Bulge- and Basal Layer-Specific Expression of Fibroblast Growth Factor-13 (FHF-2) in Mouse Skin

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A variety of polypeptide growth factors are involved in the dynamic maintenance of the skin and hair. Here, we demonstrate the presence of high levels of fibroblast growth factor (FGF)-13 in the bulge region of hair follicles. Using real-time PCR, we found that expression of FGF-13 mRNA is comparable to, or higher than, that of other FGF known to regulate hair growth and wound healing. To gain additional insight into the function of FGF-13, we evaluated its distribution using *in situ* hybridization and immunohistochemical staining. Unlike other FGF, the distribution of FGF-13 mRNA and protein in adult mice was mainly restricted to cells in the bulge region of hair follicles, although lower levels were detected with less frequency in keratinocytes in the basal layer of the epidermis. FGF-13 protein was detectable in the bulge region throughout the hair growth cycle, but its distribution was especially wide during telogen and early anagen. During hair follicle morphogenesis in newborn mice, FGF-13 protein was first detected in the bulge region and basal layer keratinocytes 3 d after birth. These findings suggest that FGF-13 may play a role in regulating the function of cells in the bulge region and basal layer of the epidermis.

Key words: FGF-13/hair follicle/hair growth cycle/morphogenesis/stem
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The fibroblast growth factor (FGF) family of ligands is encoded by 22 distinct genes in mice and humans (Ornitz and Itoh, 2001), whereas four genes encode transmembrane FGF receptor (FGFR) tyrosine kinases (Jaye *et al*, 1992). Moreover, both the ligand and receptor gene products are subject to mRNA splicing variations and post-translational changes in their binding specificities and affinities. The result is a large group of ligands and receptors that, in combination, mediate a wide variety of biological activities. Among them, FGF-1, -2, -5, -7, -10, and -22 are known to be expressed in dermal and hair follicular cells and to regulate the hair growth and skin regeneration (Diana and DuCros, 1993; DuCros, 1993; DuCros *et al*, 1993; Hebert *et al*, 1994; Danilenko *et al*, 1995; Marchese *et al*, 1995; Guo *et al*, 1996; Petho-Schramm *et al*, 1996; Rosenquist and Martin, 1996; Mitsui *et al*, 1997; Ortega *et al*, 1998; Suzuki *et al*, 2000; Nakatake *et al*, 2001; Stenn and Paus, 2001; Beyer *et al*, 2003). FGF-7, -10, and -22 function in concert with the FGFR-2 IIIb to stimulate proliferation of keratinocyte in the normal and wounded skin (Werner *et al*, 1994; Marchese *et al*, 1995; Igarashi *et al*, 1998; Ohuchi *et al*, 2000; Beyer *et al*, 2003). Collectively, these studies clearly demonstrate the importance of the concerted activities of FGF in the skin. Yet the expression levels of these molecules in the skin have never been systemically compared or quantified. In this study, therefore, we established an experimental system with which to quantify levels of FGF mRNA expression. This approach enabled us to show for the first time that FGF-13 (FHF-2: Smallwood *et al*, 1996) is specifically expressed in the cells of the bulge region of hair follicles and in the basal layer of the epidermis, suggesting its possible role in regulating the function of those cells.

Abbreviations: FGF, fibroblast growth factor; FHF, FGF homologous factor; PBS, phosphate-buffered saline; SSC, standard saline citrate

Results

High levels of FGF-13 mRNA are expressed in murine skin Previous studies have shown that FGF-1, -2, -5, -7, -10, and -22 are all highly expressed in the skin and play important roles in the skin and/or hair dynamics (DuCros *et al*, 1993; Hebert *et al*, 1994; Danilenko *et al*, 1995; Guo *et al*, 1996; Rosenquist and Martin, 1996; Mitsui *et al*, 1997; Ortega *et al*, 1998; Nakatake *et al*, 2001; Beyer *et al*, 2003). Using RT-PCR, we discovered that FGF-13 mRNA is also expressed in the skin (Fig 1*A*, arrow). As expression of FGF-13 in the skin has not been studied previously, we first established an experimental system to quantitate FGF-13 expression and compare it with that of other FGF involved in hair growth and wound healing. This entailed designing a set of highly specific primers and using real-time PCR to measure the absolute copy number of the mRNA. In this analysis, cloned cDNA fragments served as references for copy number (Suzuki M, *et al*, unpublished data). We found that on day 8 of the hair cycle, the level of FGF-13 expression was high, about 1500 copies/20 ng total RNA, which was similar to the levels of FGF-5 (including two forms of FGF-5 mRNA; Ozawa *et al*, 1998), -7, -10, and -22, and higher than the levels of FGF-1 (or acidic FGF) and -2

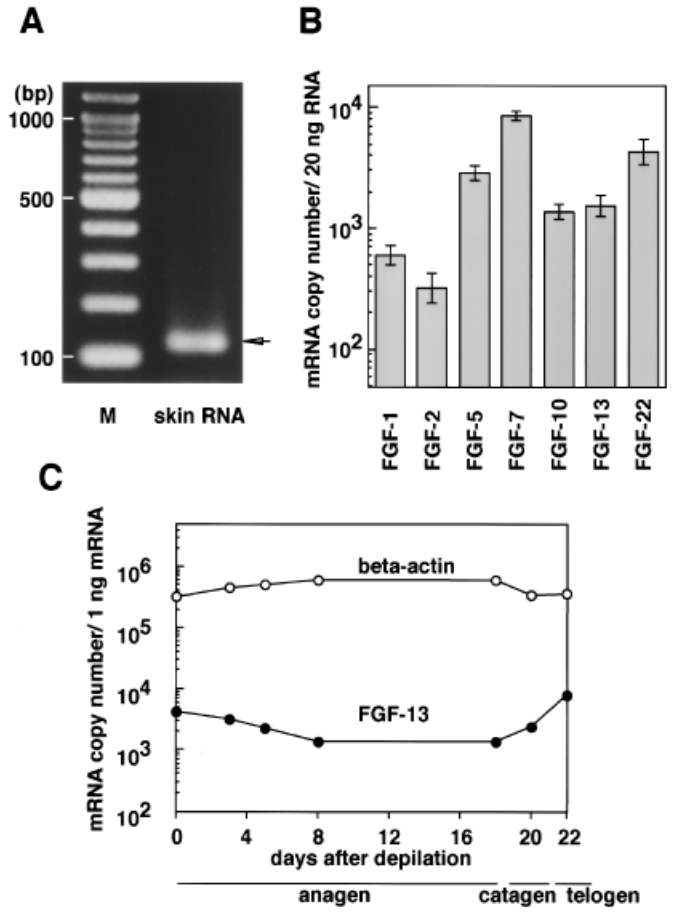


Figure 1
High levels of FGF-13 mRNA are expressed in mouse skin. (*A*) A portion of the dorsal skin of five C3H/HeN male mice was depilated during telogen (at 8 wk of age). Eight days later (anagen V phase of the hair cycle), the skin samples were harvested and total RNA was extracted. After reverse transcription using $(dT)_{12-18}$ as a primer, the cDNA pool was amplified with specific primers for FGF-13. The PCR reaction product was run on an agarose gel and photographed; M, 100-bp ladder markers. The arrow indicates the amplified fragment of FGF-13 cDNA. (*B*) The cDNA from the skin sample in panel A was amplified with specific primers for various FGF members. With the aid of serial dilutions of standard plasmids, absolute copy numbers for each FGF were calculated. Bars represent mean ± SD of results obtained from five mice. (*C*) A portion of the dorsal skin of C3H/HeN male mice was depilated during telogen (at 8 wk of age) and then at selected times thereafter (various stages of the hair cycle), the skin samples were harvested. Total RNA was extracted and further purified to mRNA, after which reverse transcription and quantification of the FGF-13 mRNA was carried out as in panel B.

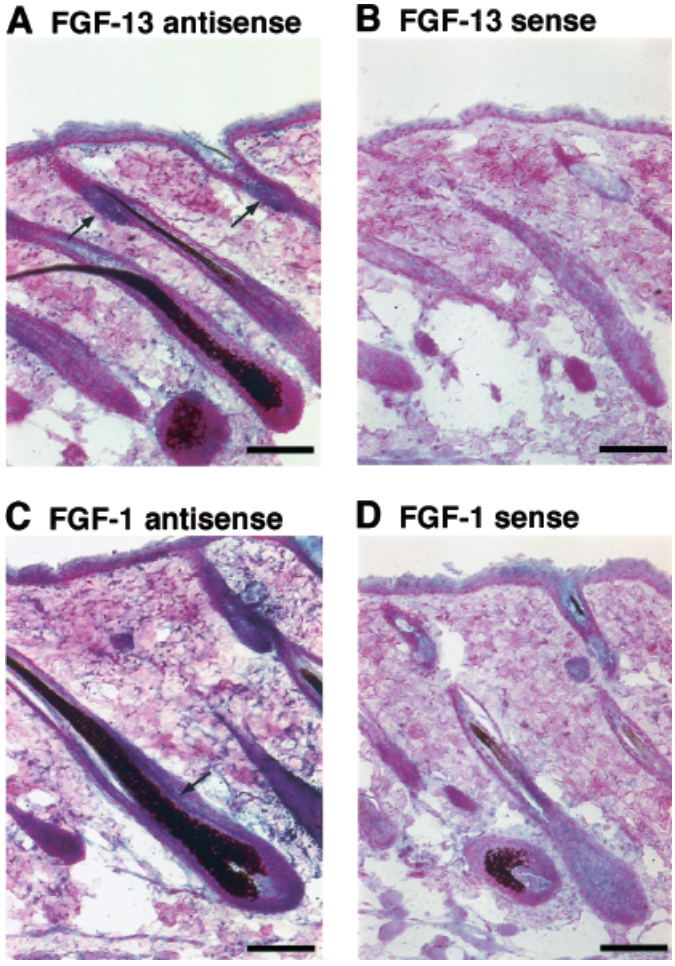


Figure 2
Localization of FGF-13 mRNA in the bulge region of the anagen hair follicle. A portion of the dorsal skin of C3H/HeN mice was depilated during telogen phase (8-wk old), and 8-d later skin samples were isolated from the same area, frozen and sectioned. *In situ* hybridization was carried out using digoxygenin-labeled antisense riboprobes specific for FGF-13 (*A*) or FGF-1 (*C*); the arrows indicate the respective signals. As a control, the respective sense probes (*B* and *D*, respectively) were also prepared and used for hybridization. The bound riboprobe was detected with anti-digoxygenin antibody conjugated with alkaline-phosphatase; the signals were visualized using BM Purple (purple signals). Bars, 100 μm.

(or basic FGF) (Fig 1*B*). Moreover, using RT-PCR to quantify FGF-13 mRNA expression over the course of the hair cycle, we found that the transcript was most abundant during telogen (days 0 and 22) and early anagen (days 3 and 5) and less so during mid-late anagen (days 8 and 18) (Fig 1*C*).

***In situ* hybridization shows bulge- and basal layer-specific expression of FGF-13 mRNA** To gain additional insight into the function of FGF-13 in murine skin, we next determined its distribution using *in situ* hybridization and immunohistochemical analysis. Although the focus of this study was on FGF-13, for comparison we also examined the expression of FGF-1 mRNA, which has been well characterized (DuCros *et al,* 1993). In mid-anagen (anagen V; day 8 after dipilation), FGF-13 mRNA expression was largely limited to the bulge region of hair follicles (Fig 2*A*; arrows). This expression pattern was clearly different from that of FGF-1 (Fig 2*C*). As previously reported (DuCros *et al*, 1993), FGF-1 was much more widely distributed in the hair bulb, especially in the inner root sheath cells (Fig 2*C*; arrow) and in dermal papilla. The sense probes produced no signal (Fig 2*B* and *D*).

Specificity of the immunohistochemical staining of FGF-13 To analyze the distribution of FGF-13 protein in the skin, we initially verified the specificity of the anti-FGF-13 antibody used for immunohistochemical staining. Mid-anagen skin (day 8 after dipilation) was used for this purpose because each portion of the hair follicle—that is, the permanent portion, bulge region, and transient portion—can be readily resolved at this stage. Consistent with the *in situ* hybridization, FGF-13 protein was detected

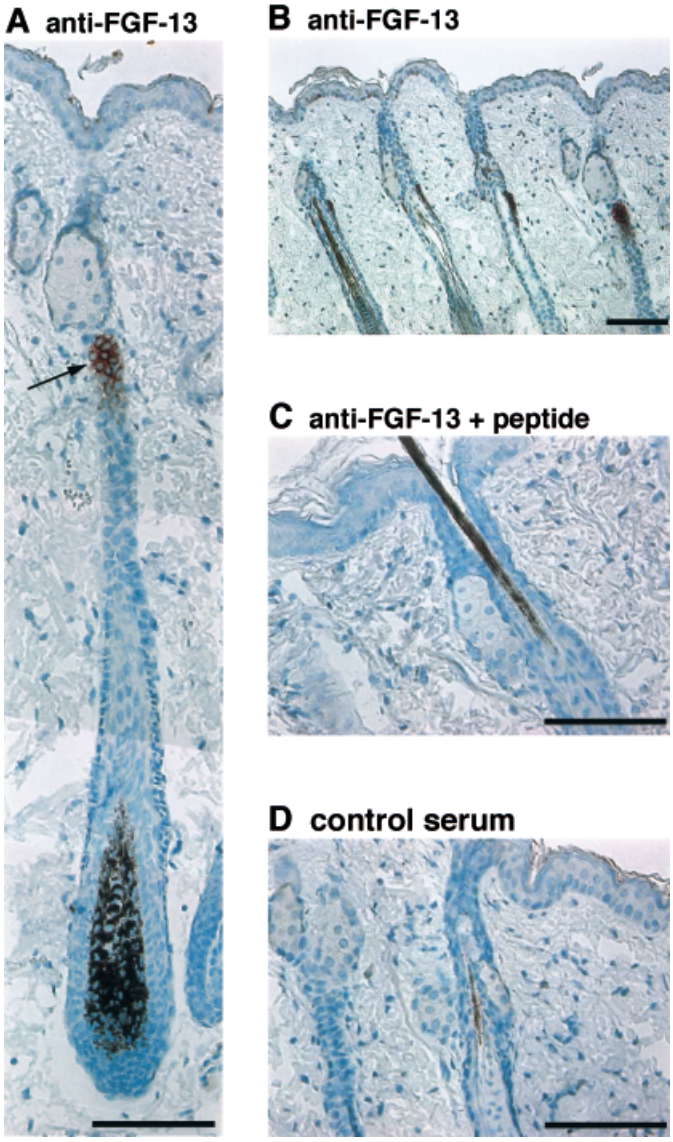


Figure 3
Specificity of the anti-FGF-13 antibody used for immunohistochemical staining. (*A*, *B*) To examine the region- and epitope-specific labeling of FGF-13, skin samples harvested from five C3H/HeN mice during the anagen V phase of the hair cycle (day 8 after dipilation) were fixed in formalin, embedded in paraffin, sliced into 4-μm sections, and labeled with the rabbit anti-FGF-13 antibody (diluted 1:1000) generated as described in Materials and Methods. The bound antibody was detected with biotinylated goat anti-rabbit IgG (diluted 1:100), and the signals were visualized using a HISTOSTAIN-SP kit (brown red signals). The sections were counterstained with hematoxylin. (*A*) Representative labeling of FGF-13 protein in the entire hair follicle at anagen V (day 8). The image is a composite of three micrographs taken at high magnification (× 40 objective lens). (*B*) Labeling of FGF-13 protein around the bulge region. (*C*) Labeling in the presence of excess epitope peptide. This skin section was incubated with rabbit anti-FGF-13 in the presence of excess epitope peptide (approx. 10,000-fold molar concentration) and processed as in panel A. (*D*) Control labeling. This skin section was incubated with normal rabbit serum and processed as in the other panels. Bars, 100 μm.

mainly in the bulge region of the hair follicles (Fig 3*A*, arrow and *B*). No labeling was observed in the hair bulb, dermal papilla, inner root sheath, or general outer root sheath, except in the bulge region (Fig 3*A*). The intense labeling in the bulge region was confirmed to be specific for FGF-13, as it was not observed when sections were stained with the same antibody in the presence of excess (approx. 10,000-fold molar concentration) epitope peptide (Fig 3*C*), or when non-immune (normal) rabbit serum was used as the primary antibody (Fig 3*D*).

Immunohistochemical staining shows bulge- and basal layer-specific expression of FGF-13 protein throughout the hair cycle in adult mice Once the specificity of the antibody was confirmed, we examined the distribution of FGF-13 protein at selected stages during the hair cycle in adult mice, that is, anagen II, III, V, and VI (days 3, 5, 8, and 18 after depilation, respectively), catagen IV (day 20 after dipilation), and telogen (days 0 and 22 after depilation). Interestingly, we found it to be present throughout the cycle, mainly in the bulge region (Fig 4*A–I*). Especially wide distributions of staining were observed during telogen (day 0, Fig 4*A*; day 22, Fig 4*G* and *H*) and early anagen (day 3, Fig 4*B* and *I*; day 5; Fig 4*C*); during telogen, cells located at the bottom part of the retarded hair follicles—that is, in the bulge region—were intensely stained for FGF-13. Further examination of the day-22 skin revealed FGF-13-positive cells to be distributed in the inner and outer parts of the bulge region during telogen (Fig 4*H*).

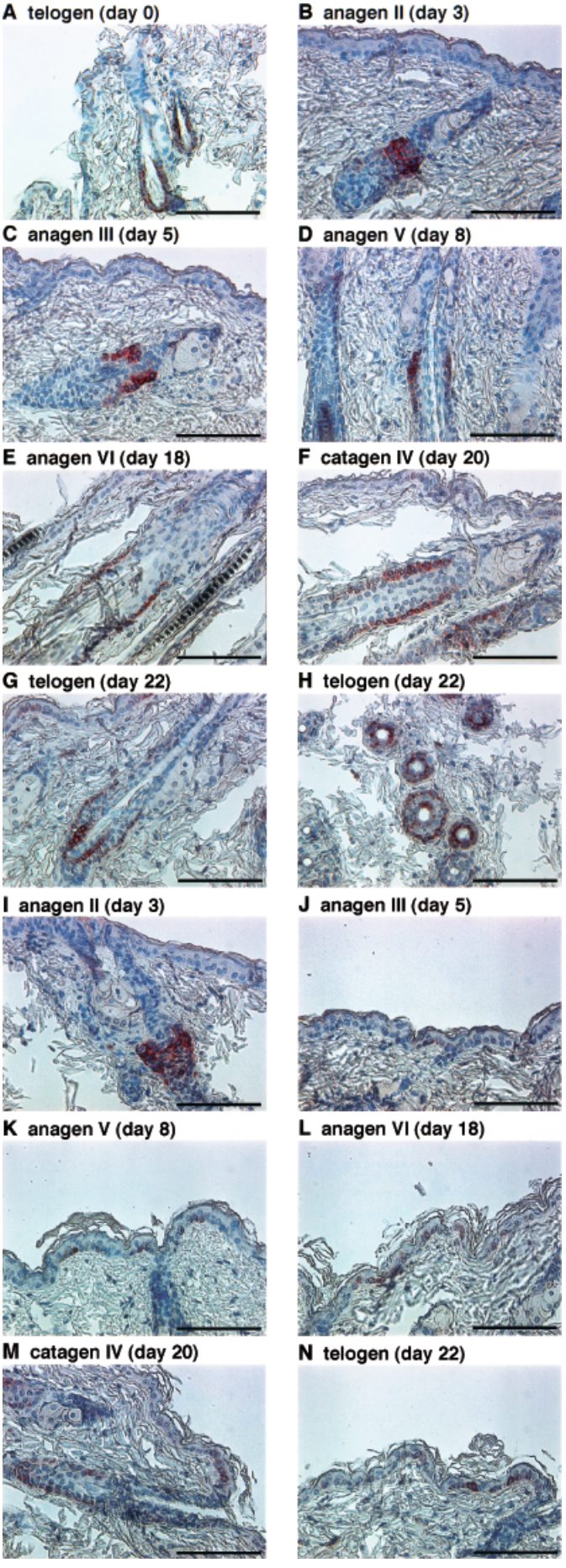
We found that some cells in the basal layer of the epidermis also harbored the protein (Fig 4*J–N*), although the signal was weaker and appeared less frequently than in the bulge region.

Expression of FGF-13 protein during hair follicle morphogenesis in newborn mice Finally, we examined expression of FGF-13 protein during hair follicle morphogenesis in 1-, 3-, 7-, 13-, 16-, and 18-d-old mice (Fig 5). Little or no FGF-13 was detected in the skin of 1-d-old mice (hair follicles of stages 3–5; Paus *et al*, 1999) (Fig 5*A*), and only weak labeling of FGF-13 was detected in the bulge region of 3 (stages 4–8)- and 7 (stage 8 and later)-d-old mice (Fig 5*B* and *C*). By day 13 and later (days 13, 16, and 18), however, the labeling of FGF-13 had become very intense in both the bulge region and basal layer of the epidermis (Fig 5*D–F*). Moreover, we noted that the distribution of FGF-13-positive cells in the basal layer of the epidermis was more apparent in newborn mice than in adult mice.

Discussion

We have shown that throughout the hair cycle of adult mice, FGF-13 is specifically distributed in the bulge region of the follicle and the basal layer of the epidermis. During hair follicle morphogenesis in newborn mice, the presence of FGF-13 protein in these regions was first detected 3 d after birth (stages 4–8) and became apparent about 7 d after birth (stage 8 and later). Although characterization of FGF-13's activity in the skin has not been completed, these findings suggest that FGF-13 may play a role in regulating the function of the cells in the bulge region and in the basal layer of the epidermis.

Although there have been studies of the expression and activity of other FGF in dermal cells and hair follicles (DuCros *et al*, 1993; Hebert *et al*, 1994; Danilenko *et al*, 1995; Guo *et al*, 1996; Rosenquist and Martin, 1996; Mitsui



et al, 1997; Ortega *et al*, 1998; Nakatake *et al*, 2001; Beyer *et al*, 2003), ours is the first description of FGF-13 expression in the skin.

Within the skin, FGF-1 is expressed in inner root sheath cells and other follicular cells and appears to induce cells to secrete growth promoting factors for outer root sheath cells (Ota *et al*, 2002). FGF-2 is also expressed in inner root sheath cells and stimulates the proliferation of hair follicle cells (Mitsui *et al*, 1997); moreover, during wound healing it stimulates proliferation of dermal fibroblasts (Ortega *et al*, 1998). Both translation products of *Fgf-5* gene, FGF-5 and FGF-5S (Ozawa *et al*, 1998), play important roles in the regulation of the anagen phase and the onset of the catagen phase during the hair cycle (Suzuki *et al*, 1998, 2000). FGF-7, -10, and -22 are specific ligands for a keratinocyte-specific form of FGFR (FGFR2 IIIb) and stimulate keratinocyte proliferation for wound healing (Guo *et al*, 1996; Beyer *et al*, 2003). Thus, by acting on different cell types under specific sets of conditions, FGF appear to regulate the hair/skin system dynamically.

The function of FGF-13 in the bulge region and in the basal layer of the epidermis is not yet known. As its expression was not observed in the temporary portion of the anagen hair follicle, FGF-13 may play a role in the regulation of keratinocytes committed to the epidermis. If so, FGF-13 may be a useful marker of epidermal keratinocyte stem cells and their progeny (Taylor *et al*, 2000). During embryonic organogenesis, FGF-13 is known to be involved in development of the nervous system and heart, where its expression pattern suggests that it performs functions distinct from other known FGF (Hartung *et al*, 1997). FGF-13 is also expressed in the brains and hearts of adults, but the levels are significantly lower than in other tissues (e.g., the kidney and ovary) (Hartung *et al*, 1997), which suggests that expression may correlate with stem cell activity in the expressing organ.

As a member of FGF family, FGF-13 may exert its effects through actions on one or more of the FGFR tyrosine kinases. At present, however, the binding specificity of FGF-13 is not known. Alternatively, it is also possible that FGF-13 acts via as yet uncharacterized pathways—for example, by recruiting intracellular binding proteins (Smallwood *et al*, 1996; Schoorlemmer and Goldfarb, 2001), or by binding to scaffold proteins, as do FGF-12 (or FHF-1) and FGF-14 (or FHF-4) (Olsen *et al*, 2003); like FGF-1, -2, -11, -12, and -14; FGF-13 lacks a secretion signal peptide. Finally, FGF-13 may be inefficiently exported from cells, despite the

Figure 4
Localization of FGF-13 protein in adult mice during various stages of the hair cycle. Skin samples from C3H/HeN mice harvested at various stages of the hair cycle were fixed in formalin, embedded in paraffin, cut into 4-μm-thick sections, and analyzed for FGF-13 protein as in this figure. Five mice were examined at each stage, and representative results are shown. (*A–I*) Hair follicles: (*A*) telogen skin (day 0); (*B*) anagen II skin (day 3); (*C*) anagen III skin (day 5); (*D*) anagen V skin (day 8); (*E*) anagen VI skin (day 18); (*F*) catagen IV skin (day 20). (*G*, *H*) Telogen skin (day 22): (*G*) the open arrow indicates FGF-13-positive cells located between the bulge region and the epidermis; (*H*) hair follicles were cut into round slices and immunostained for FGF-13. (*I–N*) Epidermis: (*I*) anagen II skin (day 3); (*J*) anagen III skin (day 5); (*K*) anagen V skin (day 8); (*L*) anagen VI skin (day 18); (*M*) catagen IV skin (day 20); (*N*) telogen skin (day 22). Bars, 100 μm.

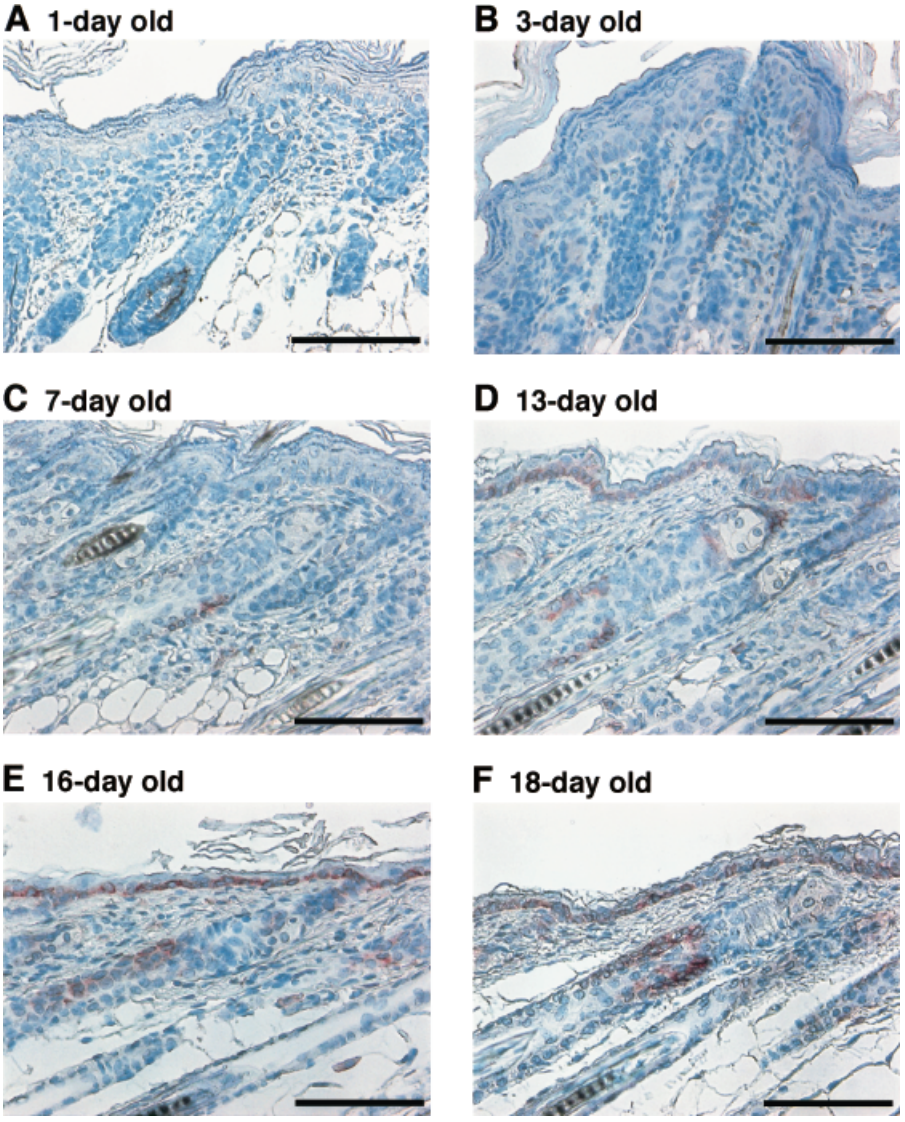


Figure 5
Localization of FGF-13 protein in newborn mice during hair follicle morphogenesis. Dorsal skin samples from C3H/HeN newborn mice were harvested on days 1, 3, 7, 13, 16, and 18 after birth. The samples were processed and analyzed for FGF-13 protein as in Fig 3. Representative distribution of FGF-13 protein in 1-d (*A*: stage 3–5)-, 3-d (*B*: stage 4–8)-, 7-d (*C*: stage 8 and later)-, 13-d (*D*)-, 16-d (*E*)-, and 18-d (*F*)-old skin. Bars, 100 μm.

absence of a signal peptide, in a manner similar to FGF-1 (Landriscina *et al*, 2001) or FGF-2. In any case, it appears to us that the likely targets of FGF-13 are the expressing cells themselves or those nearby. All these questions await further investigation.

Materials and Methods

Mice and skin sample preparation Throughout this study, all animals received humane care that was in accordance with the guidelines of AIST. All protocols were approved by the animal experiment committee of AIST. For hair cycle experiments, 35 7-wk-old male C3H/HeN mice (*Tlr4*$^{+/+}$) were obtained from Japan SLC (Hamamatsu, Japan) and maintained on a standard laboratory diet and water *ad libitum*. After conditioning for 1 wk, five mice were euthanized, the dorsal hair was cut short, and the skin samples were isolated and examined as the "day 0 (telogen)" sample. In the remaining 30 mice, the anagen phase of the hair growth cycle was induced by gently depilating their dorsal hair shafts manually without using wax or dipilatory chemicals. Then, at selected stages of hair follicle transformation—that is, on days 3 (anagen II), 5 (anagen III), 8 (anagen V), 18 (anagen VI), 20 (catagen IV), and 22 (telogen) after depilation—the mice were euthanized, and the skin samples were isolated from the depilated area and processed for either mRNA isolation, *in situ* hybridization, or immunohistochemical staining. Five mice were examined for each stage of hair follicle transformation. The experiment was then carried out once again using essentially the same design to confirm the reproducibility of the results.

For hair morphogenesis studies, the dorsal skin samples were isolated from untreated newborn C3H/HeN mice (male and female) on days 1 (stages 3–5), 3 (stages 4–8), 7 (stage 8 and later), 13, 16, and 18 after birth, examined and processed for immunohistochemical staining. At least five mice were examined at each stage of hair morphogenesis. For *in situ* hybridization, the skin samples were immediately frozen in optimal cutting temperature (OCT) compound using liquid nitrogen. For immunohistochemical staining, the samples were first fixed for 24 h at room temperature in 10% formaldehyde and then embedded in paraffin using standard procedures (Suzuki *et al*, 1998).

mRNA preparation and reverse transcription Total RNA was prepared from the skin samples using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. In some cases, the mRNA was purified from the total RNA using an Oligotex-dT30 Super mRNA purification kit (Takara Bio Inc., Osaka, Japan). Samples of the isolated RNA (2 μg of total RNA or 100 ng of mRNA) were then reverse-transcribed in a total volume of 20 μL

using SuperScript II with Oligo $(dT)_{12-18}$ as a primer (Gibco BRL, Tokyo, Japan).

Design of PCR primers and cloning of cDNAs for FGF-13 and other FGF family members Specific primer sets were designed so that the levels of FGF mRNA could be quantitatively analyzed using real-time PCR. The same sets of primers were also used for cloning respective cDNA fragments for use as copy number references in the quantification. The primers used for FGF-13 amplification were 5′-GGCAATGAACAGCGAGGGATACTTGTACAC-3′ (sense) and 5′-CGGATTGCTGCTGACGGTAGATCATTGATG-3′ (antisense). The RT mixture was diluted 10 times with dH_2O, after which PCR amplification was carried out using Pfu polymerase according to the manufacturer's (Stratagene, La Jolla, California, USA) instructions. After PCR, aliquots of the reaction solution were run on 2.0% agarose gels to confirm the size of the amplified products. To clone the respective cDNAs, the PCR products were ligated into pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, California, USA), which was then used to transform *Escherichia coli*. The nucleotide sequences of the recombinant plasmids were verified using a BigDye terminator cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster city, California, USA).

Quantification of mRNA copy number by real-time PCR Real-time PCR amplification was carried out in a Light Cycler (Roche Diagnostics, Basel, Switzerland). Two μL of the 1:10 diluted RT product was used as a template. To obtain the absolute copy numbers of each FGF, plasmid DNAs for each FGF were used for copy number references. After the quantification procedure, the yielded products were resolved by 2.0% agarose gel electrophoresis to confirm that the reaction had amplified the right DNA fragments.

***In situ* hybridization** The *in situ* hybridization experiments were performed using OCT-embedded frozen tissue sections and digoxygenin-labeled riboprobes essentially as described previously (Komminoth, 1992). Sense and antisense riboprobes were synthesized using SP6 and T7 RNA polymerases, respectively, in the presence of digoxygenin-dUTP (Roche Applied Science, Mannheim, Germany).

All possible effort was made to avoid RNA degradation during the hybridization procedure. The frozen tissues were cut into 10-μm sections using a Cryostat (Microm, model HM500OM Microm International GmbH, Walldorf, Germany), immediately placed on slide glasses and dried at 50°C for 1 h, and then stored at −80°C with a desiccant. Before hybridization, sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and then washed with PBS for 5 min. Thereafter, the sections were permeabilized with proteinase K (0.01 μg per mL in × 2 TE 10 mM Tris-Hcl, 1 mM EDTA, pH8.0 (TE)) for 5 min at 37°C, rinsed with PBS, again fixed with 4% paraformaldehyde in PBS for 5 min, and washed with PBS for 5 min. The fixed sections were methylated by soaking them twice in 0.1% diethylpyrocarbonate in PBS for 15 min each, rinsed with PBS, and soaked in × 5 saline-sodium citrate (SSC) for 15 min. Finally, hybridization was carried out for 16–18 h at 58°C using 500 ng per mL of heat-denatured (70°C for 10 min) probe in 100 μL of hybridization buffer consisting of 50% (vol/vol) formamide, × 5 SSC, 500 μg per mL salmon testes DNA, 250 μg per mL t-RNA, × 5 Denhardt's solution and 1 mM dithiothreitol (DTT). After hybridization, the slides were rinsed and washed with × 2 SSC for 30 min at room temperature, with × 2 SSC at 65°C for 20 min, and then three times with × 0.1 SSC at 65°C for 20 min. The bound riboprobe was detected with anti-digoxygenin antibody conjugated with alkaline-phosphatase (1:500, Roche). For visualization, a colorimetric reaction with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (NBT/BCIP) was carried out using BM Purple (Roche) for 72–75 h at room temperature in the dark.

Immunohistochemical staining Immunohistochemical analysis was carried out using paraffin-embedded tissue sections with anti-FGF-13 antibody. The antibody was generated in rabbits injected with a mouse FGF-13 fragment conjugated with Keyhole Limpet hemocyanin (KLH). After evaluating the titer of this antibody using an enzyme-linked immunosolvent assay (results not shown), we confirmed the specificity by immunoblotting. We found that this antibody specifically recognized FGF-13 among a large number of unrelated proteins also present in the *in vitro* translation mixture (see Fig 6).

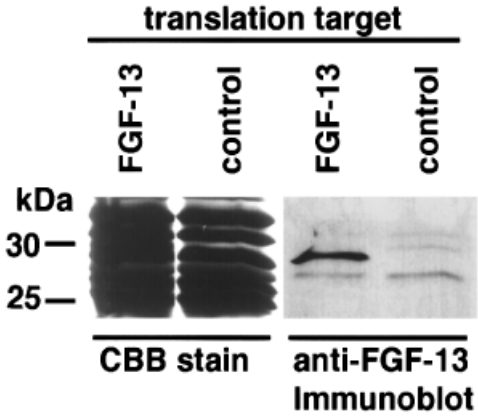


Figure 6
Specificity of the anti-FGF-13 antibody. *In vitro* translation of FGF-13 was carried out. As a control, the reaction was run without target template DNA. After termination of the reaction, the mixture, which contained a variety of proteins involved in transcription/translation, was resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were either stained with CBB dye, or further transferred to a nitrocellulose membrane, and immunoblotted with the anti-FGF-13 antibody. The signal was visualized using ECL-western blotting detection reagents.

Formalin-fixed mouse skin samples were sliced into 4-μm sections, placed on slide glasses, and deparaffinized. The skin sections were then subjected to antigen retrieval by soaking them for 2 min in boiled 0.01 M citrate adjusted to pH 6.0, after which they were blocked with 10% normal goat serum and incubated with anti-FGF-13 rabbit antiserum (1:1000 dilution) or the same dilution of non-immune rabbit serum for 2 h at room temperature. After washing three times with PBS for 2 min with shaking, the sections were incubated with biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA; 1:100 dilution) for 10 min at room temperature, and the signals were visualized using a HISTOSTAIN-SP kit according to the manufacturer's (Zymed Laboratories Inc, San Francisco, California, USA) instructions. Aminoethylcalbazole (red) served as the chromogen; the sections were counterstained with hematoxylin (blue). In each experiment, we stained at least five slides for every stage of the hair cycle. The experiments were repeated at least twice to confirm that the results were reproducible.

***In vitro* translation and immunoblotting** *In vitro* translation of FGF-13 was carried out using a Puresystem Classic Mini kit (Post Genome Institute, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the template cDNA to be translated was prepared by subjecting it to PCR using a protocol that incorporated the T7 promoter. It was then translated in a reaction mixture containing all the factors necessary for transcription/translation. As a control, the reaction was carried out without a template. After termination of the reaction, the mixture was resolved by SDS-15% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blotted with the anti-FGF-13 antiserum (1:500 dilution). The signal was visualized using enhanced chemiluminescence (ECL)-western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and X-O mat XB-1 film (Kodak, Rochester, New York, USA).

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