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Fibroblast Growth Factor 5 Inhibits Hair Growth by Blocking Dermal Papilla Cell Activation

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Fibroblast growth factor (FGF) 5 inhibits hair growth and induces catagen in mouse hair follicles, *in vivo*. Given that FGF-5 receptor (FGFR1) is expressed in dermal papilla cells (DPCs), which are known to stimulate outer root sheath cell (ORSC) proliferation, we hypothesized that FGF-5 attenuates DPC-mediated ORSC proliferation. In the present study, DPCs and ORSCs were isolated from rat vibrissae, after which the effects of FGF-5 on proliferation of ORSCs cultured in DPC-conditioned medium were assessed. We first confirmed that FGFR1 was expressed in cultured DPCs and detected FGFR2-4 as well. ORSC proliferation was increased approximately twofold when the cells were cultured in DPC-conditioned medium, and the effect was unaltered by FGF-5. In addition, FGF-5 did not directly inhibit ORSC proliferation; indeed, it actually promoted proliferation of both DPCs and ORSCs. When DPCs were first activated by exposure to FGF-1 and FGF-2, which are expressed in hair follicles during anagen, ORSC proliferation observed in the resultant conditioned medium was substantially greater than in medium conditioned by unstimulated DPCs. The FGF-1-induced enhancement was reversed by FGF-5, diminishing ORSC proliferation to control levels. By contrast, the enhancement of DPC-mediated ORSC proliferation by FGF-2 was not suppressed by FGF-5. Proliferation of ORSCs did not depend on DPC proliferation, nor did FGF-1 directly promote ORSC proliferation. Dermal papillae thus appear to require activation before they will efficiently stimulate hair growth, and FGF-5 appears to inhibit hair growth and induce catagen by blocking that activation.

Key Words: **dermal papilla cell; outer root sheath cell; conditioned medium; FGF receptor; FGF-1, FGF-2.**

The hair growth cycle in mammals is composed of three phases: anagen, during which follicles grow, hair synthesis takes place, and skin thickness increases; catagen, during which follicles involute and skin thickness decreases; and telogen, during which both follicles and skin are at rest. This cycle is regulated by a variety of mediators, including several members of the fibroblast growth factor (FGF) family (1, 2).

The FGF family consists of at least 23 members, exhibiting a diverse array of biological activities (3–31). The *FGF-5* gene was originally reported to be one of the human oncogenes (32), but because of its high homology with both acidic and basic FGF, it was later classified as belonging to the FGF family (33). Hébert *et al.* (34) reported that deletion of the *Fgf-5* gene abnormally prolongs anagen in mice, suggesting that its expression leads to termination of anagen and induction of catagen. The gene products include both full-length and short forms of the protein (FGF-5 and FGF-5S, respectively); the latter being less than half the size of the former as a consequence of alternative splicing and a frame shift (35, 36). We previously found that FGF-5, but not FGF-5S, inhibits hair growth and induces catagen (37).

Hair is thought to grow as a result of proliferation of outer root sheath cells (ORSCs) induced by humoral factors synthesized and released by dermal papilla cells (DPCs). This is based on the observations that proliferation of isolated ORSCs is stimulated when they are co-cultured with DPCs (38) and that DPCs produce hepatocyte growth factor/scatter factor (HGF/SF) (39) and insulin-like growth factor (IGF)-I (40), both of which stimulate proliferation of ORSCs. In addition, DPCs express FGF-5 receptors (41), and FGF-5 positive cells collect near DPCs during catagen and telogen (42). In the present study, therefore, we tested the hypothesis that FGF-5 inhibits hair growth by reducing the capacity of DPCs to stimulate ORSC proliferation, thereby triggering induction of catagen.

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We also investigated whether FGF-5 interacts with FGF-1 and FGF-2, which are expressed in hair follicles during anagen (1). Our findings indicate that hair growth is regulated by a system that is much more complicated than previously thought.

MATERIALS AND METHODS

Preparation of recombinant FGF-5. Recombinant FGF-5 protein was obtained as previously reported (35). Briefly, an FGF-5 cDNA fragment was amplified using the primer set, 5′-CGGAATTC-CATATGGGTGAAAAGCGTCTCGCCCCCAAA-3′ (sense) and 5′-CG-CCATATGTTTATCCAAAGCGAAACTT-3′ (antisense), and a pLTR122 template (33); the N-terminal hydrophobic signal sequence of the original FGF-5 protein was not included in this construct (43). The amplified fragment was cloned into pBluescript SK^+, sequenced, digested with *Nde*I, and inserted into the NdeI site of the bacterial vector pET-3c. The recombinant plasmid was then transfected into *E. coli* (strain BL21(DE3)pLysS) (44), and the desired protein was expressed and extracted as described previously (43). FGF-5 protein was dissolved to a concentration of 100 or 200 μg/ml in phosphate buffered saline (PBS; pH 7.5) and stored at −20°C before use.

Isolation and culture of DPCs and ORSCs DPCs and ORSCs were isolated from the labial vibrissae of 3-week-old Wistar rats (SLC Japan, Hamamatsu, Japan) using the methods of Messenger *et al.* (45) and Kobayashi *et al.* (46). The left and right labial regions of each rat were removed with a scalpel under sterile conditions, and vibrissa follicles in the anagen phase were visualized under a stereoscopic microscope. Each follicle was then cut at the top of the hair bulb, and the dermal papilla was removed and cultured using the static culture technique. DPCs were maintained in Eagle minimum essential medium (MEM) (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) (Trace Biosciences, Victoria, Australia) at 37°C under an atmosphere of 5% CO_2. After 4 days in culture, the medium was replaced with MEM plus 10% FBS, and culture was continued for an additional 4 weeks. The cultures were then washed in Dulbecco's PBS (−) (Nissui, Tokyo, Japan) and incubated for 1 h at 37°C in 0.25% trypsin plus 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) (Gibco, BRL) to disperse the cells. The resultant cell suspensions were centrifuged, and the pelleted cells were subcultured.

To obtain ORSCs, the follicular sheath was carefully removed using a scalpel and needle, and after incubation with 0.1% collagenase/dispase (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C, the dermal sheath was removed. The sheath was then incubated with 0.05% trypsin plus 0.53 mM EDTA for one hour at 37°C, dispersing the ORSCs. The dispersed cells were plated in culture dishes coated with type IV collagen (Becton, Bedford, MA) and maintained in S-MEM (Gibco BRL) supplemented with 15% FBS, 10 ng/ml recombinant human epidermal growth factor (EGF) (Gibco BRL), 4 μg/ml insulin (Wako, Osaka, Japan), and 0.4 μg/ml hydrocortisone (Sigma, St. Louis, MO).

Detection of FGF receptors in DPCs Before examining the effects of FGF-5 on cultured DPCs, we used immunohistochemical techniques to test for the presence of its receptor, FGFR1 (47), as well as FGFR2, FGFR3 and FGFR4. DPCs were isolated as described above, seeded onto chamber slides (Nunc, Naperville, IL), and cultured overnight at 37°C. The following day the cells were fixed for 20 min in 10% formalin in PBS and treated for 5 minutes with 0.5% Triton X-100 in PBS. They were then incubated for 30 min with 10 μg/ml anti-FGFR1, anti-FGFR-2, anti-FGFR3, anti-FGFR4 (Santa Cruz) or rabbit IgG (Chemicon, Temecula, CA), for 30 min with biotin-conjugated goat anti-rabbit IgG (1:50 dilution; Biosource, Camarillo, CA), and then for 30 min with fluorescein-conjugated streptavidin (1:100 dilution; Biosource). After each treatment, the cells were washed three times for 5 min each with PBS. All incubations and washes were carried out at room temperature. Fluorescent emissions from the immunolabeled cells were then observed under a microscope using a narrow-band absorption filter centered at 515 nm.

Effects of FGF-5 on the ability of DPC-conditioned medium to stimulate ORSC proliferation After subculturing DPCs and ORSCs twice, subconfluent cells were seeded into 24-well plates (DPCs: $4 \times 10^4/cm^2$; ORSCs: $2 \times 10^2/cm^2$). The following day, 0, 10 or 100 ng/ml FGF-5 was added to the DPCs, which were then cultured for an additional 24 h. The resultant DPC-conditioned medium was collected, filtered and used to replace the medium in which the ORSCs were being cultured. The appropriate fresh medium was promptly added to the DPCs, and 24 h later, the conditioned medium was again recovered to replace the old DPC-conditioned medium added to the ORSCs the day before. This procedure was repeated three times until on the fourth day, 1 μCi/well of [*methyl*-^{3}H]thymidine (Moravek, Brea, CA) was added to the ORSCs. After labeling the cells for 5 h, ORSC proliferation was measured in terms of [*methyl*-^{3}H]thymidine uptake. For comparison (control), proliferation of cells incubated in MEM plus 10% FBS was measured in the same way.

Direct effects of FGF-5 on the proliferation of DPCs and ORSCs. DPCs and ORSCs were cultured as described above, except that one day after seeding, the culture media were replaced with MEM plus 0.3% FBS or S-MEM plus 3% FBS, respectively. The cells were then cultured for 4 more days, after which 0, 10, 100, or 1000 ng/ml FGF-5 was added, followed one day later by 1 μCi/well of [*methyl*-^{3}H]thymidine. Cells proliferation was then assessed as described above.

Effects of FGF-1 and FGF-2 on the capacity of DPC-conditioned medium to stimulate ORSC proliferation and the interaction of FGF-5 with FGF-1 or FGF-2. Then, we examined the effect of FGF-5 on FGF-1- and FGF-2-induced DPC activity. DPCs and ORSCs were seeded into 24-well plates as described above. The following day, FGF-1 or FGF-2 (0, 0.1, 1, or 10 ng/ml) (Progen, Heidelberg, Germany) was added to DPCs with or without 10 ng/ml FGF-5. The ORSC medium was subsequently replaced with DPC-conditioned medium for 3 days; on the fourth day ORSC proliferation was measured as described above. As a control, proliferation of ORSCs cultured in conventional medium was also measured. In addition, after collecting the DPC-conditioned medium for the third time, FGF-1 or FGF-2 was added to the DPCs, with or without FGF-5, and their proliferation was measured the following day.

Effect of heparin on the ability of FGF-1 and FGF-5 to activate DPCs. We also evaluated the effect of heparin on the ability of FGFs to activate DPCs. DPCs and ORSCs were seeded into 24-well plates as described above, and the following day 10 ng/ml FGF-1 (Progen) or FGF-5 was added to the DPCs, with or without 5 μg/ml heparin (Sigma, St. Louis, MO). The concentration of heparin used was selected based on previous studies of FGF-1 and FGF-5 (35, 44, 48). In some plates, the ORSC medium was subsequently replaced with the DPC-conditioned medium, and ORSC proliferation in DPC-conditioned or control medium was measured as described above. DPC proliferation was also measured in similar fashion.

Direct effects of FGF-1 and FGF-5 on ORSC proliferation. FGF-1 (0, 0.1, 1, or 10 ng/ml), with or without FGF-5 (10 ng/ml), was added to MEM plus 10% FBS and incubated in the absence of DPCs for 24 h at 37°C, after which the FGF-containing medium was used to replace the normal ORSC culture medium. This procedure was repeated daily for 3 days, and on the fourth day ORSC proliferation was measured as above.

RESULTS

Detection of FGF Receptors in DPCs

Anti-FGFR1 antibody reacted with cultured DPCs (Fig. 1a), which is consistent with the earlier detection

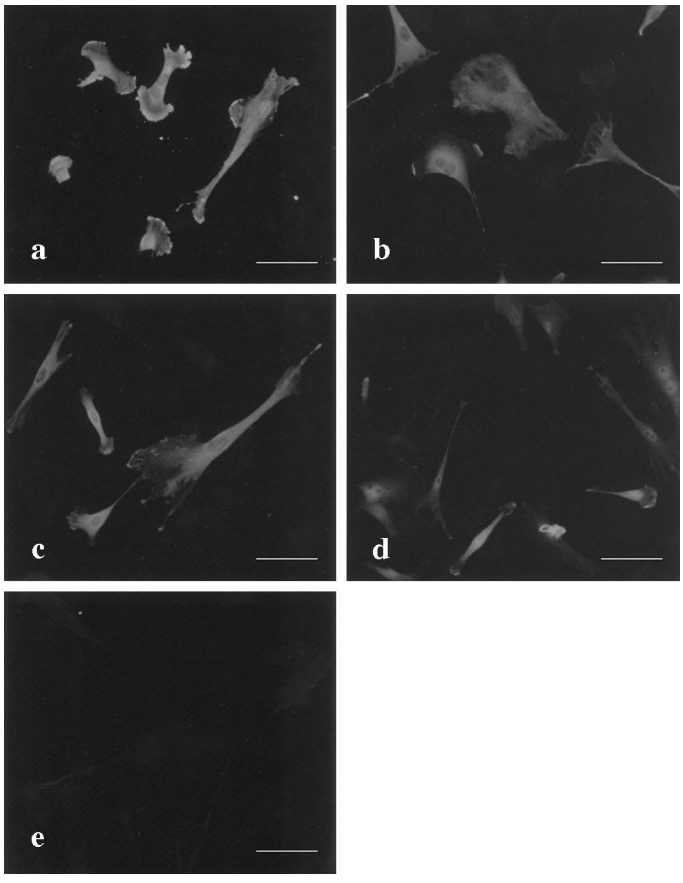

FIG. 1. Detection of FGF receptors in DPCs. DPCs were labeled with anti-FGFR1 (a), anti-FGFR2 (b), anti-FGFR3 (c), or anti-FGFR4 (d) antibodies. Control cells were treated with rabbit IgG (e). The immunolabeling was visualized by incubation with biotin-conjugated anti-rabbit IgG, followed by fluorescein-conjugated streptavidin. Scale bar, 100 μm.

of FGFR1 mRNA in mouse dermal papillae using *in situ* hybridization (41), and indicates that cultured DPCs express a specific FGF-5 receptor. In addition, FGFR2, FGFR3, and FGFR4 receptors were also detected in the cultured DPCs, though the level of immunolabeling was less than for FGFR1 (Fig. 1b–1d). By contrast, control rabbit IgG did not bind to the cells (Fig. 1e).

Effects of FGF-5 on the Capacity of DPC-Conditioned Medium to Stimulate ORSC Proliferation

As shown in Fig. 2, ORSCs cultured in FGF-5-free, DPC-conditioned medium exhibited significantly higher levels of proliferation than cells cultured in normal ORSC medium (control), which is consistent with earlier findings that DPCs release growth factors that stimulate ORSC proliferation (38). Addition of FGF-5 to the DPCs had no effect on their ability to stimulate ORSC proliferation.

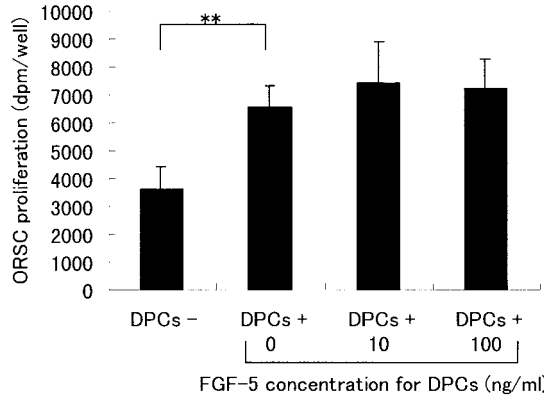

FIG. 2. Effects of FGF-5 on the capacity of DPC-conditioned medium to stimulate ORSC proliferation. Conditioned medium was collected from DPCs exposed to the indicated concentrations of FGF-5, and the proliferation of ORSCs cultured in that medium was measured. For comparison (control), ORSCs were cultured in standard culture medium. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by ORSCs in four wells; DPCs−, without conditioned medium of DPCs; DPCs+, with conditioned medium of DPCs; $^{**}P < 0.01$ (Student's *t* test).

Direct Effects of FGF-5 on the Proliferation of DPCs and ORSCs

FGF-5 dose-dependently stimulated DPC proliferation, with significant increases being achieved at concentrations of 100 or 1000 ng/ml (Fig. 3). In addition, 1000 ng/ml FGF-5 induced a small but significant increase in ORSC proliferation (Fig. 4).

Effects of FGF-1 and FGF-2 on the Capacity of DPC-Conditioned Medium to Stimulate ORSC Proliferation and the Interaction of FGF-5 with FGF-1 and FGF-2

FGF-1 strongly enhanced the ORSC proliferation seen in DPC-conditioned media, with significant in-

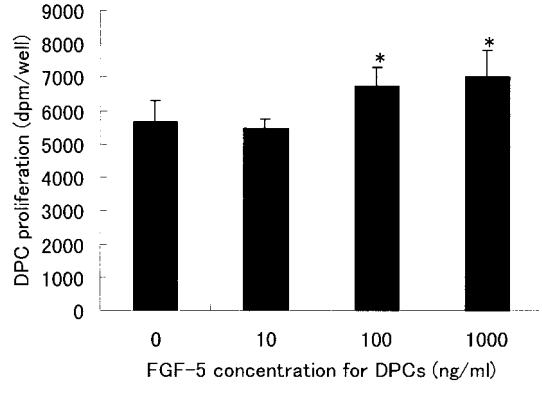

FIG. 3. Direct effects of FGF-5 on DPC proliferation. Proliferation of DPCs exposed to the indicated concentrations of FGF-5 was measured. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by DPCs in four wells; $^{*}P < 0.05$ vs culture without FGF-5 (Student's *t* test).

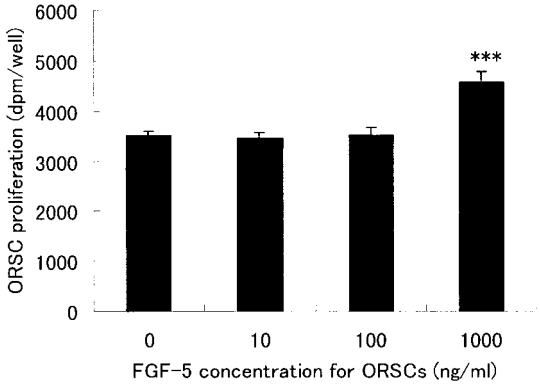

FIG. 4. Direct effects of FGF-5 on ORSC proliferation. Proliferation of ORSCs exposed to the indicated concentrations of FGF-5 was measured. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by ORSCs in four wells; ***$P < 0.001$ vs culture without FGF-5 (Student's *t* test).

creases being achieved at concentrations of 1 and 10 ng/ml (Fig. 5a). Moreover, the enhanced proliferation induced by 1 ng/ml FGF-1 was significantly attenuated by 10 ng/ml FGF-5, and although the effect was not statistically significant, FGF-5 also substantially inhibited proliferation stimulated by 10 ng/ml FGF-1. DPC-mediated ORSC proliferation was also enhanced by FGF-2; however, in this case, the enhancement was unaffected by FGF-5 (Fig. 5b).

In parallel experiments, we investigated the effects of FGF-1 and FGF-2 on proliferation of DPCs in the presence and absence of FGF-5. As shown in Fig. 6, DPC proliferation was enhanced by 10 ng/ml FGF-1 and this enhancement was significantly blocked by 10 ng/ml FGF-5. In contrast, DPC proliferation was unaffected by FGF-2. Addition of 10 ng/ml FGF-5 did not modulate DPC cell proliferation when FGF-1 or FGF-2 did not affect DPC proliferation.

Effect of Heparin on the Ability of FGF-1 and FGF-5 to Stimulate DPCs

Heparin enhanced the ability of 10 ng/ml FGF-1 to stimulate DPC proliferation (Fig. 7a). On the other hand, it inhibited both the DPC-mediated ORSC proliferation and the ability of FGF-1 to enhance the DPC activity (Fig. 7b). Heparin had no effect on FGF-5's ability to stimulate DPC and DPC-mediated ORSC proliferation (Fig. 7). Because heparin inhibited DPC-mediated facilitation of ORSC proliferation in the presence or absence of FGF-1, the effects of FGF-1 and FGF-2 on ORSC and DPC proliferation and their interaction with FGF-5 (Figs. 5 and 6) were evaluated in the absence of heparin.

Direct Effects of FGF-1 and FGF-5 on ORSC Proliferation

Finally, we investigated the direct effects of FGF-1 and FGF-5 on ORSC proliferation. By following the same procedure as the other experiments, when FGF-1, with or without FGF-5, was diluted in culture medium, incubated overnight at 37°C, and then added to the cells, the direct effects of FGF-1 and FGF-5 on ORSC proliferation were no longer apparent (Fig. 8). When either FGF-1 or FGF-5 was added directly to ORSCs, however, proliferation was enhanced (data not shown).

DISCUSSION

Simokawa *et al.* showed that HGF/SF (39) produced by DPCs in human hair stimulate ORSC proliferation, while Itami *et al.* (40) showed that, when exposed to androgen, human beard DPCs secrete IGF-I, stimulating ORSC proliferation. Thus, DPCs appear to synthesize and secrete growth factors that affect hair growth

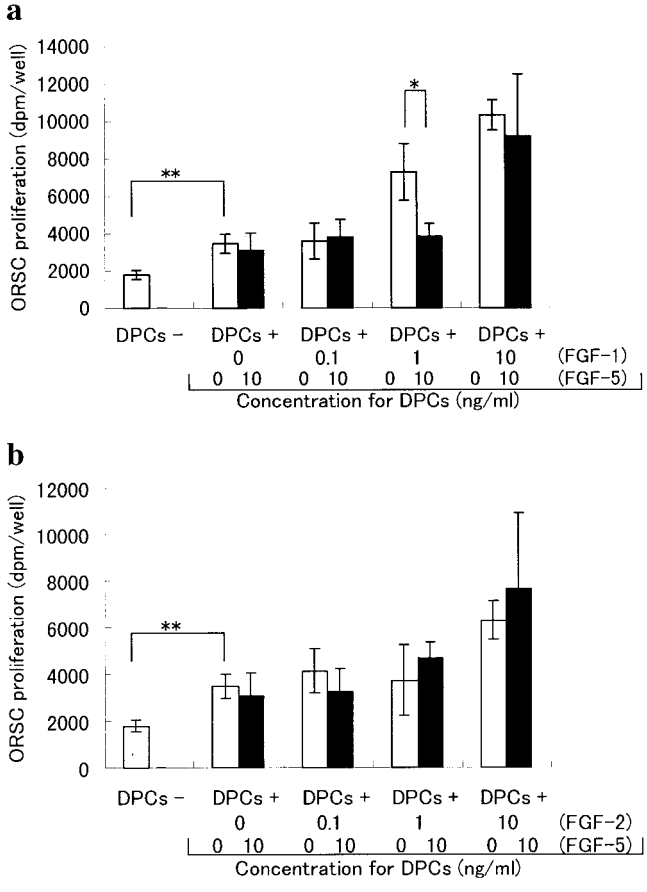

FIG. 5. Effects of FGF-1 and FGF-2 on the ability of DPC-conditioned media to stimulate ORSC proliferation and the interaction of FGF-5 with FGF-1 or FGF-2. ORSCs were cultured in medium conditioned by DPCs treated with the indicated concentrations of FGF-1 (a) or FGF-2 (b) in the presence or (black bars) absence (white bars) of 10 ng/ml FGF-5. Control ORSCs were cultured in the standard culture medium. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by ORSCs in four wells; DPCs−, without conditioned medium of DPCs; DPCs+, with conditioned medium of DPCs; *$P < 0.05$, **$P < 0.01$ (Student's *t* test).

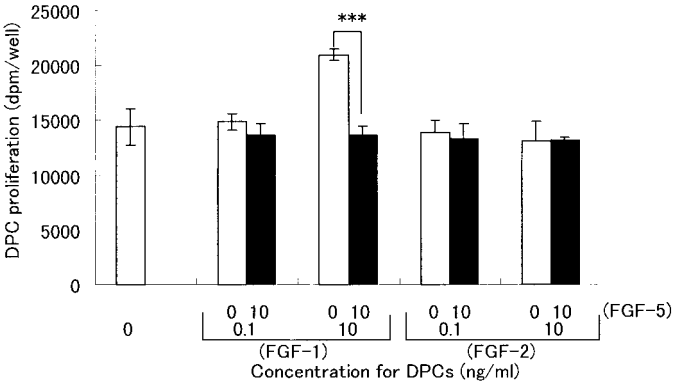

FIG. 6. Effects of FGF-1 and FGF-2 on DPC proliferation in the presence and absence of FGF-5. DPCs were exposed to the indicated concentrations of FGF-1 or FGF-2 in the presence (black bars) and absence (white bars) of 10 ng/ml FGF-5, after which DPC proliferation was assessed. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by ORSCs in four wells; $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

in vivo (38). In addition, we have shown that mouse FGF-5 inhibits hair growth and is associated with induction of catagen (37), and that during both catagen and telogen, macrophage-like cells containing FGF-5 migrate from the dermis to the panniculus adiposus, where the dermal papilla is located (42). In that regard, Resenquist and Martin showed that FGFR1, the high-affinity FGF-5 receptor (47), is expressed in dermal papillae and that DPCs are targets of FGF-5 (41). We therefore hypothesized that FGF-5 inhibits hair growth by suppressing production of growth factors in the dermal papillae. However, the findings of the present study indicate that, *in vitro,* FGF-5 does not reduce the capacity of DPCs to stimulate ORSC proliferation (Fig. 2), and that direct addition of FGF-5 to DPCs and ORSCs slightly enhances their proliferation (Figs. 3 and 4). How then does FGF-5 suppress hair growth and induce catagen (37) *in vivo*? To explain this discrepancy, we hypothesized that, *in vivo,* levels of growth factor produced by unstimulated DPCs are insufficient to induce hair growth, and that hair growth only occurs when higher levels are produced following activation of DPCs by one or more factors. We further suggested that FGF-5 attenuates the effects of such activators on DPCs. Since du Cros *et al.* (1) reported that FGF-1 and FGF-2 are produced in follicles during the growth phase, we tested this hypothesis by examining the effects of FGF-1 and FGF-2 in the presence and absence of FGF-5.

We found that FGF-1 and FGF-2 enhance DPC-mediated ORSC proliferation; that the effect of FGF-1 is blocked by FGF-5 (Fig. 5a); that enhancement of DPC proliferation by FGF-1 at higher concentrations is blocked by FGF-5 (Fig. 6); and that incubation of FGF-1 in medium in the absence of DPCs blocks its effect on ORSC proliferation (Fig. 8). Taken together, these findings suggest that the effects of FGF-1 on DPC-mediated ORSC proliferation is not due to induction of DPC proliferation, nor does FGF-1 stimulate ORSC proliferation directly. Instead it appears likely that, when DPCs are stimulated by an activator such as FGF-1, release of growth factors is enhanced, thereby inducing hair growth. FGF-5 in turn inhibits hair growth by suppressing the activation of DPCs by FGF-1.

It is known that heparin stabilizes FGF-1, enhances its biological activity, and increases its affinity for FGFR1 (43, 48). Consistent with those findings, we found that heparin enhances FGF-1's ability to stimulate DPC proliferation (Fig. 7a). On the other hand, heparin inhibited DPC-mediated ORSC proliferation and the ability of FGF-1 to enhance that effect. Thus, it is possible that DPC-mediated induction of ORSC pro-

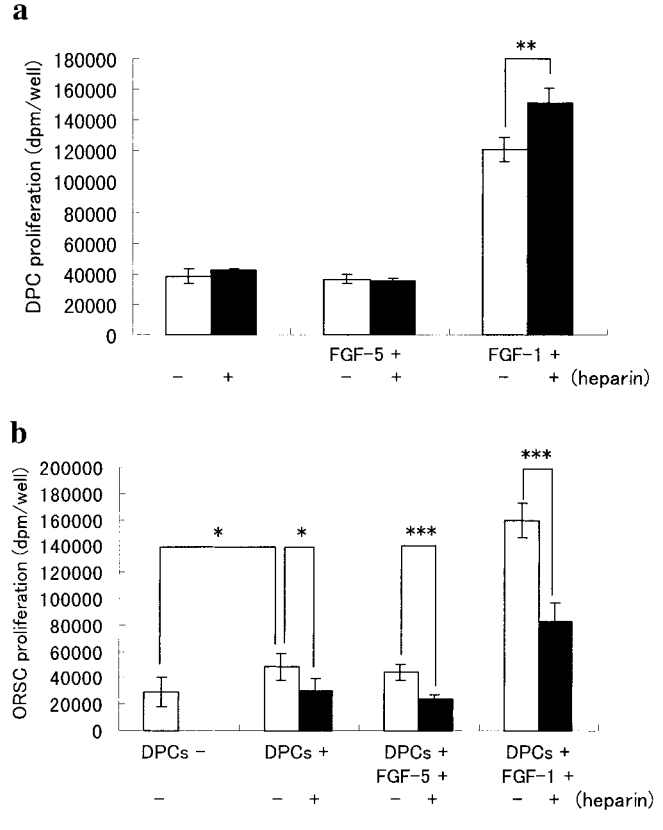

FIG. 7. Effect of heparin on the ability of FGF-1 and FGF-5 to stimulate DPCs. FGF-5 (10 ng/ml) or FGF-1 (10 ng/ml) was added to DPCs cultured with (white bars) or without (black bars) 5 μg/ml heparin. As a control, DPCs were also cultured with or without heparin in the absence of FGF. Standard ORSC medium was subsequently replaced with the DPC-conditioned medium, after which proliferation of DPCs (a) and ORSCs (b) was measured. Control ORSCs were cultured in the standard culture medium. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by the cells in four wells; DPCs−, without conditioned medium of DPCs; DPCs+, with conditioned medium of DPCs; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

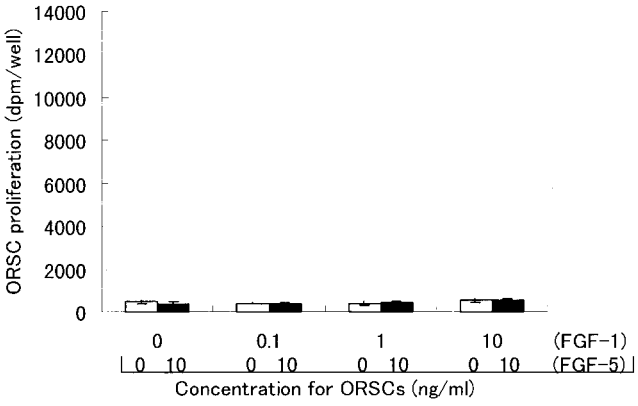

FIG. 8. Direct effects of FGF-1 and FGF-5 on ORSC proliferation. The indicated concentrations of FGF-1, alone (black bars) or in combination with 10 ng/ml FGF-5 (white bars), were added to culture medium and incubated overnight at 37°C. The resultant medium was added to ORSCs, and their proliferation was measured. Numerical data show means ± SD of [*methyl*-^{3}H]thymidine uptake by ORSCs in four wells.

liferation is likely achieved by a signaling molecule(s) that is inhibited by heparin.

FGF-1 has a high affinity for all four receptor isoforms identified in DPCs (Fig. 1) (49). It is therefore possible that FGF-5 interacts with FGF-1 by competitively inhibiting its binding to FGFR1. There is, however, no conclusive evidence that FGF-1 is the DPC activator involved in hair growth *in vivo*. Other proteins that may interact with DPCs and stimulate hair growth include EGF (50), bone morphogenic protein (BMP)-2 (51, 52) and FGF-7 (53). We also confirmed that, like FGF-1, FGF-2 enhances DPC-mediated ORSC proliferation, but in this case, the effect is unaltered by FGF-5 (Fig. 5b). This suggests FGF-2 activates DPC via a different receptor from FGF-5, indicating that some factors may promote hair growth via pathways not regulated by FGF-5.

FGF-7 is yet another FGF family protein known to stimulate hair growth (53). It is expressed in DPCs and activates ORSCs, but not DPCs. FGF-7 specifically binds to FGFR2 IIIb isoform, and as FGF-5 does not bind to this receptor, we suggest that FGF-7 is not involved in FGF-5-mediated regulation of hair growth. It is nevertheless possible that FGF-7 is one of factors whose expression is upregulated in DPCs by stimulation with FGF-1 or FGF-2. We have reported that administration of FGF-5 to mice induces catagen (37), which is associated with cessation of hair growth and induction of apoptosis in ORSCs (54). The FGF-5S, a short form of FGF-5 protein that is endogenously expressed in the hair follicle during mid-late anagen, abolished this catagen-inducing activity of FGF-5 possibly by inhibiting FGF-5 binding to its receptor (35, 37). FGF-5 may be directly involved in the induction of catagen, as suppression of DPC activators might directly activate apoptotic pathways in ORSCs of matured hair follicles. Alternatively or in addition, other proteins synthesized during catagen may bind to dermal papillae and interact with FGF-5 to activate apoptotic pathways. Either scenario is consistent with the idea that FGF-5 does not inhibit hair growth or induce catagen on its own, and provides further evidence that regulation of hair growth is more complicated than expected and involves multiple factors, including FGF-1 and FGF-5.

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