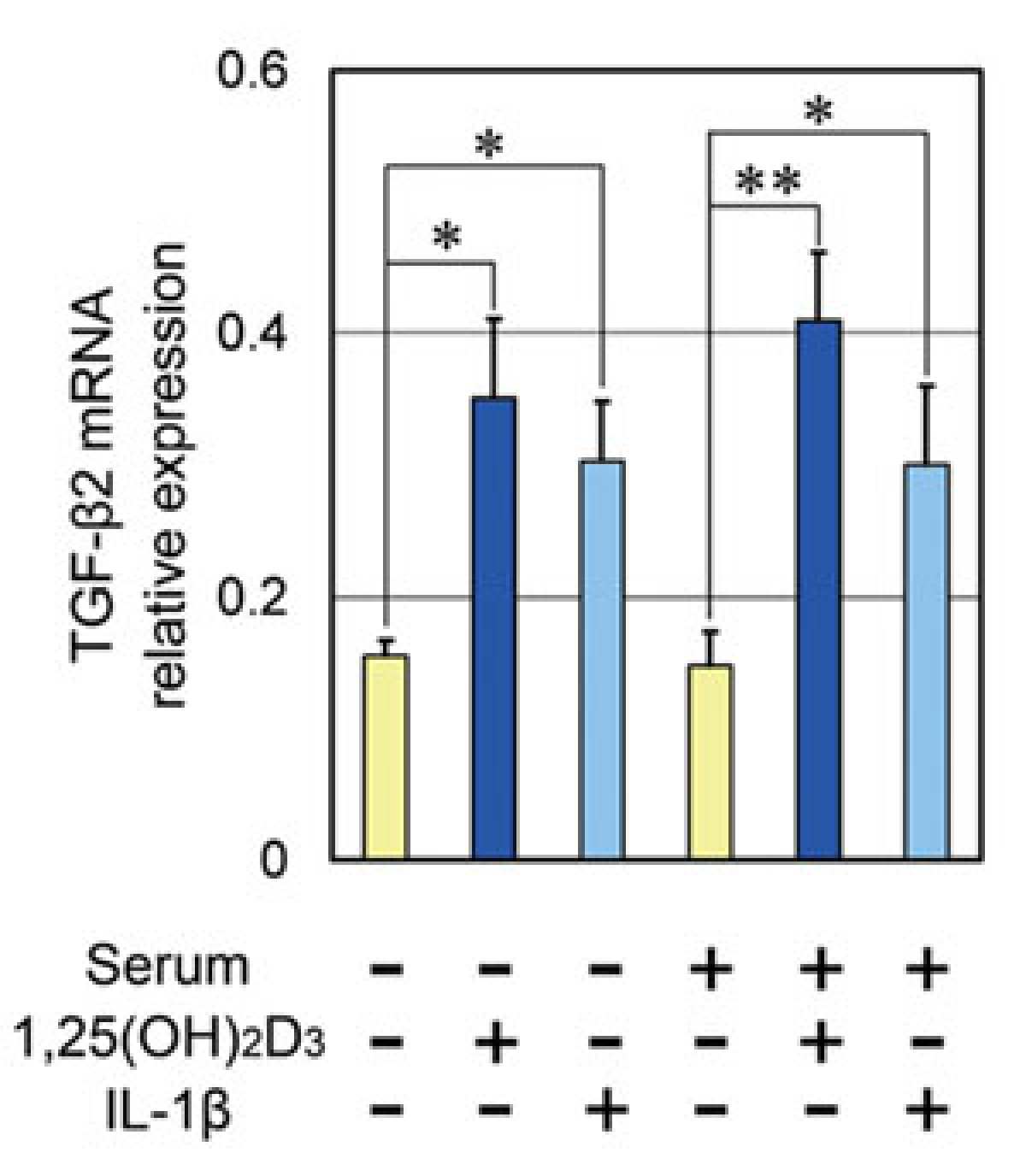
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- - - + + +
- + - - + -
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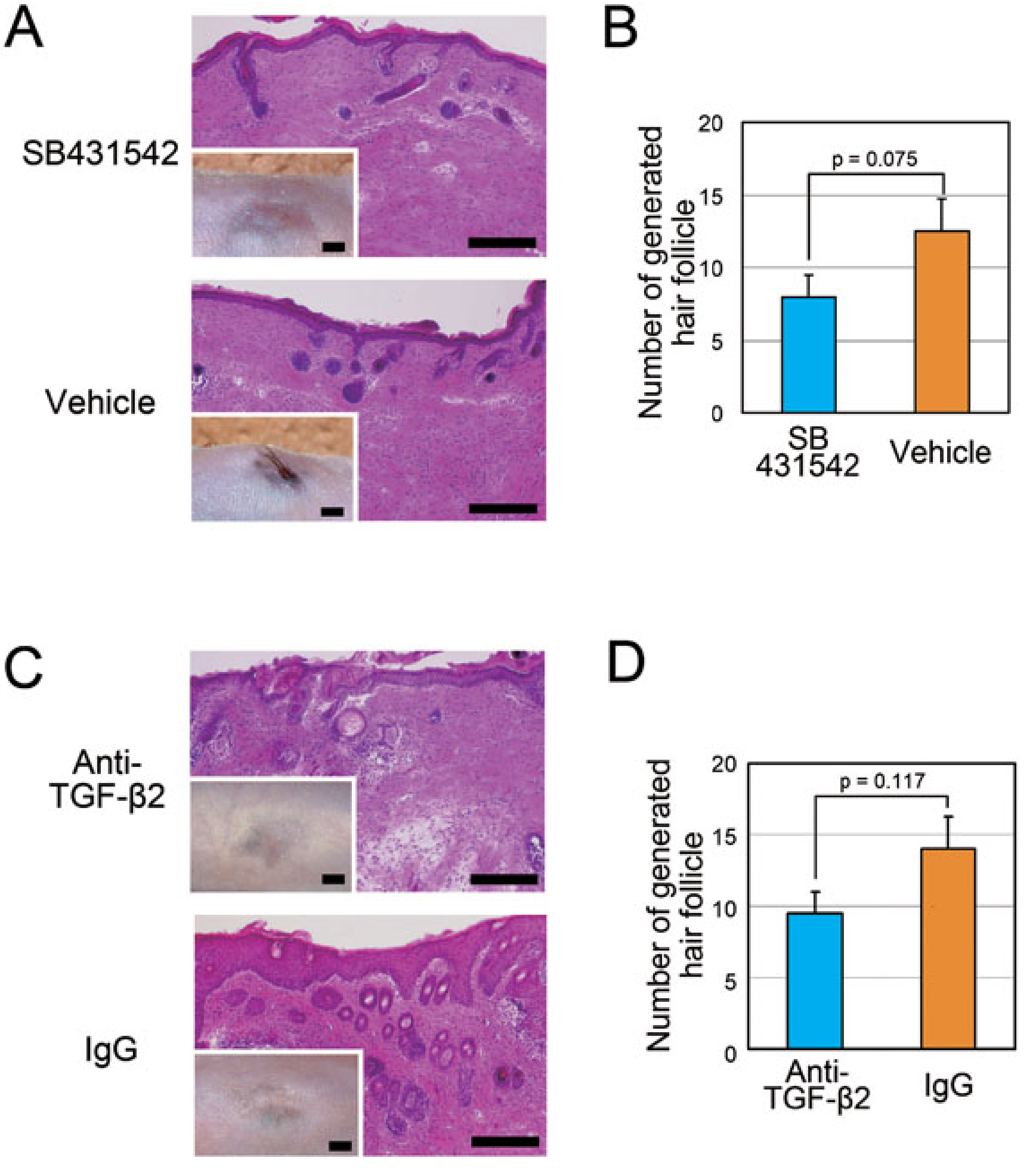
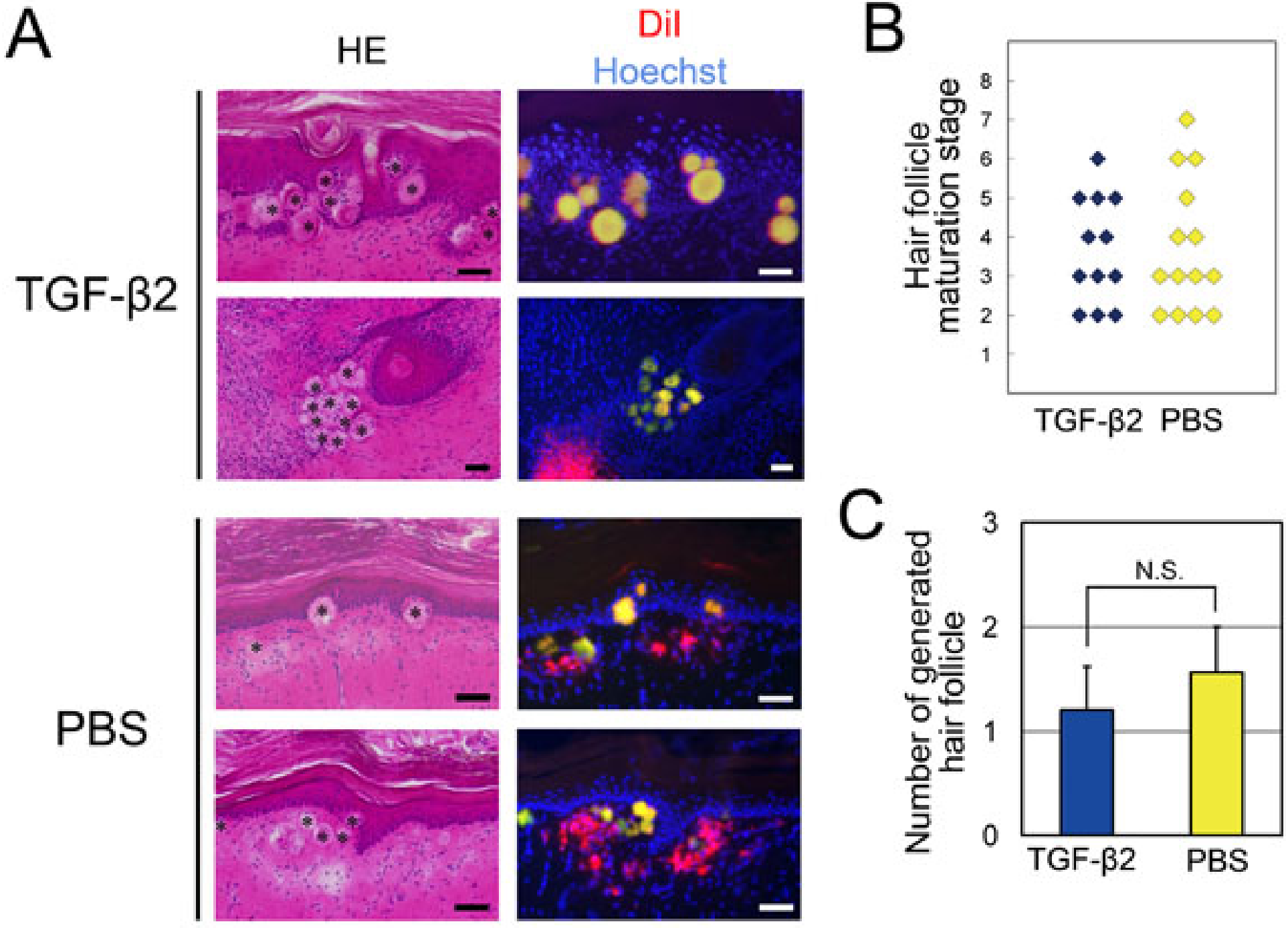

A
SB431542
Vehicle
B
Number of generated hair follicle
20
15
10
5
0
p = 0.075
SB 431542
Vehicle
C
Anti-TGF-β2
IgG
D
Number of generated hair follicle
20
15
10
5
0
p = 0.117
Anti-TGF-β2
IgG


A
HE
DiI
Hoechst
TGF-β2
PBS
B
Hair follicle maturation stage
8
7
6
5
4
3
2
1
TGF-β2
PBS
C
Number of generated hair follicle
3
2
1
0
N.S.
TGF-β2
PBS