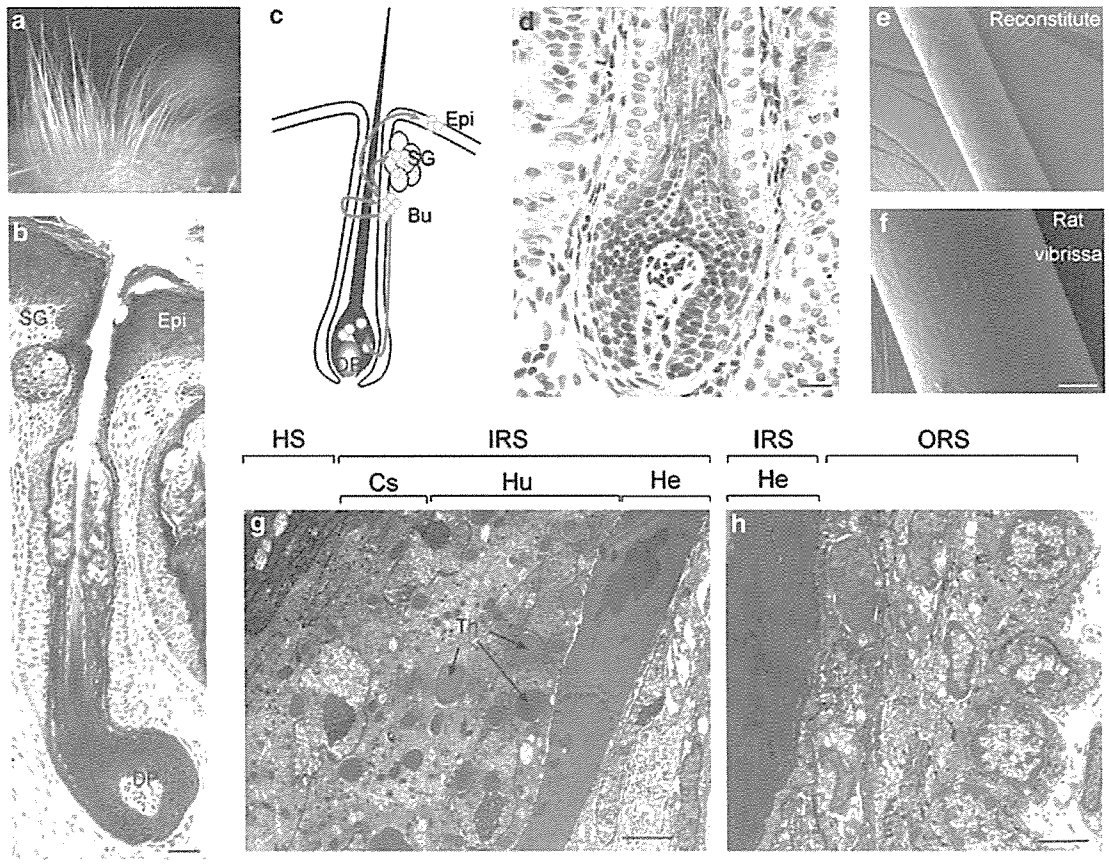

Figure 2 Intact, mature HFs were reconstituted from the cultured, bulge-derived stem cells. (**a**) Hairs that were reconstituted from bulge-derived cells and rat vibrissa dermal papilla cells. Bulge cells were isolated and cultured in Defined-Keratinocyte Serum Free Medium (Invitrogen). Primary dermal papilla cells were microdissected from vibrissae of new born SD rats (Clea) and cultured in Amino MAX medium (Invitrogen). Engraftment was performed as previously described.[22] Equal numbers of dermal papilla cells and epithelial cells were combined at a final density of 2–3 × 10^6 cells and these cells were injected into a silicon chamber attached to the back of an anesthetized SCID mouse (Clea). After 1 week, the wounds had healed, and chamber tops were removed. Hair typically appeared 2–3 weeks thereafter. (**b**) X-gal staining of the reconstituted skin demonstrated that all the epithelial components were produced from the bulge-derived cells from Rosa 26 mice. Equal numbers of rat dermal papilla cells and Rosa 26 mouse bulge cells were injected into a silicon chamber attached to the back of an anesthetized SCID mouse (Clea). After 4 weeks, the reconstructed skin was immediately frozen in OCT compound (Sakura Finetechnical), and cut at a thickness of 6 μm. To detect the transgene product expression, sections were fixed for 1 h in 0.2% glutaraldehyde and stained *en face* with 1 mg/ml X-gal in 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, 0.02% NP-40 and 0.01% sodium deoxycholate at 37°C for 5 h. Sections were counterstained with H&E. The cultured bulge-derived cells differentiated to all epithelial cell types within the cutaneous epithelia. Epi; Epidermis, SG; Sebaceous gland, DP; Dermal papilla cells. (**c**) Schematic view of HF stem cell behavior during the skin reconstitution processes. Bulge-derived cells differentiate into all the epithelial components. Epi; Epidermis, SG; Sebaceous gland, DP; Dermal papilla cells, Bu; bulge (**d**) H&E staining of reconstituted HF showed normal bulb region morphology in the reconstituted HF. (**e** and **f**) Scanning electron microscopy of reconstituted hair shaft; hairs for scanning electron microscopy (EM) analysis were fixed in 5% glutaraldehyde in PBS at room temperature for 30 min, processed using standard techniques, and visualized using a HITACHI S-4500 electron microscope. (**e**) The reconstituted hair shaft was demonstrated intact, mature shaft morphology similar to a normal rat vibrissa (**f**). (**g** and **h**) For transmission EM, fresh biopsies of engrafted skin were fixed in 2% glutaraldehyde solution, postfixed in 1% OsO_4, dehydrated, and embedded in Epon 812. All the samples were ultrathin sectioned at a thickness of 70 nm, and stained with uranyl acetate and lead citrate. Photographs were taken using a Hitachi H-7100 transmission electron microscope. A longitudinal section of the inner layers of a reconstituted HF (**g**) exhibited the Henle's (He) layer flanked by the trichohyalin (Th)-rich Huxley's (Hu) layer, and the thin IRS cuticle (Cs). The hair shaft (HS) was internal to the IRS. In the outer layers (**h**), there were normal ORS cells. Scale bars: 50 μm (**b** and **d**), 15 μm (**f**), 3 μm (**g** and **h**).

Finally, we reconstituted HFs and interfollicular epidermis *in vivo* from cultured bulge-derived cells. At first, we tried to follow the behavior of transplanted cells using a retrovirus harboring EGFP gene as a marker, because GFP was generally used in several reports as a real-time marker in the living mice.[19–21] However, we could not demonstrate the required signals *in vivo*, although we could detect the signals in the differentiated epidermal regions that is stratum corneum and IRS cells from skin tissue sections. We speculate that the reason we could not obtain sufficient EGFP signal *in vivo* or from epidermal sections might be that scattered or diffuse EGFP cannot generate a sufficiently strong detectable signal *in vivo* or tissue sections. Indeed, 1 month after transplantation, the *in vivo* EGFP expression was diffuse and its signal was only weakly detected. Thus, to demonstrate the transgene expression clearly, we decided to use cultured bulge-derived cells harboring the retrovirus vector-*LacZ* marker as follows. Cultured SD rat vibrissa bulge-derived cells were infected with

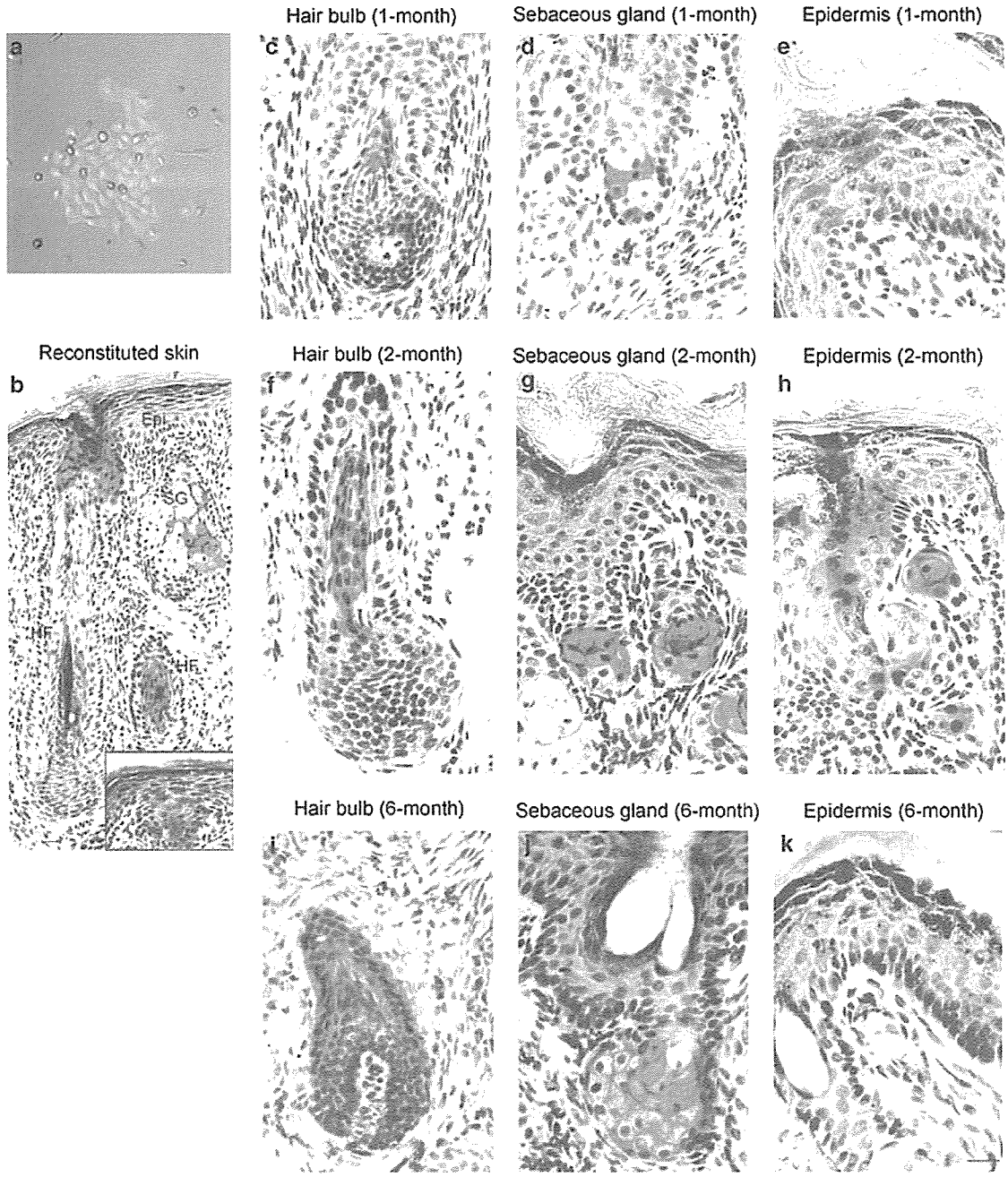

Figure 3 Expression of transgene in reconstituted skin. (**a**) Gene-transfected HF stem cells were used for hair reconstruction. Expression of the transgene was confirmed by the presence of fluorescent enhanced green fluorescent protein (EGFP) in cultured cells. Gene expression was performed using a pDON-AI retrovirus vector system (Takarashuzo). (**b–k**) Histochemical staining demonstrated the transgene expression in HFs reconstituted from bulge-derived cells harboring the transgene. Plasmid pNASSβ was obtained from Clontech. Plasmid pDON-AI was obtained from Takarashuzo. In pDON-AI retroviral vector, the U3 region was replaced by a heterologous human cytomegalovirus immediately early promoter. Plasmid pNASSβ was digested with *Not*I to obtain the 3.5 kb β-galactosidase (β-gal) fragment. Plasmid pDON-AI was digested with *Not* I. The β-gal fragment was then ligated into the linearized pDON-AI. GP2–293 cells (Clontech) were treated with 25 μM chloroquine for 2 h before transfection. The cells were transfected with pVSV-G and pDON plasmid DNA using a calcium phosphate transfection kit (Invitrogen), according to the manufacturer's protocol. After 48 h, the supernatant was collected, filtered (0.45 μm; Millipore), and concentrated by ultracentrifuge. The pellet was resuspended in TNE buffer and incubated at 4°C overnight. The concentrated virus were infected into the cells isolated from the bulge with polybrene (8 μg/ml). Concentrated virus preparations with titers of 2×10^8 β-gal-positive colony-forming units (CFU)/ml, as determined on 293 cells. After 5 days culture, bulge-derived cells harboring transgene and DP cells were mixed and grafted onto SCID mouse. Expression of marker transgene *LacZ* (Blue) was retained in the reconstituted HFs and the epidermis after 1, 2 or 6-month follow-up. (**b**) *LacZ* -positive cells were seen in every part of epithelial component including the interfollicular epidermis (inset), hair follicle including the bulge region and sebaceous glands. Epi; Epidermis, SG; Sebaceous gland, Bu; bulge. (**c–k**) High power views of reconstituted tissues demonstrated that *LacZ* marker expression had been maintained in every epithelial compartment of reconstituted skin during a 6-month follow-up. (**c–e**) At 1-month after transplantation; (**f–h**) 2-month after transplantation; (**i–k**) 6-month after transplantation; (**c**, **f**, **i**) hair bulb; (**d**, **g**, **j**) sebaceous gland; (**e**, **h**, **k**) epidermis. Scale bars; 50 μm.

retrovirus carrying the *LacZ* gene under the CMV promoter. At 4 or 5 days after infection, cells were grafted as previously described. We did not use FACS to enrich the cells expressing transgene because we had not used the EGFP system as a transgene expression marker. Hair shaft budding was detected at 4 weeks after grafting and these mice were killed. Transgene expression in the HF epithelium and the epidermis was analyzed by X-gal histochemical staining in tissue sections from the reconstituted skin. β-Gal-positive keratinocytes were found in several parts of the *de novo* reconstituted skin including the HF epithelium, the sebaceous glands and the interfollicular epidermis (Figure 3b). In HFs, β-gal-positive cells could be observed in any area of the HF, that is ORS, IRS and matrix (Figure 3c). β-Gal expression was also seen in the basal cells and differentiated cells of the sebaceous gland (Figure 3d). The clonal pattern of β-gal-positive epidermal keratinocytes was indicative of epidermal proliferation units (EPU) (Figure 3e). These results showed that any epithelial skin element including HFs, sebaceous glands and the interfollicular epidermis could be reconstituted from bulge-derived stem or progenitor cells carrying the *LacZ* transgene. We then followed the fate of these gene-transduced cells. We observed the reconstituted HFs, sebaceous glands and interfollicular epidermis 2 months after transplantation. β-Gal-positive cells were seen in the reconstituted tissues and the areas covered by the β-gal-positive cells appeared to expand compared with the skin 1 month after transplantation (Figure 3f–h). Furthermore, we were able to detect the transgene expression in the reconstituted tissues as late as 6 months after transplantation (Figure 3i–k).

To summarize the data in the present study, we have obtained a stem cell pool from the HF bulge of not only neonatal but also adult mice and rats and performed gene transfer into the cultured bulge-derived cells. We have succeeded in reconstituting HFs from these cultured bulge-derived cells harboring the transgene. In addition, the transgene was expressed in all reconstituted epithelial skin compartments including the HF epithelium, sebaceous gland epithelium and the epidermis for at least 6 months after transplantation.

In the present HF reconstitution study, we did not use EGFP as a transgene expression marker. Thus, we were unable to use FACS to enrich the cells expressing transgene. For practical application of this system to gene therapy, using FACS to maximize the percentage of targeted, transgene expressing cells would be important, especially if targeted cells are at a growth disadvantage relative to wild type cells.

HF units have the potential to become a source of proteins for possible use in protein therapy. In addition, HF stem cells are able to provide cells that are able to form the vast majority of the epidermis. Thus, HF-targeted gene therapy might be applicable not only to skin diseases, but also to systemic diseases, for example, hormone deficiency.

Therefore, a 'hair follicle stem cell-targeted gene transfer and reconstitution system' may enable the stable expression of transgene in any part of the cutaneous epithelium and provide longstanding therapeutic benefits if used for gene therapy. In addition, this system has the potential to produce a reliable means for gene-function analysis and gene therapy. We have now applied this system to a clinical setting and the results are currently under analyses in our laboratory.

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Two cases of folliculosebaceous cystic hamartoma

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Summary

Folliculosebaceous cystic hamartoma (FSCH) is a rare cutaneous hamartoma composed of dilated folliculosebaceous units associated with mesenchymal elements. Two cases of FSCH with typical histopathological features are reported. Patient 1 was a 60-year-old man presented with a normal skin-coloured asymptomatic nodule on his scalp. Patient 2 was a 70-year-old man with an asymptomatic nodule on his right auricle that had persisted for the previous 15 years. In all, 34 cases of FSCH have been reported in the English literature. Clinically, the lesions are asymptomatic, usually rubbery to firm in consistency, and usually occur on or above the neck (> 90%). Most lesions do not exceed 25 mm in diameter (> 90%). Histopathologically, FSCH shares several similar features to sebaceous trichofolliculoma, but it is usually possible to differentiate these two tumours.

Introduction

Folliculosebaceous cystic hamartoma (FSCH) is widely considered to be a hamartoma composed of multiple tissue elements, including both ectodermal and mesodermal components. Epithelial components comprise adnexal and folliculosebaceous cystic proliferations, while mesenchymal components exhibit variable fibroplasias with vascular and adipose tissue. Here, we report two cases of FSCH with typical histopathological features.

Case reports

Patient 1

A 60-year-old man presented with a tumour on his scalp, which had become apparent some 10 years previously. Physical examination showed a normal skin-coloured, elastic soft asymptomatic nodule, 25 mm in diameter, with a smooth surface covering of skin (Fig. 1a). Histopathological examination (Fig. 1b, c) revealed a markedly dilated follicular cystic structure lined by stratified squamous epithelium. Abundant, mature, sebaceous lobules radiated outward from the cystic structure. The cysts showed a predominant infundibular keratinization pattern, and were surrounded by a dense fibrovascular stroma with mucin deposition. These features were characteristic of FSCH.

Patient 2

A 70-year-old man visited our clinic for an asymptomatic nodule on the right auricle that had persisted for 15 years. The lesion was a normal skin-coloured pedunculated soft nodule, 10 mm in diameter (Fig. 2a). We made an initial clinical diagnosis of the lesion as a soft fibroma and excised it completely. Histopathologically, a dilated follicular cystic structure was observed (Fig. 2b,c), with numerous sebaceous lobules arising from its wall in the dermis and subcutis. There were excess fibrous components around these structures, with adipocyte hyperplasia, small venules and sweat

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Conflict of interest: none declared.

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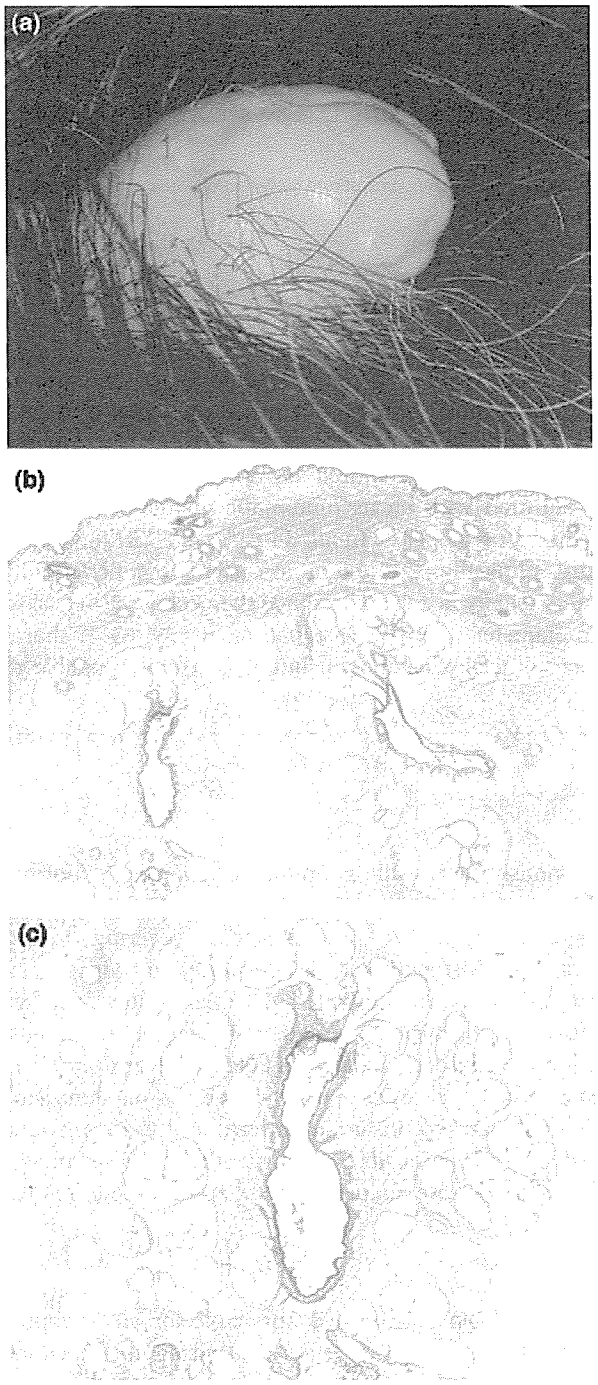

Figure 1 (a) A normal skin-coloured, 25-mm asymptomatic nodule with a smooth skin surface; (b) low-power view showing a dilated folliculosebaceous unit; (c) multiple sebaceous lobules radiate outward from the cystic structure, predominantly showing infundibular keratinization. Haematoxylin and eosin; original magnification (b) × 6; (c) × 40.

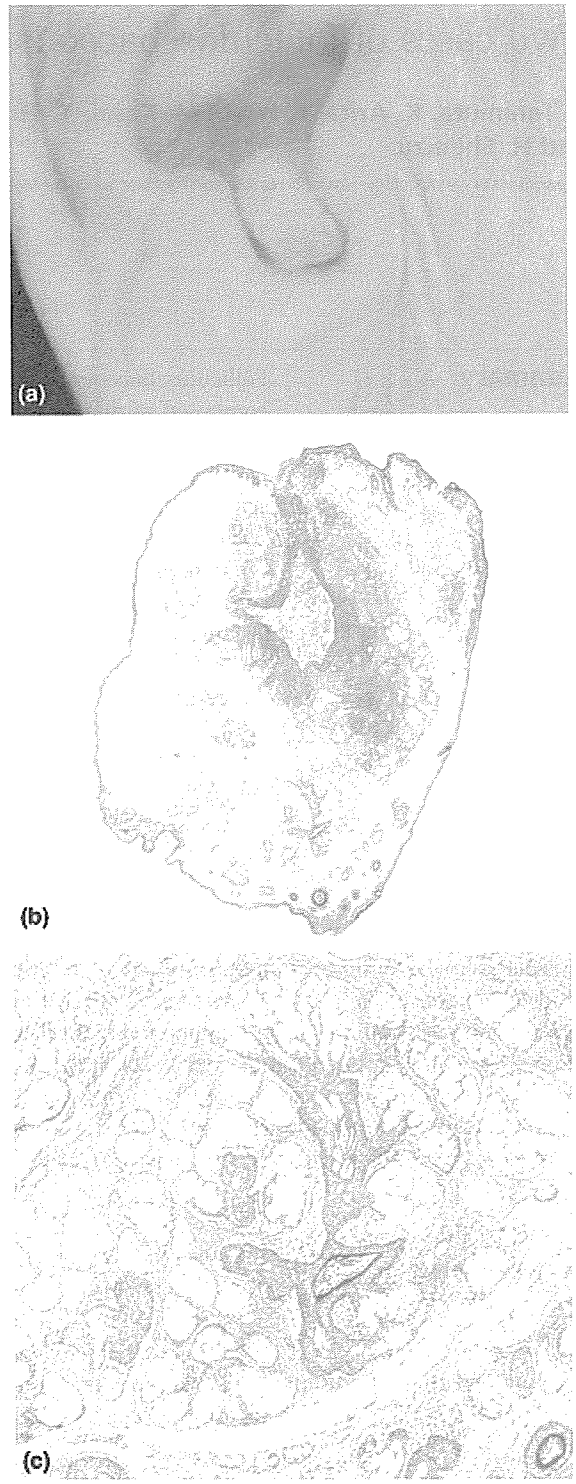

Figure 2 (a) Normal skin-coloured, 10-mm pedunculated nodule on the right auricle; (b) excess fibrous components (around the cystic structure and sebaceous lobules; (c) clefts can be seen between the pericystic fibrous tissue and the rest of the dermis. Haematoxylin and eosin, original magnification (b) ×4; (c) ×40).

glands. Clefts were observed between the pericystic fibrous tissue and the surrounding dermis. These features were characteristic of FSCH.

Discussion

FSCH was originally described by Kimura *et al.* in 1991,[1] and since then, 34 patients, including our two, have been reported in the English literature. The size of the lesions in all reported cases on the head and neck did not exceed 25 mm in diameter. In three extremely large growths (giant variants), the lesions were located on the upper back, labia majora and upper arm.[2–4]

Physical examination shows FSCH lesions to be asymptomatic, usually rubbery to firm in consistency. They tend to grow slowly with no change in colour or texture over time.[5] FSCH lacks distinctive clinical features, and the initial diagnoses in all reported cases included disorders other than FSCH, such as intradermal naevus, sebaceous hyperplasia, basal cell carcinoma, lipoma, and neurofibroma.[5] Our two cases were misdiagnosed as neurofibroma and soft fibroma, respectively.

Histopathological features of FSCH include: (i) an infundibular cystic structure adjacent to the sebaceous glands; (ii) laminated fibroplasia around the epithelial component; (iii) mesenchymal changes around these fibroepithelial units (collagen bundles, adipocytes and small venules); (iv) clefts between the fibroepithelial units and the surrounding altered stroma, and the adjacent normal skin structures; (v) confinement of these processes primarily to the dermis.[1]

FSCH is sometimes difficult to differentiate from trichofolliculoma, especially those variants with marked sebaceous components, termed sebaceous trichofolliculoma (STF) by Plewig.[6] Although both exhibit a widely dilated cavity or sinus lined by stratified squamous epithelium with numerous sebaceous lobules, there are many differential points. Clinically, STF is a centrally depressed lesion that exhibits protruding hairs of nasal apex and tends to occur at a young age, often in neonates. Histopathologically, STF contains numerous hair follicles, with the lower portion composed of terminal hairs, vellus hairs and trichoids arising from the cyst wall. In addition, no mesenchymal changes are found in STF. Recently, Schulz and Hartschuh have proposed that FSCH could actually be a late stage in trichofolliculoma formation.[7] However, their conclusion fails to explain congenital FSCH cases sufficiently;[2,5,8] the idea of FSCH being a late stage of STF with mesenchymal changes is difficult to reconcile with its documented appearance in neonates.

Interestingly, several cases of FSCH with abnormal neural components have been reported.[5,9] These variations indicate that the FSCH is intrinsically a skin hamartoma including mesenchymal differentiation, which is not seen in STF.

FSCH usually occurs on the head or neck as a single, nodular lesion usually < 25 mm in diameter. Clinical diagnosis of FSCH is remarkably difficult because it can have a varying clinical appearance, in many cases similar to intradermal naevi. Histopathologically, FSCH shares several similar features with STF, but it is usually possible to differentiate these two tumours.

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PDGF isoforms induce and maintain anagen phase of murine hair follicles

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KEYWORDS
Hair cycle;
Hair growth;
PDGF-AA;
PDGF-BB

Summary

Background: It is known that platelet-derived growth factor (PDGF) receptors are expressed in hair follicle (HF) epithelium.
Objectives: The aim of the present study was to clarify the effects of PDGF-AA and -BB on the cyclic growth of HFs.
Methods: PDGF-AA or -BB was injected into the dorsal skin of C3H mice during the second telogen phase once daily for five consecutive days, or PDGF-AA or -BB dissolved in hyaluronic acid was injected only once. In order to confirm the effects of different PDGF isoforms, anti-PDGF-AA antibody or anti-PDGF-BB antibody was injected just after each injection of PDGF-AA or -BB. In addition, anti-PDGF antibodies were injected into the skin of C3H mice during the second anagen phase once daily for 5 days. We studied expression of signaling molecules in the skin where anagen phase had been induced by PDGF injection by real-time RT-PCR.
Results: Both PDGF-AA and -BB injection experiments immediately induced the anagen phase of the hair growth cycle at the injection sites.

The induction of anagen was interfered by anti-PDGF antibody treatment. Real-time RT-PCR using extracted RNA from the PDGF injected sites of skin samples showed upregulated expression of HF differentiation-related key signaling molecules, Sonic hedgehog (Shh), Lef-1 and Wnt5a.
Conclusions: These results indicate that both PDGF-AA and -BB are involved in the induction and maintenance of the anagen phase in the mouse hair cycle. Local application of PDGF-AA and -BB might therefore prove to be an effective treatment option for alopecia associated with early *catagen induction* and elongated telogen phase.

Abbreviations: HF, hair follicle; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; Shh, sonic hedgehog

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1. Introduction

Platelet-derived growth factor (PDGF) is a potent mitogen produced in a variety of cell types including keratinocytes and endothelial cells, and is important for cell growth, proliferation and differentiation [1]. PDGF-AA and platelet-derived growth factor receptor (PDGFR)-alpha are expressed in human hair follicles during fetal development [2], and a role for PDGF-AA in hair follicle development has been suggested by the fact that hair formation was perturbed by injection of PDGFR-alpha antibodies into newborn mice [3]. There are several different isoforms of PDGF (PDGF-AA, -AB, -BB and -CC) that exert their biological activities by binding to two distinct cell surface receptors, which were shown to be ligand-induced tyrosine kinase proteins possessing intrinsic tyrosine kinase activity during differentiation [1].

The PDGF receptor-alpha (PDGFR-alpha) has been shown to bind to all three PDGF chains (PDGF-A, -B and -C), whereas the PDGF receptor-beta (PDGFR-beta) is specific for the PDGF-B polypeptide. Both types of PDGF receptors are expressed in human follicular keratinocytes, although the mesenchymal dermal papilla cells only express PDGFR-alpha. It is known that the expression levels of PDGF isoforms in vitro can be influenced by treatment with cytokines known to be positive and negative regulators of hair follicle growth activity, including IL-1beta, IL-4, interferon-gamma [4].

PDGF-A null mice were reported to develop a skin and hair phenotype characterized by progressive loss of dermal mesenchymal cells, thereby causing an impaired formation of dermal papilla components giving rise to the hair follicle [5]. They suggested that the cutaneous phenotype of PDGF-A null mice might also be the result of progressive depletion of multipotent dermal mesenchymal progenitor cells, due to their reduced proliferation caused by an absence of PDGF-A.

From these facts, we hypothesized that PDGFs might be a positive regulator of hair growth in vivo. In this study, we analyzed the effect of cutaneous injections of recombinant human PDGF-AA and -BB on hair follicle growth in mice. As a result, local injections of PDGF-AA and -BB have anagen inducible effects on murine hair follicles. We confirmed that the PDGF-AA hair growth effect was blocked by simultaneous injection of anti-PDGF-AA antibody. In addition, we showed hair growth restraint effects after injection with anti-human PDGF-AA antibody and anti-human PDGF-BB antibody into mouse skin during the second anagen phase of HFs. We have clearly demonstrated that local injection of PDGF isoforms can induce and maintain the anagen phase of murine hair follicles, and these effects might be associated with the upregulation of Sonic hedgehog (Shh), Wnt5a and Lef-1. These results suggest the possibility that a local application of PDGF might be a worthwhile treatment for alopecia.

2. Materials and methods

2.1. Reagents

We used recombinant human PDGF isoforms and antibodies as follows: recombinant human PDGF-AA (CHEMICON International, CA, Temecula, USA), recombinant human PDGF-BB (CHEMICON International, Temecula, USA), rabbit polyclonal anti-human PDGF-AA antibody (CHEMICON International, Temecula, USA), rabbit polyclonal anti-human PDGF-BB antibody (CHEMICON International, Temecula, USA), rabbit polyclonal anti-PDGFR-alpha (C-20) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-PDGFR-beta (958) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), monoclonal anti-human/mouse Shh N-terminal peptide antibody (R&D systems, Minneapolis, MN, USA) and anti-Stat3 antibody (Epitope specific rabbit antibody (LAB vision, Fremont, CA, USA).

2.2. Animals

Mice used in this study were male mice C3H/HeN (47-day-old, second telogen phase; body weight, 19—20 g) (CLER, Japan) for anagen induction experiments and 28-day-old male C3H/HeN mice (second anagen phase; body weight, 16—17 g) for telogen induction experiments.

2.3. Anagen induction by PDGF local injection

All the animal experiments were performed under the approval of the ethical committee on animal study in Hokkaido University. Recombinant human PDGF-AA and -BB were dissolved in sterile and toxin-free phosphate-buffered saline containing 0.1% bovine serum albumin (0.1% BSA—PBS). PDGF-AA or -BB (1 μg) dissolved in 100 μl of 0.1% BSA—PBS and 0.1% BSA—PBS for controls were intradermally injected into the dorsal skin of 47-day-old male C3H mice (second telogen) once daily for 5 consecutive days (total 5 μg of PDGF isoforms) (PDGF-AA, $n = 5$; PDGF-BB, $n = 5$; control, $n = 5$). All mice were sacrificed 10 days after the first injection, and skin samples were prepared for further studies. In order to confirm the effects of different PDGF isoforms,

anti-PDGF-AA antibody or -BB antibody was injected just after each injection of PDGF-AA or -BB (anti-PDGF-AA antibody following PDGF-AA, $n = 5$; anti-PDGF-BB antibody following PDGF-BB, $n = 5$).

We also dissolved PDGF-AA or -BB in 1% hyaluronic acid for single injection. 5 μg of PDGF-AA or -BB in 1% hyaluronic acid was injected into the dorsal skin of 47-day-old male C3H mice (second telogen) only once. Only 1% hyaluronic acid was injected as a negative control (PDGF-AA in hyaluronic acid, $n = 5$; PDGF-BB in hyaluronic acid, $n = 5$; control, $n = 5$).

2.4. *Catagen induction* by injections of anti-PDGF antibodies

Anti-human PDGF-AA polyclonal antibody (1 μg) or anti-human PDGF-BB polyclonal antibody dissolved in 100 μl of 0.1% BSA—PBS were injected into the dorsal skin of five C3H/HeN male mice (28-day-old, second anagen phase; body weight, 16—17 g) once daily for 5 consecutive days (total 5 μg of PDGF isoforms), and then sacrificed 10 days after the first injection. Control mice at the same age were injected with 100 μl of 0.1% BSA—PBS once daily for 5 consecutive days (anti-PDGF-AA antibody, $n = 5$; anti-PDGF-BB antibody, $n = 5$; control, $n = 5$).

2.5. Evaluation of hair growth activity

Hair growth activity of the mouse skin samples was evaluated in H&E sections. The skin sample resected from the injection site was fixed in 10% buffered formalin solution and paraffin embedded. A 5 μm thick section was stained with hematoxylin and eosin. Skin thickness was defined as the distance from the epidermal granular layer to the top of the panniculus carnosus and was used as a parameter indicating hair follicle length [6]. Measurements were carried out in three fields per mouse, and their average value was expressed in micrometers.

The data from the samples were compared with those of the 0.1% BSA—PBS injected control samples, and analyzed by Student's paired *T*-test. Data with p values less than 0.05 were taken as significant.

It is known that alkaline phosphatase staining appears in anagen phase outer root sheath [7]. For the alkaline phosphatase staining, fresh mouse skin specimens embedded in OCT compound (Tissue-Tek, Sakura, Japan) were quick-frozen using an isopentane cooled bath with dry ice, and sections were cut in the cryostat. The skin samples were stained with alkaline phosphatase and the hair cycle phase was identified from the staining patterns.

2.6. Expression of signaling molecules

We studied expression of signaling molecules, Shh, Wnt5a, Lef-1 and Stat3, by immunohistchemical staining in the skin where anagen phase had been induced by PDGF injections, and by using real-time RT-PCR.

For the real-time RT-PCR analysis, pieces of skin tissue (about 60 mg/mouse) were treated with 1.5 ml Trizol (Invitrogen, Karlsruhe, Germany) and were crushed and mixed using a mortar. The RNA was then extracted using a standard protocol. After RNA preparation, the total RNA was treated with DNase I (Invitrogen, Karlsruhe, Germany) 1 U/1 μg-RNA for 15 min at room temperature followed by inactivation with EDTA (Sigma, Munich, Germany). After the DNase treatment, genomic DNA was not detected in the sample by PCR. First-strain cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco, Karlsruhe, Germany).

Real-time PCR was used to obtain quantitative data on differences between Shh, Lef-1, Wnt5a and Stat3 mRNA of the control group and mice injected PDGF-AA or -BB. Real-time RT-PCR assays using TaqMan probes (Taqman(R) Gene Expression Assays: Lef1, Assay ID Mm00550265m1; Shh, Assay ID Mm00436527m1; Stat3, Assay ID Mm00456961m1; Wnt5, Assay ID Mm00437347m1; GAPDH, Assay ID Mm99999915g1) (Roche Molecular Systems Inc., Pleasanton, CA, USA) were carried out in a micro reaction tube. The reaction mixture for each one tube TaqMan reaction mix consisted of 5 μl of Universal 5× buffer, 1 μl of 10 mM dNTP, 1 μl of enzyme mix (Qiagen, Germany), 1 μl of 20 pM forward primer, 1 μl of 20 pM reverse primer, 1 μl of 25 pM fluorogenic FAM labeled JEV probe, and 15 of RNA sample to a total volume of 25 μl. Thermo-cycling conditions were as follows: 30 min at 50 °C for reverse transcription; 5 min at 95 °C to activate DNA polymerase and to deactivate reverse transcriptase; 45 or 50 cycles of 10 s at 95 °C to denature, 20 s at 55 °C to anneal and 20 s at 72 °C for extension, and 5 min at 72 °C for final extension. The reverse transcription and PCR amplification were performed by the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, USA). Each analysis was normalized to cellular mGAPDH levels, by calculating the difference between the Ct for mGAPDH and the Ct for Shh, Lef-1, Wnt5a, Stat3. ΔCt represents the mean Ct value of each sample and was calculated for Shh, Lef-1, Wnt5a, Stat3. The gene copy numbers of the samples were determined by the following formula: ΔΔCt = ΔCt target sample −ΔCt calibrator sample (negative control sample).

The relative gene copy numbers were calculated by the expression $2^{-\Delta\Delta Ct}$. The data from the

target samples was compared with those of the 0.1% BSA—PBS injected control sample, and analyzed by Student's paired *T*-test. Data with *p* values less than 0.05 were evaluated as significant.

For the immunohistochemical observations, frozen sections from fresh skin specimens were used. All 6-μm sections were stained by the streptoavidin—biotin—peroxidase complex method according to the manufacturer's manual (Vector Laboratories Inc., Burlingame, CA, USA). The dilution for anti-PDGFR-alpha antibody was 1/100 and the dilutions for anti-PDGFR-beta antibody, anti-Stat3 antibody, anti-Shh antibody were 1/100, 1/50 and 1/20, respectively. Sections were incubated in normal goat serum, and endogenous peroxidase activity was blocked in 0.01% hydrogen peroxidase solution, then incubated in monoclonal antibody (polyclonal antibody) for 2 h. Color was developed by incubation in freshly prepared substrate solution containing 50 mM Tris—HCl, pH 7.6, 3,3′-diaminobenzidine—HCl (0.05 mg/ml), and 0.01% hydrogen peroxide at room temperature for 5 min. Counterstaining with hematoxylin was performed when it was necessary to identify the morphology.

3. Results

3.1. Either PDGF-AA or -BB local injections induced anagen phase in murine hair follicles

The area in close proximity to the injection sites in three out of five mice became darkened in color, indicating that HFs were in the anagen hair cycle phase (Fig. 1a and d), whereas the injection sites using just the vehicle solution alone (0.1% BSA—PBS) all five mice retained their normal white color, suggesting that they remained in telogen phase (Fig. 1g). Histologically, a large number of HFs in the anagen phase were observed in both the PDGF-AA or -BB injected sites. Quantitatively, PDGF-AA or -BB injected mice showed significant increases in skin thickness at the injected sites (Fig. 1b and e). The Student's *T*-test demonstrated highly significant differences in skin thickness between controls and PDGF-injected groups ($p < 0.05$) (Fig. 4). Using alkaline phosphatase staining, we confirmed that HFs of PDGF injected mice were in anagen phase (Fig. 1c and f). There was no significant increase in skin thickness in the vehicle solution alone injection sites (Fig. 1h).

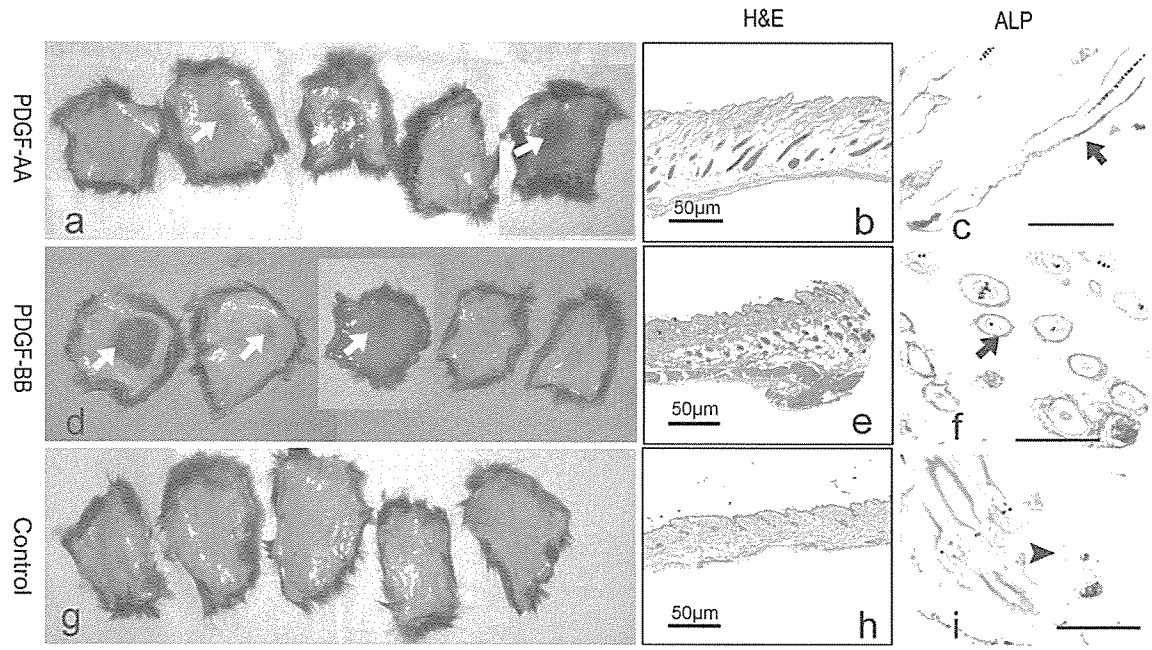

Fig. 1 Local injections of either PDGF-AA or -BB induced and maintained hair follicle anagen phase. PDGF-AA, PDGF-BB or vehicle solution (0.1% BSA—PBS) was injected intradermally into 47-day-old male C3H mice (second telogen) for 5 consecutive days (total 5 μg of PDGF-AA or -BB), and the mice were then sacrificed on day 10. The photographs showed dark grey areas indicating anagen induction on the reverse side of the resected murine skin (a, PDGF-AA; d, PDGF-BB). No darken areas were seen in the negative controls (g, 0.1% BSA—PBS injected skin). Both PDGF-AA (b) and PDGF-BB (e) injected mice showed histologically larger hair follicles in the dermis and thicker skin (h, negative control, 0.1% BSA—PBS injected skin). Both PDGF-AA (c) and PDGF-BB (f) injected mice had anagen phase hair follicles with alkaline phosphatase-positive outer root sheath (arrows). Outer root sheaths of hair follicles in the negative control (i) specimens were negative for alkaline phosphatase staining (arrowhead). Scale bars, 50 μm.

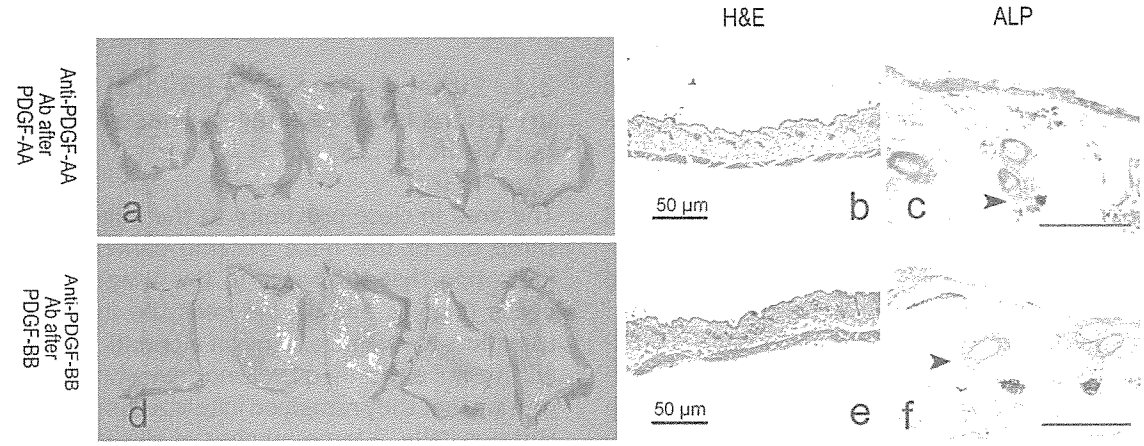

Fig. 2 Anti-PDGF antibody injections following PDGF injections interfered with the anagen induction effect of PDGF isoforms. (a) In the PDGF-AA injected mouse skin treated with anti-PDGF-AA antibody, no darkened areas indicating anagen induction were observed on the reverse side of the resected murine skin. (b and c) Histologic sections stained with hematoxylin and eosin (b) or alkaline phosphatase (c) showed hair follicles still in the telogen phase. (d) In mouse skin injected with PDGF-BB and anti-PDGF-BB antibodies, blackened areas indicating anagen induction were not seen in the reverse side of the skin. (e and f) Hair follicles at telogen phase were observed in the histologic sections stained with hematoxylin and eosin (e) or alkaline phosphatase (f). Arrowheads: alkaline phosphatase-negative outer root sheath. Sale bars: 50 μm.

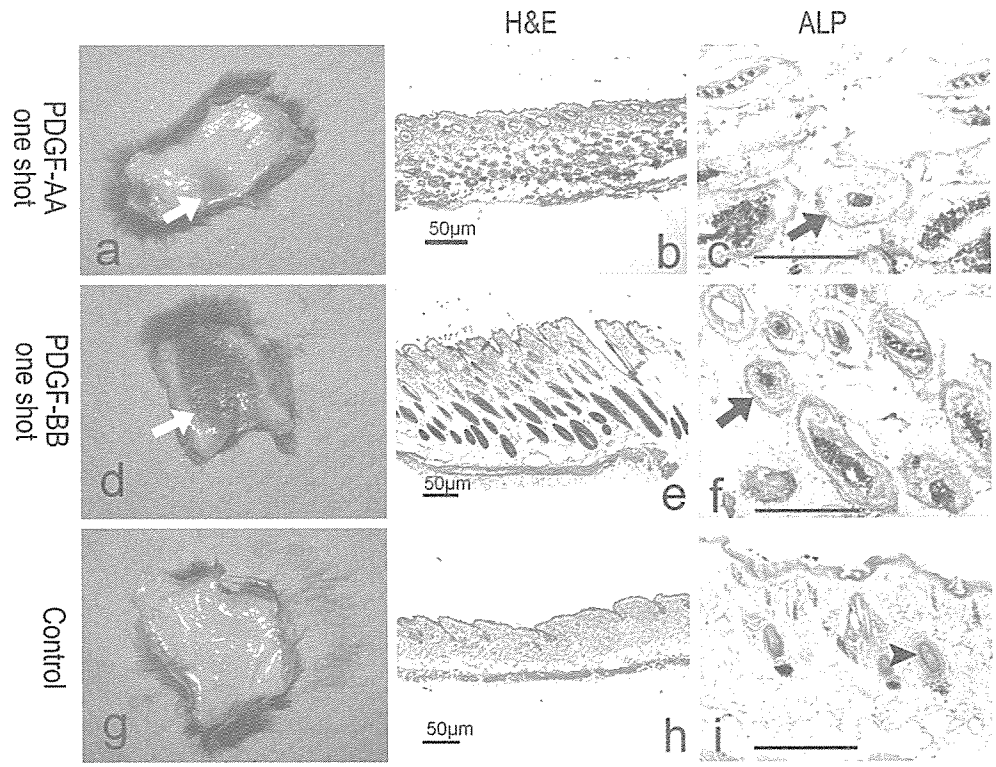

Fig. 3 Induction of anagen phase was obtained even with single injection of PDGF isoforms in hyaluronic acid. In the mouse skin in which one-shot PDGF-AA/hyaluronic acid (a) or PDGF-BB/hyaluronic acid (d) was injected, dark grey areas indicating anagen induction (arrows) were observed in the reverse side of the skin. No darken areas were seen in the negative controls (g, 1% hyaluronic acid only injected skin). Histologic sections stained with hematoxylin and eosin (b and e) or alkaline phosphatase (c and f) confirmed the anagen induction of hair follicles in the PDGF-AA (b and c) or PDGF-BB (e and f) injected sites. Alkaline phosphatase-positive outer root sheath (arrows) suggested hair follicles were at anagen phase (c and f). Outer root sheaths of hair follicles in the negative control (i) specimens were negative for alkaline phosphatase staining (arrowhead). Scale bars, 50 μm.

Using alkaline phosphatase staining, we confirmed that mice were in telogen phase (Fig. 1i).

3.2. Anti-PDGF antibody injections interfered with the anagen induction effect of PDGFs

In the present experiment, anti-PDGF-AA antibody was injected several minutes after the PDGF-AA injection and similarly anti-PDGF-BB antibody was injected after the PDGF-BB injection. Areas in close proximity to the injection site of all mice remained white in color, indicating that the HFs were in the telogen phase (Fig. 2a and d). There was no significant increase in skin thickness in the injection sites (Figs. 2b and e and 4). By alkaline phosphatase staining, we confirmed that HFs in each mouse remained in telogen (Fig. 2c and f). These findings clearly indicated that effects of injected PDGF-AA or -BB were inhibited by the respective injections of anti-PDGF-AA antibody or -BB antibodies.

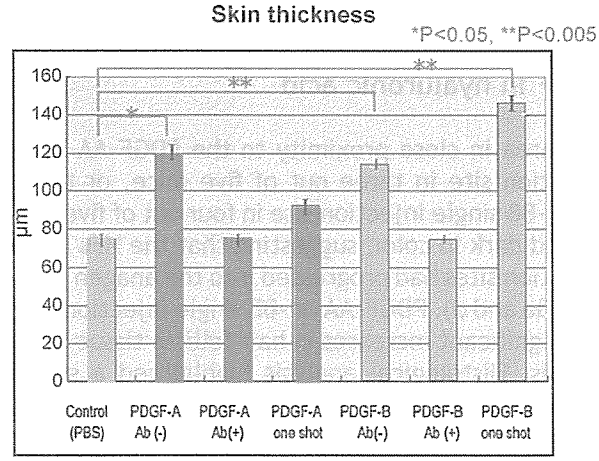

Fig. 4 Local injections of either PDGF-AA or -BB resulted in increased skin thickness suggesting anagen induction in 47-day-old male C3H mice at the second telogen. The *T*-test provided highly significant differences in skin thickness between control groups and PDGF-injected groups ($^{*}p < 0.05$, $^{**}p < 0.005$).

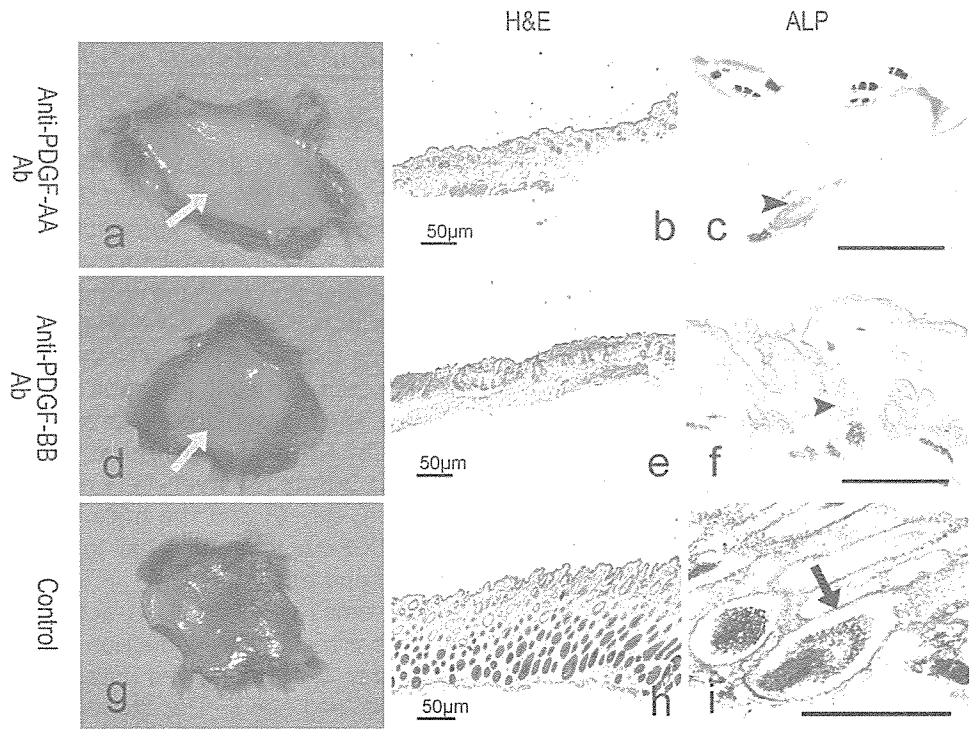

Fig. 5 Local injections of either anti-PDGF-AA or -BB antibodies induced catagen phase. Anti-PDGF-AA, anti-PDGF-BB or vehicle solution (0.1% BSA—PBS) was injected intradermally into 28-day-old male C3H mice (second anagen) for 5 consecutive days (total 5 μg of anti-PDGF-AA or -BB). (a) In mouse skin injected with anti-PDGF-AA antibody, the reverse side of the resected murine skin at the injection site seemed to be brighter in color (arrow). (b and c) Histologic sections stained with hematoxylin and eosin (b) or alkaline phosphatase (c) showed hair follicles at telogen phase. (d) In the mouse skin injected with anti-PDGF-BB antibody, color of the reverse side of the skin was whiter (arrow). (e and f) Hair follicles in telogen phase were observed in the histologic sections stained with hematoxylin and eosin (e) or alkaline phosphatase (f). (g) Control 0.1% BSA—PBS-injected skin showed darkened color indicating hair follicles in the anagen phase. (h and i) Histologically, anagen hair follicles were observed in the control skin by hematoxylin and eosin stain (h) and alkaline phosphatase stain (i). Arrowheads, alkaline phosphatase-negative outer root sheath; arrow, alkaline phosphatase-positive outer root sheath. Sale bars: 50 μm.

3.3. Similar induction effect of anagen phase was obtained by a single injection of PDGF in hyaluronic acid

The area in close proximity to the PDGF-AA single injection site in three out of five mice, or to the PDGF-BB single injection site in four out of five mice turned dark in color, suggesting that the HFs at the injection sites had progressed into the anagen phase (Fig. 3a and d). PDGF-AA or -BB single injection mice had significant increases in hair follicle tissue in the dermis. Histological analysis highlighted a significant increase in skin thickness ($p < 0.05$) (Figs. 3b and e and 4). Using alkaline phosphatase staining, we confirmed HFs in each mouse at the injection sites were in the anagen phase (Fig. 3c and f). Whereas the injection sites using just the vehicle solution alone (1% hyaluronic acid) all five mice retained their normal white color, no significant increases in hair follicle tissue in the dermis, from the result of alkaline phosphatase staining, suggesting that they remained in telogen phase (Figs. 3g–i).

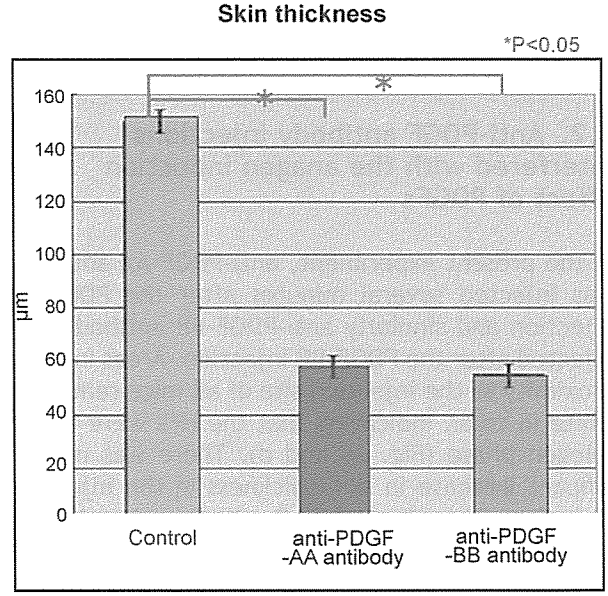

Fig. 6 Local injections of either anti-PDGF-AA or -BB antibodies resulted in decreased skin thickness suggesting *catagen induction*. The *T*-test provided significant differences in skin thickness between controls and PDGF-injected groups ($^{*}p < 0.05$) in 28-day-old male C3H mice at the second anagen phase.

3.4. Local injections of anti-PDGF-AA antibody or anti-PDGF-BB antibody *induced catagen* phase

The tissue in close proximity to the anti-PDGF-AA or -BB injection sites in all mice remained bright in color, indicating that the HFs were in telogen (Fig. 5a and d), although the injection sites of the vehicle solution alone (0.1% BSA–PBS) in all five mice were dark in color, suggesting that they were still in anagen phase (Fig. 5g). Anti-PDGF-AA or -BB injected mice showed significant decreases in hair follicle tissue in the dermis around the injection sites. Histological analysis demonstrated a significant decrease in skin thickness at the injection sites, compared with control group ($p < 0.05$) (Figs. 5b, e and h and 6). Using alkaline phosphatase staining, HFs at the injection sites of each mouse was confirmed to be in telogen phase (Fig. 5c and f). These findings show that local injections of both anti-PDGF-AA and -BB antibodies *induced catagen* phase.

3.5. Expression of the HF differentiation-related signaling molecules, Shh, Wnt5a and Lef-1, was upregulated in skin samples in which anagen had been induced by PDGF local injections

Immunohistological observation of the anti-Shh antibody revealed that Shh expression was seen in the dermal papilla, hair matrix, inner root sheath and outer root sheath of the hair follicles in the anagen phase 10 days after the PDGF injections (Fig. 7b and c). Conversely, in skin tissue obtained from the mice before injection, only weak Shh expression was seen restricted in the secondary hair germ, sebaceous gland and outer root sheath of hair follicles in the telogen phase (Fig. 7a). Real-time RT-PCR using extracted mRNA from the anagen phase HF inducted by local injection of PDGF (day 10) showed the upregulated expression of Shh. The Student's *T*-test demonstrated significant differences in Shh mRNA levels between control (before injection, day 0) and the mice skin after 10 days both PDGF-AA and -BB local injection ($p < 0.05$) (Fig. 7d and e).

Real-time RT-PCR using extracted mRNA from skin samples after anagen induction by local injection PDGF (day 10) also showed upregulated expression of Wnt5a and Lef-1. The Student's *T*-test demonstrated significant differences in Wnt5a and Lef-1 mRNA level between control (before injection, day 0) and the mice skin after 10 days both PDGF-AA and -BB local injection ($p < 0.05$) (Fig. 8).

Stat3 immunostaining in the dermal papilla, hair matrix and inner root sheath was present in mice 10 days after the injection (Fig. 9b and c). Before the injection, only the secondary hair germ, sebaceous gland and inner root sheath showed Stat3 expression (Fig. 9a). There was no significant difference in Stat3 mRNA levels between control samples (day

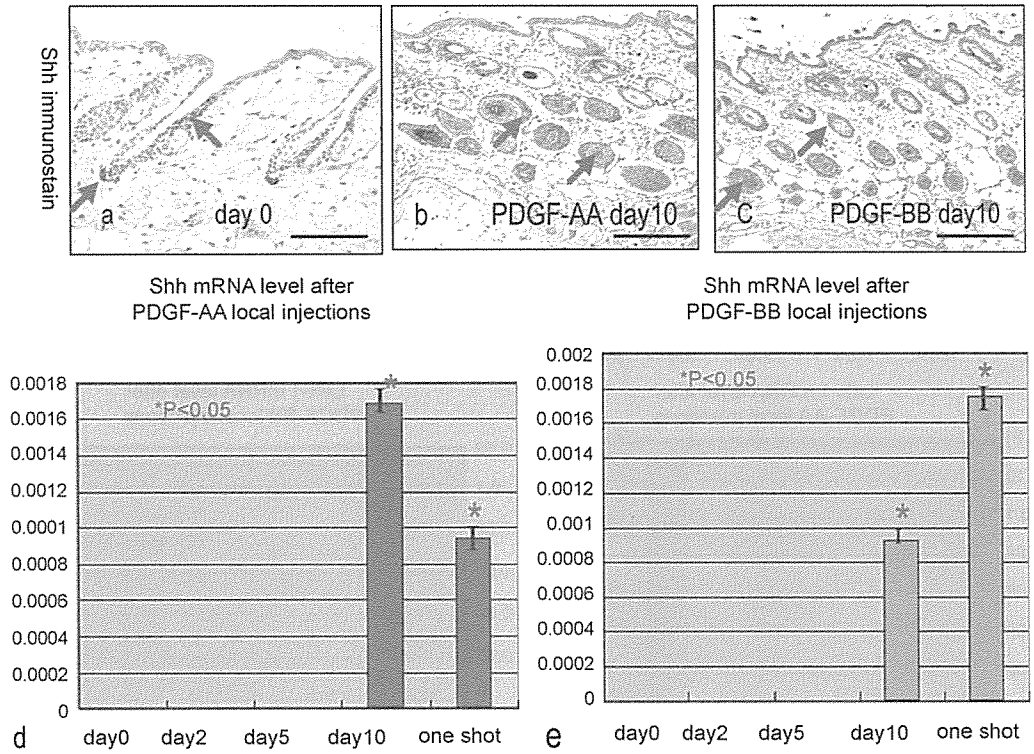

Fig. 7 Shh expression in the hair follicle was upregulated by either PDGF-AA or -BB injection. (a) In the skin of control non-injected mice (day 0), Shh immunoreactivity was weakly observed in the secondary hair germ, sebaceous gland and outer root sheath of hair follicles (arrows). (b and c) Strong Shh immunolabeling was observed in the dermal papilla, hair matrix, inner and outer root sheath of hair follicles in the PDGF-AA (b) or PDGF-BB (c) injected skin (day 10). Sale bars: 50 μm. (d and e) By real-time RT-PCR analysis, Shh mRNA expression was upregulated at day 10 after consecutive 5 days injection or one-shot injection. The *T*-test demonstrated significant differences in Shh expression ($^{*}p < 0.05$). Day 0, before injection; day 2, after consecutive 2 days' injection; day 5, after consecutive 5 days' injection; day 10, 5 days after consecutive 5 days' injection; one shot, 10 days after one-shot injection.

0) and the mouse skin after 10 days of either local injection with PDGF-AA or -BB (Fig. 9d and e).

4. Discussion

PDGF-AA and its receptor PDGFR-alpha are expressed in human HFs and the HF associated mesenchyme, respectively, during human fetal hair follicle morphogenesis [2]. It has previously been reported that injection of anti-PDGFR-alpha antibodies into newborn mice perturbed hair formation [3]. It had also been reported that both types of PDGF receptors are expressed in human follicular keratinocytes, whereas the mesenchymal dermal papilla cells only express PDGFR-alpha and that these PDGF expression levels isoforms can be influenced in vitro by treatment with positive and negative regulator cytokines affecting hair follicle activity [4]. PDGF-A null mice develop a specific skin and hair phenotype characterized by a progressive loss of dermal mesenchyme, and impaired formation of the dermal components of HF [5]. These facts suggested a role for PDGF-AA in HF development. We think PDGFs bind to PDGF receptors on follicular keratinocytes and dermal papilla cells and induce anagen phase via Shh, Lef-1 and Wnt pathways [5].

In this study, we analyzed the effect of cutaneous injections of recombinant human PDGF-AA and -BB on HF growth in mice. We showed that both PDGF-AA and -BB local injections resulted in induction of anagen phase in mice HFs (Fig. 1). We also demonstrated that anti-PDGF antibody injections following PDGF injections interfered with the anagen induction effect of PDGFs (Fig. 2). Furthermore, we showed that local injections of both anti-PDGF-AA and -BB antibodies induced catagen hair cycle phase (Fig. 5). These results indicated that both PDGF-AA and -BB play a role in the induction and maintenance of the anagen hair cycle phase in mice.

Our present data are comprised first report that has analyzed the effect of cutaneous injections of recombinant human PDGF-AA and -BB on HF growth using adult mice in the telogen hair cycle stage. In the present study, we have clearly demonstrated the

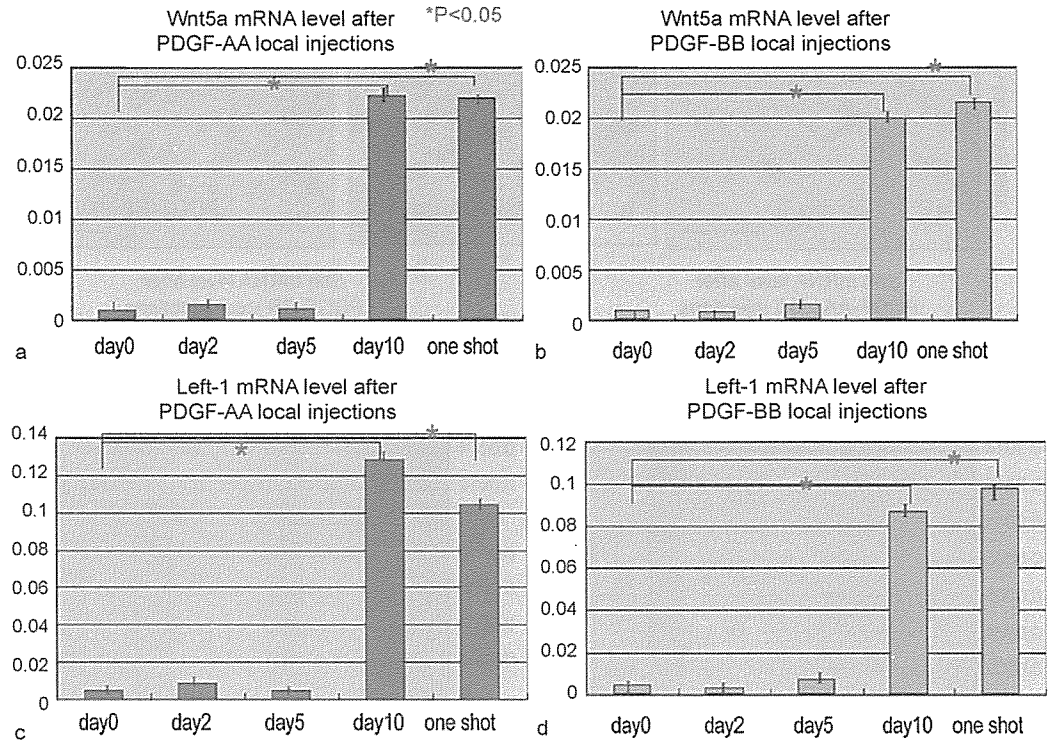

Fig. 8 Both Wnt5a and Lef-1 expressions in the hair follicle were upregulated by PDGF-AA or -BB injection. (a and b) By real-time RT-PCR analysis, Wnt5a mRNA expression was upregulated at day 10 after consecutive 5 days injection or single injection. The *T*-test demonstrated significant differences in Wnt5a expression ($^{*}p < 0.05$). (c and d) Lef-1 mRNA expression was also upregulated at day 10 after consecutive 5 days injection or single injection. The *T*-test demonstrated significant differences in Lef-1 expression ($^{*}p < 0.05$). Day 0, before injection; day 2, after consecutive 2 days' injection; day 5, after consecutive 5 days' injection; day 10, 5 days after consecutive 5 days' injection; single, 10 days after single injection.

effects of induction of anagen phase and maintenance of murine HFs by PDGF-AA and -BB. Local injection of hepatocyte growth factor was reported to result in a delayed transition from anagen to telogen but only weak anagen induction activity [8]. Subcutaneous application of slow-release gelatin hydrogel beads containing basic fibroblast growth factor or hepatocyte growth factor, and collagen hydrogel containing vascular endothelial growth factor led to the appearance of dark areas on the reverse (dermal) subcutaneous side of skin tissue and increased hair shaft elongation in mice [9]. From these findings, the effects of PDGF-AA and -BB local injection appeared to be strong both in the induction and maintenance of anagen phase, although it is difficult to compare these results with ours because the two detailed experimental designs were different.

To determine the optimal treatment method, we tried two different injection methods. One involved the administration of a solution of PDGF on 5 consecutive days and the other is single injection administration using 1% hyaluronic acid as a slow-release vehicle. We obtained similar anagen phase induction effects using both methods (Figs. 1 and 3). Due to the in vivo instability and short half-life of PDGF, if administered in solution form into skin, the expected biological effects may not always be obtained or effect may be short-lived. It has been suggested that hyaluronic acid has a slow-release effect if injected with PDGF [10]. PDGF formulated with hyaluronic acid as a vehicle is thought to be a potentially useful treatment for alopecia.

Previous experimental data suggest that HF transition from telogen to anagen is associated with the activation of the Shh [11], Wnt [12]/beta-catenin [13]/Lef-1 [13] and STAT3 [14] signaling pathways. Thus, we studied the expression of Shh, Wnt5a, Lef-1 and Stat3 in the anagen PDGF induced skin samples using immunohistochemical staining and real-time reverse transcriptase (RT)-PCR. Our results showed that expression of Shh, Wnt5a and Lef-1 was upregulated in the skin samples in which anagen had been induced by PDGF local injections (Figs. 7 and 8). It was suggested that PDGF isoforms induce anagen phase via upregulation of Shh, Wnt5a and Lef-1 in mice HFs. Conversely, there were no significant differences in the Stat3 mRNA levels between control and the mice skin 10 days after both PDGF-AA and -BB treatment

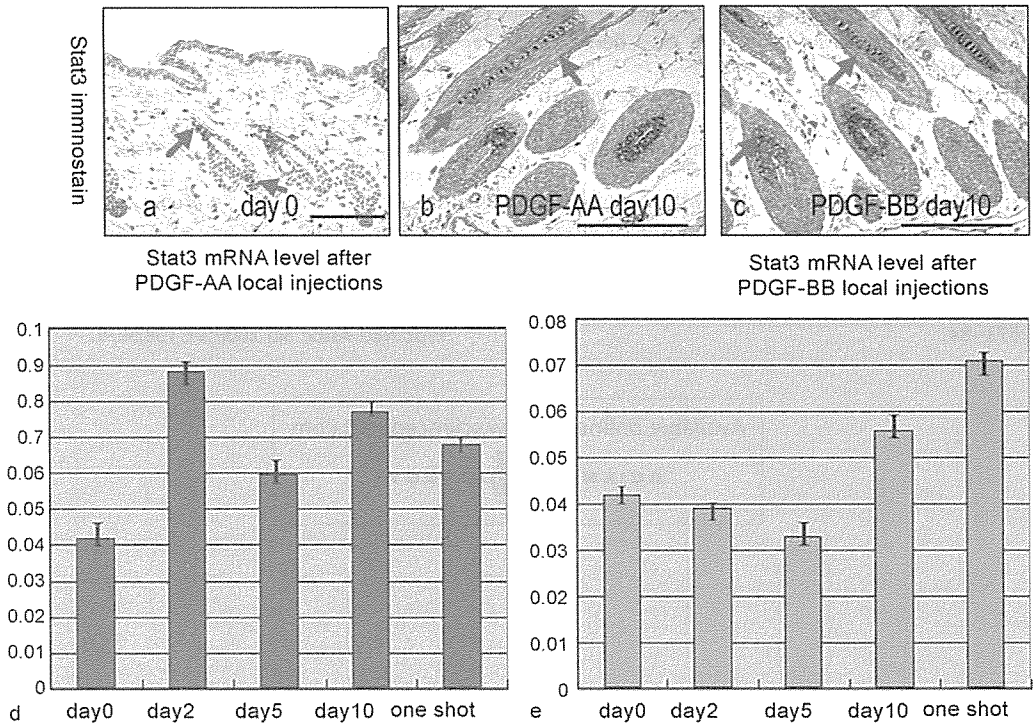

Fig. 9 Stat3 expression in the hair follicle was not significantly upregulated by PDGF-AA or -BB injection. (a) In the skin of control non-injected mice (day 0), Stat3 immunoreactivity was observed in the secondary hair germ, sebaceous gland and inner root sheath of hair follicles (arrows). (b and c) Stat3 immunolabeling was also observed in the dermal papilla, hair matrix and inner root sheath of hair follicles in the PDGF-AA (b) or PDGF-BB (c) injected skin (day 10). Scale bars: 50 μm. (d and e) By real-time RT-PCR analysis, Stat3 mRNA expression was not significantly upregulated at day 10 after consecutive 5 days injection or single injection. Day 0, before injection; day 2, after consecutive 2 days' injection; day 5, after consecutive 5 days' injection; day 10, 5 days after consecutive 5 days' injection; single, 10 days after single injection.

(Fig. 9). The reason why no significant differences were detected may be due to the fact that STAT3 might also expressed in the telogen phase.

In conclusion, PDGF isoforms have been shown both induce and maintain mouse HFs anagen phase and we demonstrated that application of PDGF-AA or -BB had a significant anagen induction effect. Local application of PDGF-AA, PDGF-BB, or a cocktail of several growth factors including PDGFs might be used as an easy and effective treatment for alopecia which is caused by an early catagen phase induction and elongation of the telogen phase of the human hair cycle. Further studies will be needed to confirm the effects of PDGFs in human hair follicles.

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Dietary glucosylceramide improves skin barrier function in hairless mice

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Summary

Background: Sphingolipids are known to play an important role in both water retention and epidermal permeability barrier function in mammalian stratum corneum. However, little is known about the effects on epidermal function of orally administered sphingolipids.
Objective: We examined the effect of dietary glucosylceramide (GluCer) on the maintenance and recovery of epidermal barrier function.
Methods: Hairless mice were fed a particular diet (HR-AD) for 4 weeks to induce chronic skin perturbation. Subsequently, a normal diet supplemented with GluCer (from rice bran and germ) was provided for the next 4 weeks. Transepidermal water loss (TEWL) and stratum corneum flexibility were measured throughout this recovery phase. Additional hairless mice were fed a diet with or without a maize-extracted GluCer supplement for 5 weeks, then their skin was acutely perturbed with repeated tape-stripping, and the TEWL was measured.
Results: Although skin functions were generally lower following chronic perturbation, in GluCer-fed mice the TEWL was significantly reduced at 2 weeks and the stratum corneum flexibility was increased at 3 weeks compared to controls. Following acute barrier perturbation by tape-stripping, mice an HR-AD fed a GluCer diet exhibited enhanced recovery compared with the control diet group.

Abbreviations: GluCer, glucosylceramide; TEWL, transepidermal water loss

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Conclusion: These results demonstrate that in hairless mice skin barrier functions impaired by chronic or acute perturbations were improved by dietary GluCer. The oral administration of GluCer may be useful for the preservation and recovery of epidermal barrier functions an HR-AD.

1. Introduction

The stratum corneum is the extreme outer layer of the skin and is responsible for protection against external stimuli. This tissue prevents excessive transepidermal water loss (TEWL) from the body to the external environment, which is indispensable for the survival of mammals. The lamellar structure of the stratum corneum is composed of mainly lipids, including cholesterol, fatty acids, and ceramide, a fundamental constituent of sphingolipids that plays a critical role in water-retention and other epidermal permeability barrier functions [1]. In skin diseases such as atopic dermatitis, the lesional skin tends to be overly sensitive and dry, exhibiting defective permeability barrier functions. There is also a markedly altered types of ceramides in the lesional skin of atopic dermatitis patients compared with normal tissue from healthy persons [1—4].

Hairless mice (HR-1) are a known animal model for observing skin abnormalities such as atopic dermatitis. Atopic dermatitis-like symptoms can be induced in these animals by feeding them a particular diet, the HR-AD [5,6]. In addition, acute perturbations in human or hairless mice skin have been induced using a typical application of an organic solvent such as acetone or tape-stripping [7,8], both of which reportedly reduce the TEWL.

GluCer is a major sphingolipid of plant tissue, including those used as food sources [9—11]. Reportedly, the application of the sphingolipid GluCer to dorsal skin of mice significantly reduced the TEWL and barrier disruption induced by ultraviolet radiation [12]. However, it is not clear what effect oral administration of GluCer would have on skin barrier function. In the present report, we examine the efficacy of dietary GluCer in restoring epidermal barrier function using a model of chronic or acute perturbation in hairless mice (HR-1 mice). Our results indicate that in these animals orally administered GluCer improves epidermal function previously impaired by an HR-AD diet or tape-stripping. Thus, our findings suggest the possibility that dietary GluCer could be therapeutic in treating skin disorders.

2. Materials and methods

2.1. Animals

Four-week-old male hairless mice (HR-1) and 8-week-old female hairless mice (Hr/Hr) were purchased from Hoshino Experimental Animal Center (Yashio, Japan) and Charles River Laboratories Japan, Inc. (Yokohama, Japan). Animal procedures were approved by the Ethical Committee at Hokkaido University Graduate School of Medicine and were performed in accordance with their guidelines. The mice were housed in plastic cages in a barrier facility at a temperature of 22 ± 3 °C, with 50 ± 20% relative humidity, and a 12:12 h light:dark cycle.

2.2. Reagents

Dietary glucosylceramide (GluCer), extracted from rice germ and bran (Nippn ceramide RPG) or dietary GluCe from maize (Nippn ceramide CP-K6) were purchased from Nippon Flour Mills Co., Ltd. (Tokyo, Japan). HPLC analysis indicated a purity of greater than 6%.

2.3. Diets

The Labo MR stock (the normal rodent diet), and the HR-AD (the special diet, skin-damage is induced) were purchased from Nosan Corp. (Yokohama, Japan) [5]. The experimental treatment diet included the Labo MR stock used in the normal diet supplemented with 1000 ppm GluCer (from rice germ and bran) (Nosan Corp., Yokohama, Japan). The AIN-76A rodent diet were purchased from Research Diets, Inc. (New Brunswick, NJ, USA), which contains carbohydrate (66.0%), protein (20.3%), fat (5.0%), and very little sphingolipids (<0.005%), supplemented with 1000 ppm GluCer (from maize).

2.4. TEWL and stratum corneum flexibility assessments

All measurements were carried out at 22 ± 3 °C and a humidity level of 50 ± 20%, and were performed in triplicate for each skin spot. TEWL was measured using an Electrolytic water analyzer (Meeco,