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PDGF isoforms induce and maintain anagen phase of murine hair follicles

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Short title: The effect of PDGF on the murine hair cycle

Key words: hair cycle; hair growth; PDGF-AA; PDGF-BB

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

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 five 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.

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 PDGF-BB on hair follicle growth in mice. As a result, local injections of PDGF-AA and PDGF-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, U.S.A.), recombinant human PDGF-BB (CHEMICON International,

Temecula, U.S.A.), rabbit polyclonal anti-human PDGF-AA antibody (CHEMICON International, Temecula, U.S.A.), rabbit polyclonal anti-human PDGF-BB antibody (CHEMICON International, Temecula, U.S.A.), rabbit polyclonal anti-PDGFR-alpha (C-20) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), rabbit polyclonal anti-PDGFR-beta (958) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), monoclonal anti-human/mouse Shh N-terminal peptide antibody (R&D systems, Minneapolis, MN, U.S.A.) and anti-Stat3 antibody (Epitope specific rabbit antibody (LAB vision, Fremont, CA, U.S.A.).

2.2. Animals

Mice used in this study were male mice C3H/HeN (47 day old, second telogen phase; body weight, 19-20g) (CLER, Japan) for anagen induction experiments and 28-day-old male C3H/HeN mice (second anagen phase; body weight, 16-17g) 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 PDGF-BB were dissolved in sterile and toxin-free phosphate-buffered saline containing 0.1% bovine serum albumin (0.1% BSA-PBS). 1µg PDGF-AA or PDGF-BB dissolved in 100µl 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 5µg 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 anti-PDGF-BB antibody was injected just after each injection of PDGF-AA or PDGF-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 PDGF-BB in 1% hyaluronic acid for single injection. 5 μ g of PDGF-AA or PDGF-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

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

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 immunohistochemical 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 60mg/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) 1U/1µg-RNA for 15 minutes 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 PDGF-BB. Real-time RT-PCR assays using TaqMan probes (Taqman(R) Gene Expression Assays: Lef1, Assay ID Mm00550265ml; Shh, Assay ID Mm00436527ml; Stat3, Assay ID Mm00456961ml; Wnt5, Assay ID Mm00437347ml; GAPDH, Assay ID Mm99999915g1) (Roche Molecular Systems, Inc., Pleasanton, CA, U.S.A.) were carried out in a micro reaction tube. The reaction mixture for each one tube TaqMan reaction mix consisted of 5µl of Universal 5X buffer, 1 micro l of 10 mM dNTP, 1 micro l of enzyme mix (Qiagen, Germany), 1 micro l of 20 pM forward primer, 1 micro l of 20 pM reverse primer, 1 micro l of 25 pM fluorogenic FAM labeled JEV probe, and 15 of RNA sample to a total volume of 25 micro l. 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: $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{target sample}} - \Delta\text{Ct}_{\text{calibrator sample}}$ (negative control sample).

The relative gene copy numbers were calculated by the expression $2^{-\Delta\Delta\text{Ct}}$. 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, U.S.A.). 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 PDGF-BB local injections induced anagen phase in murine hair follicles.

The area in close proximity to the injection sites in 3 out of 5 mice became darkened in color, indicating that HFs were in the anagen hair cycle phase (Figs.1a, 1d), 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 PDGF-BB injected sites. Quantitatively, PDGF-AA or PDGF-BB injected mice showed significant increases in skin thickness at the injected sites (Figs.1b, 1e). 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 (Figs.1c, 1f). There was no significant increase in skin thickness in the vehicle solution alone injection sites (Fig.1h). 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 (Figs.2a, 2d). There was no significant increase in skin thickness in the injection sites (Figs. 4, 2b, 2e). By alkaline phosphatase staining, we confirmed that HFs in each mouse remained in telogen (Figs.2c, 2f). These findings clearly indicated that effects of injected PDGF-AA or PDGF-BB were inhibited by the respective injections of anti-PDGF-AA antibody or anti-PDGF-BB antibodies.

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 3 out of 5 mice, or to the PDGF-BB single injection site in 4 out of 5 mice turned dark in color, suggesting that the HFs at the injection sites had progressed into the anagen phase (Figs.3a, 3d). PDGF-AA or PDGF-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. 4, 3b, 3e). Using alkaline phosphatase staining, we confirmed HFs in each mouse at the injection sites were in the anagen phase (Figs.3c, 3f). 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, 3h, 3i).

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 anti-PDGF-BB injection sites in all mice remained bright in color, indicating that the HFs were in telogen (Figs.5a, 5d), 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 anti-PDGF-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. 6, 5b, 5e, 5h). Using alkaline phosphatase staining, HFs at the injection sites of each mouse was confirmed to be in telogen phase (Figs.5c, 5f). These findings show that local injections of both anti-PDGF-AA antibody and anti-PDGF-BB antibody 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 (Figs. 7b,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 induced by local injection of PDGF (day10) 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 PDGF-BB local injection ($p < 0.05$) (Figs.7d, 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 PDGF-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 (Figs. 9b, 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 0) and the mouse skin after 10 days of either local injection with PDGF-AA or PDGF-BB (Figs. 9d, 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 PDGF-BB on HF growth in mice. We showed that both PDGF-AA and PDGF-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 antibody and anti-PDGF-BB antibody induced catagen hair cycle phase (Fig.5). These results indicated that both PDGF-AA and PDGF-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 PDGF-BB on HF growth using adult mice in the telogen hair cycle stage. In the present study, we have clearly demonstrated the effects of induction of anagen phase and maintenance of murine HFs by PDGF-AA and PDGF-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 PDGF-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 (Fig.1, 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 (Fig.7, 8). It was suggested that PDGF isoforms induce anagen phase via upregulation of Shh, Wnt5a and Lef-1 in mice HF. Conversely, there were no significant differences in the Stat3 mRNA levels between control and the mice skin 10 days after both PDGF-AA and PDGF-BB treatment (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 HF anagen phase and we demonstrated that application of PDGF-AA or PDGF-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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Legends

Fig.1 Local injections of either PDGF-AA or PDGF-BB induced and maintained hair follicle anagen phase.

PDGF-AA, PDGF-BB or vehicle solution (0.1% BSA-PBS) was injected intradermally into 47d old male C3H mice (second telogen) for 5 consecutive days (total 5 μ g of PDGF-AA or PDGF-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 darkened 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 micro m.

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, 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, blacked areas indicating

anagen induction were not seen in the reverse side of the skin. (e, 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. Scale bars: 50 microm.

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 darkened areas were seen in the negative controls (g, 1% hyaluronic acid only injected skin). Histologic sections stained with hematoxylin and eosin (b, e) or alkaline phosphatase (c, f) confirmed the anagen induction of hair follicles in the PDGF-AA (b, c) or PDGF-BB (e, f) injected sites. Alkaline phosphatase-positive outer root sheath (arrows) suggested hair follicles were at anagen phase (c, f). Outer root sheaths of hair follicles in the negative control (i) specimens were negative for alkaline-phosphatase staining (arrowhead). Scale bars, 50 microm.

Fig.4 Local injections of either PDGF-AA or PDGF-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 antibody or anti-PDGF-BB antibody induced catagen phase.

Anti-PDGF-AA, anti-PDGF-BB or vehicle solution (0.1% BSA-PBS) was injected intradermally into 28 d old male C3H mice (second anagen) for 5 consecutive days (total 5 μ g of anti-PDGF-AA or anti-PDGF-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, 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, 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, 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. Scale bars: 50 micro m.

Fig.6 Local injections of either anti-PDGF-AA antibody or anti-PDGF-BB antibody 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.

Fig.7 Shh expression in the hair follicle was up-regulated by either PDGF-AA or PDGF-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, 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). Scale bars: 50 micrometers. (d, e) By real-time RT-PCR analysis, Shh mRNA expression was up-regulated 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.

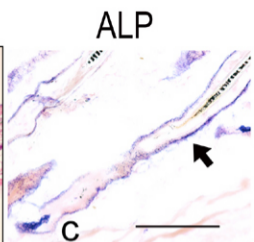
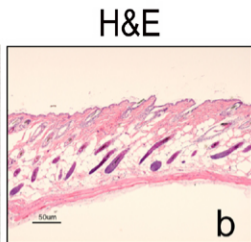
Fig.8 Both Wnt5a and Lef-1 expressions in the hair follicle were up-regulated by PDGF-AA or PDGF-BB injection.

(a, b) By real-time RT-PCR analysis, Wnt5a mRNA expression was up-regulated at day 10 after consecutive 5 days injection or single injection. The *T*-test demonstrated significant differences in Wnt5a expression (*, $p < 0.05$). (c,d) Lef-1 mRNA expression was also up-regulated 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.

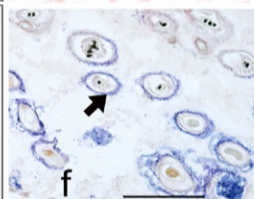
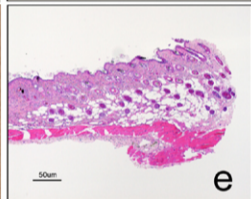
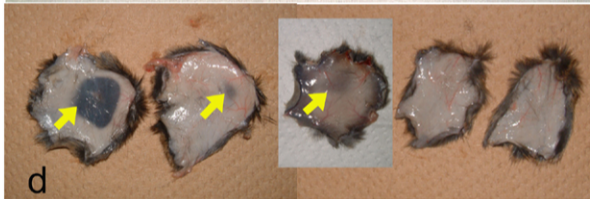
Fig.9 Stat3 expression in the hair follicle was not significantly up-regulated by PDGF-AA or PDGF-BB injection.

(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, 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 μ m. (d, e) By real-time RT-PCR analysis, Stat3 mRNA expression was not significantly up-regulated 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.

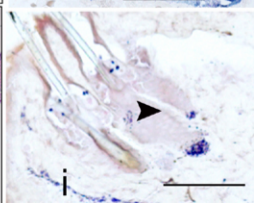
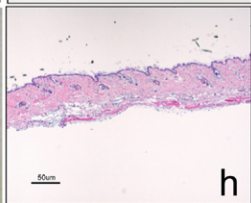
PDGF-AA



PDGF-BB



Control



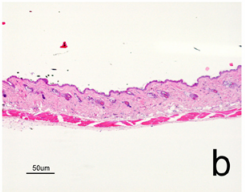
Anti-PDGF-AA
Ab after
PDGF-AA



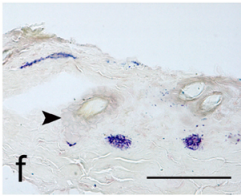
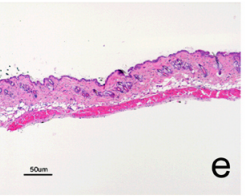
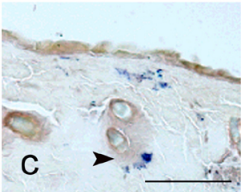
Anti-PDGF-BB
Ab after
PDGF-BB



H&E



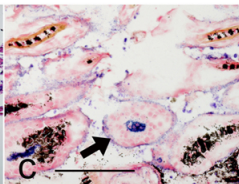
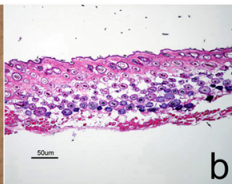
ALP



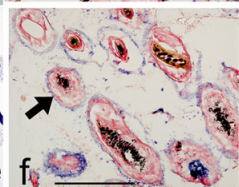
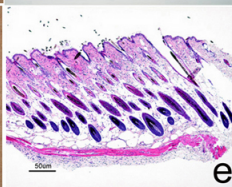
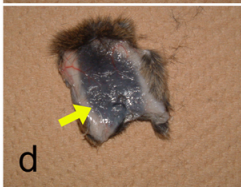
H&E

ALP

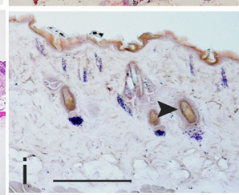
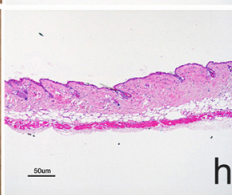
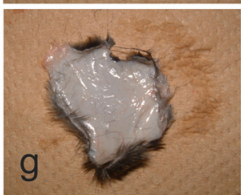
PDGF-AA
one shot



PDGF-BB
one shot

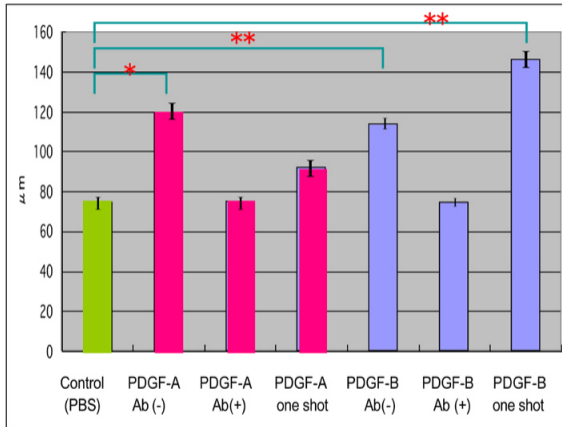


Control



Skin thickness

*P < 0.05, **P < 0.005



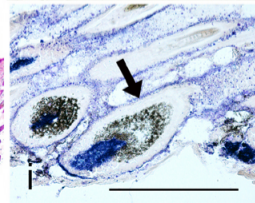
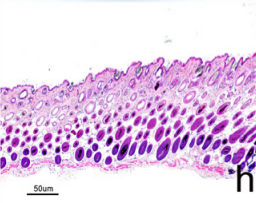
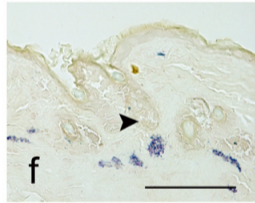
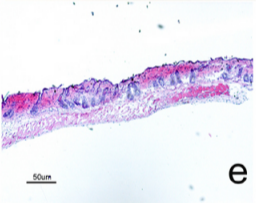
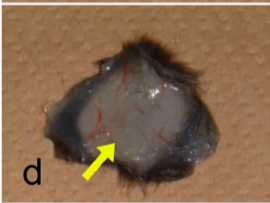
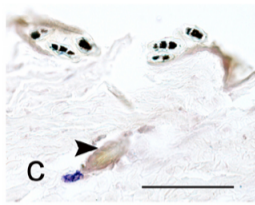
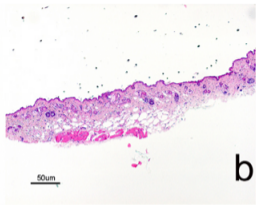
Anti-PDGF-AA
Ab

Anti-PDGF-BB
Ab

Control

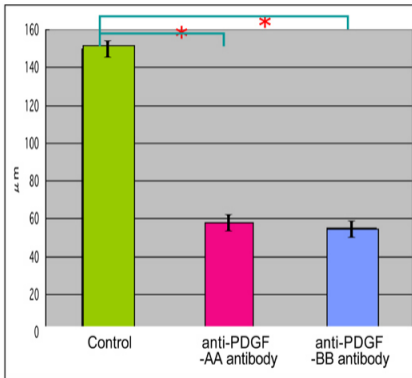
H&E

ALP

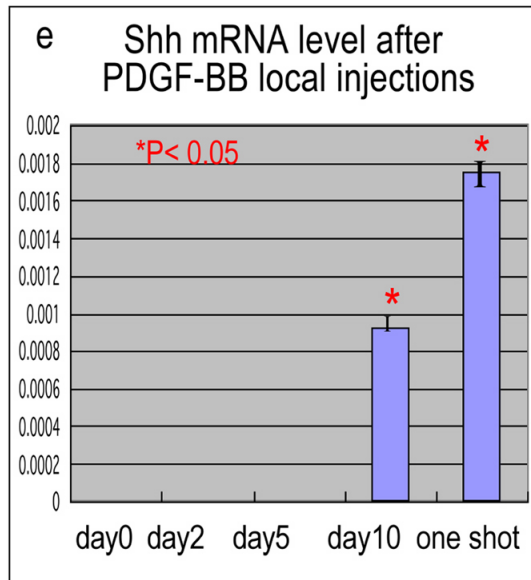
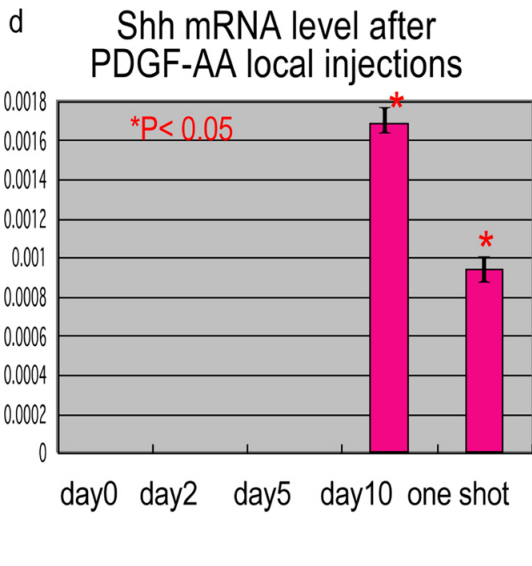
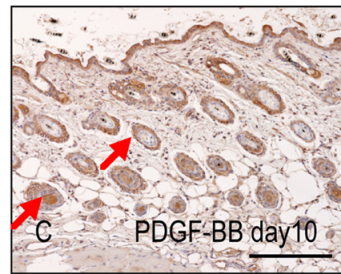
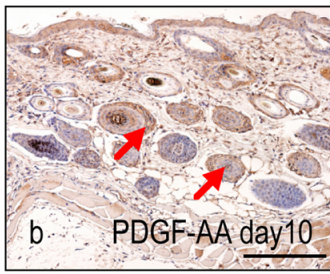
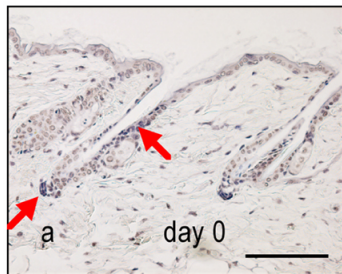


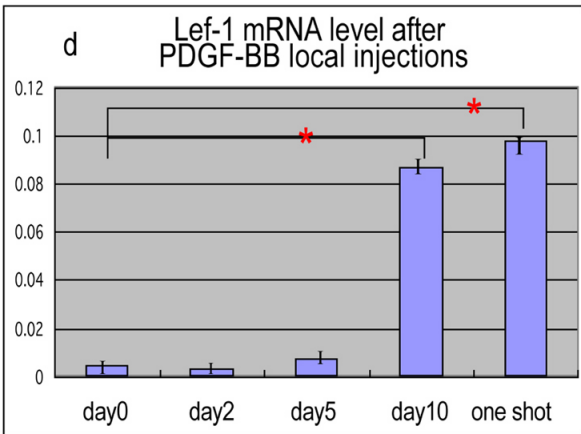
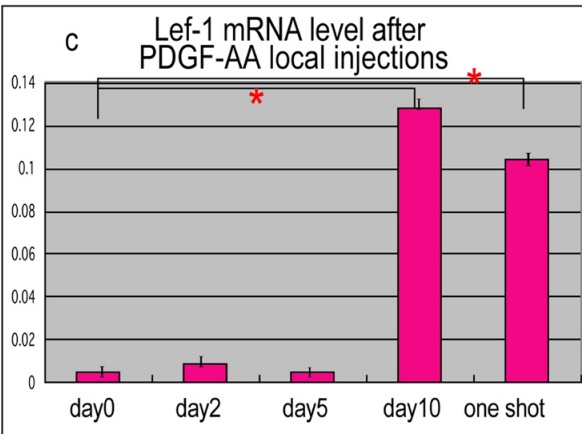
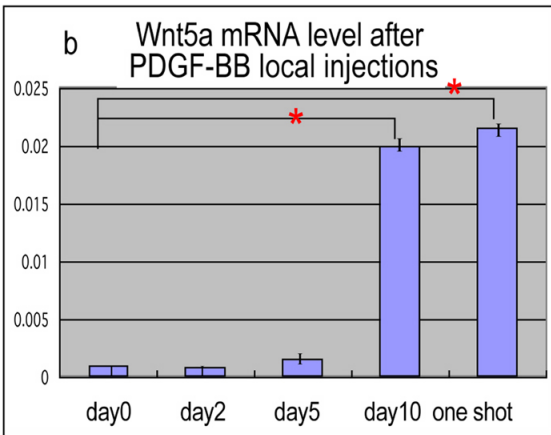
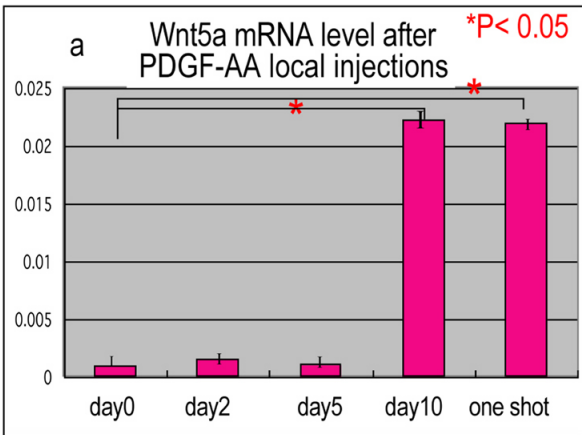
Skin thickness

*P < 0.05

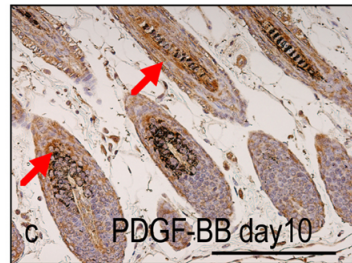
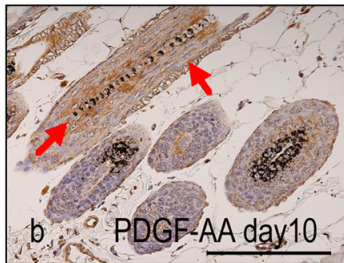
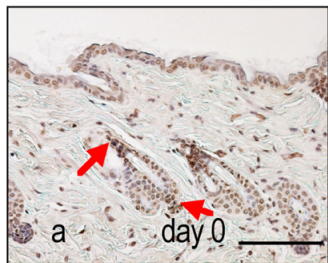


Shh immunostain

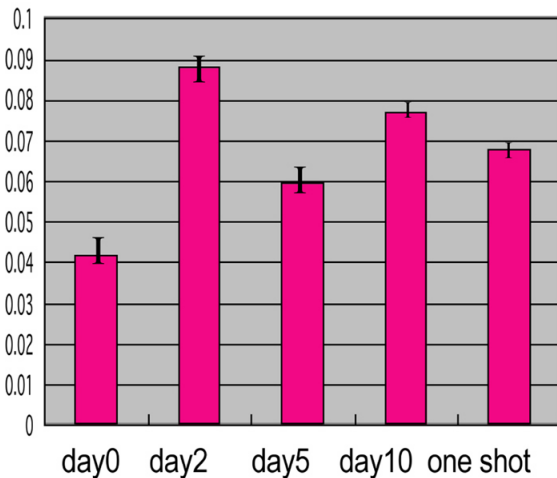




Stat3 immunostain



d Stat3 mRNA level after PDGF-AA local injections



e Stat3 mRNA level after PDGF-BB local injections

