Defective terminal differentiation and hypoplasia of the epidermis in mice lacking the *Fgf10* gene

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Abstract Here, we characterized the skin and hair phenotype of mice lacking the fibroblast growth factor 10 gene (*Fgf10*), a newly identified member of the fibroblast growth factor family. Histological examination of *Fgf10*$^{-/-}$ newborn mouse skin revealed abnormalities in epidermal morphogenesis. The number of proliferating cells in the basal layer was decreased, the granular layer was hypoplastic and lacked distinctive keratohyaline granules and tonofibrils. The expression of loricrin, a marker of epidermal differentiation, was dramatically reduced. Despite the presence of *Fgf10* transcripts in normal hair follicles, abnormalities of hair development were not observed in *Fgf10*$^{-/-}$ skin. These data suggest that *Fgf10* is required for embryonic epidermal morphogenesis but is not essential for hair follicle development.

Key words: Fibroblast growth factor 10; Loricrin; Gene knockout mouse; Skin; Hair follicle

1. Introduction

The fibroblast growth factor (Fgf) family is comprised of more than 20 structurally related polypeptides that have been implicated in multiple aspects of vertebrate embryonic development and adult tissue homeostasis, including wound healing and tumorigenesis.

The fibroblast growth factor 10 gene, *Fgf10*, originally identified based on sequence homology with *Fgf3* and *Fgf7*, is expressed in several embryonic organs, including lungs, limbs, posterior pituitary, duodenum and spinal cord [1]. In adult tissues, *Fgf10* transcripts are found in the lung, heart [1] and the dermal component of mouse skin [2]. The FGF10 protein initiates outgrowth of limb buds [3], and induces branching morphogenesis of embryonic mouse lung [4]. Two studies found that *Fgf10*$^{-/-}$ mice died at birth, primarily due to defective lung development suggesting that *Fgf10* functions as an essential regulator during organogenesis [5,6].

Among the known Fgf family members, *Fgf10* is most similar to *Fgf7* (keratinocyte growth factor) [7], which is expressed in the dermis [8] and hair follicles [9]. While an important role of FGF7 in mediating the initiation and acceleration of wound healing was suggested [10], *Fgf7*$^{-/-}$ mice have no obvious cutaneous abnormality [11].

Recently, human *Fgf10* was shown to be expressed in the dermal papilla, outer root sheath cells and epidermal keratinocytes [12]. Recombinant FGF10 protein is mitogenic for fetal rat keratinizing epidermal cells, but has essentially no mitogenic activity for established fibroblasts cell lines such as NIH/3T3 [13]. Moreover, it has been shown that the induction of *Fgf10* expression during wound healing is more prominent than that of *Fgf7* [14]. During skin morphogenesis, however, the physiological role of *Fgf10* remains to be elucidated. In this study, we characterized the skin and hair phenotype of mice lacking *Fgf10*.

2. Materials and methods

2.1. Animals

The *Fgf10*$^{-/-}$ mice and genotyping have been previously described [6].

2.2. Histological analysis and immunostaining

Newborn mice were sacrificed and 6-μm-thick sections of the fixed skin, for a total of 10 skin specimens, were stained with hematoxylin/eosin (H&E). Frozen skin sections from 18.5 days post coitum (dpc) were stained with optimal dilutions of antibodies against transglutaminase 1 (kindly provided by Dr. Takashi Hiiragi [15]), loricrin (Babco, Richmond, CA, USA) and cytokeratin 14 (K14; Cymbus Bioscience, Southampton, UK). Paraffin sections of 16.5 dpc embryonic skins were stained with rabbit anti-mouse FGF10 antibodies (sc-7917, R&D systems, CA, USA). Proliferating cells were detected using an anti-proliferating cell nuclear antigen (PCNA) (Dako Japan Co., Japan).

2.3. In situ hybridization for gene expression

In situ hybridization with digoxigenin-labeled probes was performed as described [16]. The following probes were used: *Fgf10* (kindly provided by H. Ohuchi and N. Itoh), *Fgf2* (kindly provided by G. Martin) and *Fgf7* (Riken Institute). Sense probes were used as negative controls.

2.4. Transplantation of full-thickness *Fgf10*$^{-/-}$ skin grafts onto nude mice

1.5 cm^2 full-thickness skin grafts were isolated from 18.5 dpc fetal mice and transplanted on adult BALB/c nu/nu mice (Japan SLC). Subcutaneous tissue was removed from donor trunk skin. Recipient mice were anesthetized and trunk skin equivalent to the size of the graft was excised, sparing the panniculus carnosus. Two months after the transplantation, incisional biopsies were performed under general

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Abbreviations: Fgf10, fibroblast growth factor 10; KHG, keratohyaline granule

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anesthesia. All experimental procedures were undertaken in accordance with the Guideline for Animal Experiment, Faculty of Medicine, Kumamoto University, Japan.

2.5. Electron microscopy

For transmission electron microscopy, the back skin from wild-type and mutant mice were fixed by standard procedures, dehydrated and embedded in Epon 812. Sections were double-stained with uranyl acetate and lead citrate, and examined with an Hitachi JEM-100S electron microscope. For scanning electron microscopy (SEM), hair specimens were removed from back skin and briefly washed in phosphate-buffered saline before fixing in 1% osmium tetroxide in 0.1 M cacodylate buffer. The dried samples were coated with gold, and examined with an Hitachi S-800 scanning electron microscope.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from wild-type and *Fgf10*$^{-/-}$ mice back skin at 18.5 dpc by standard procedures. RT-PCR was carried out using a One-Step RT-PCR System (Gibco BRL). The following primers were used (amplified length shown as bp): *Fgf7* 5′ GTGAGAAGACTGTTCTGTCG, *Fgf7* 3′ GCCATAGGAAGAAAATGGGC (380 bp), *Fgf2* 5′ AACGGCGGCTTCTTCCTGCG, *Fgf2* 3′ GCCCAGTTCGTTTCAGTGCC (289 bp), *Fgfr2* 5′ CCAGAAGAGCCACCAACCAAATACC, *Fgfr2* 3′ GACAAACTCCACATCCCCTCCG (729 bp), *Skn-1a* (*Oct-11*) 5′ GCTTCTCTCCATCCACTCCAGC, *Skn-1a* (*Oct-11*) 3′ CTCACCACCTCCTTCTCCATCG (713bp), *Shh* 5′ GACGGCCATCATTCAGAGGAG, *Shh* 3′ AGGAAGGTGAGGAAGTCGCTG (335 bp), *PDGF-A* 5′ GGCTCGAAGTCAGATCCACAGC, *PDGF-A* 3′ CCTCTAACCTCACCTGGACCTC (417 bp), *TGF-β2* 5′ CTTAACATCTCCCACCCAGCGC, *TGF-β2* 3′ GCAGTAGGCAGCATCCAAAGCG (400 bp).

Each primer set flanked at least one intron to discriminate the contamination with genomic DNA. Primer specificity of the PCR products was confirmed by a subsequent nested PCR. EF-1α primers were used as an internal standard.

3. Results

3.1. Expression of Fgf10 during skin and hair follicle morphogenesis

Fgf10 transcripts were detected in the basal layer of the epidermis at 17.5 dpc (Fig. 1B). Correspondingly, FGF10 protein was detected in the epidermis (Fig. 1F). Within hair follicles, *Fgf10* mRNA was detected in the dermal papilla (Fig. 1D; white arrow) and in the hair matrix (Fig. 1D; white arrowheads). Dynamic changes of gene expression of several regulatory factors have been reported during the hair follicle development. Low level *Fgf10* expression was detected in embryonic hair follicles at earlier stages between 16.5 and 18.5 dpc (data not shown). Although it was previously reported that human *Fgf10* is expressed in the dermal papilla and outer root sheath cells [12], we could not detect its expression in the developing murine outer root sheath.

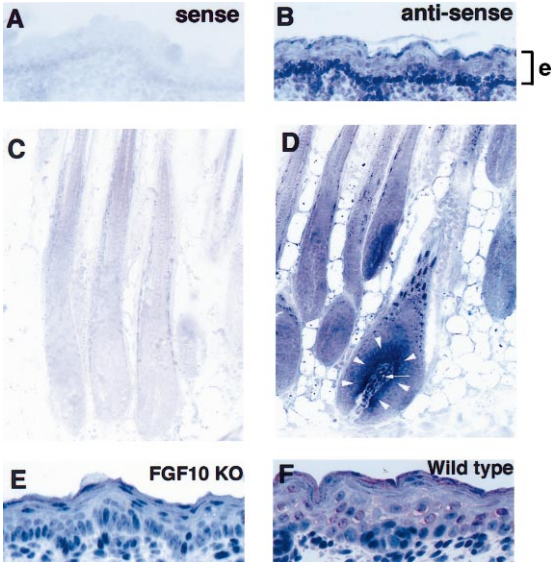

Fig. 1. Expression of *Fgf10* during skin and hair follicle morphogenesis. In situ hybridization of *Fgf10* in 17.5 dpc fetal mouse skin (B) and hair follicles 11 days after birth (D). An *Fgf10*-sense probe was used as a negative control (A and C). Immunohistochemical analysis of the FGF10 protein in fetal mouse skin tissue sections of wild-type mice (positive control) (F) and mutant (*Fgf10*$^{-/-}$) (E). Magnifications: ×400 (A, B, E and F), ×200 (C and D). e, epidermis.

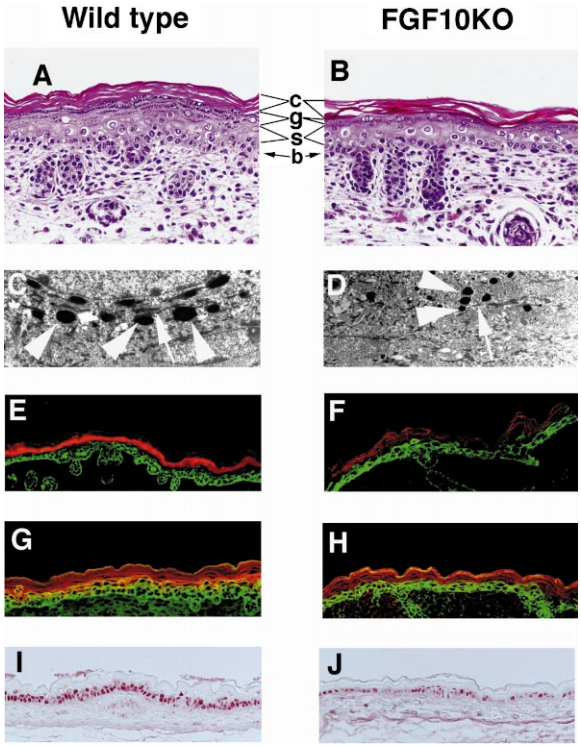

Fig. 2. Histological analysis of *Fgf10*$^{-/-}$ mouse skin. H&E staining of skin from newborn wild-type (A) and mutant mice (B). Ultrastructure of the wild-type (C) and mutant mice epidermis (D). Transverse sections of dosal skin from wild-type (E, G and I) and mutant fetuses (F, H and J) at 18.5 dpc stained with antibodies to loricrin (visualization with Texas red) (E and F), TGase 1 (visualization with Cy3) (G and H), K14 (visualization with FITC) (E–H) and PCNA (visualization with 3-amino-9-ethylcarbazol) (I and J). Magnifications: ×400 (A and B), ×3000 (C and D), ×200 (E-J). b, basal layer of epidermis; s, spinous layer; g, granular layer; c, cornified layer.

3.2. Analysis of the skin from Fgf10$^{-/-}$ *newborn mice*

Histological examination of *Fgf10*$^{-/-}$ newborn mouse skin revealed that the granular layer of the epidermis was thinner than that of control mice, and granular cells were formed without distinctive keratohyaline granules (KHGs) and tonofibrils (Fig. 2B). Moreover, ultrastructural analysis of the mutant granular layer revealed that the KHGs (Fig. 2C,D; white arrowheads) were immature and surrounded by loosely packed tonofibril bundles (Fig. 2C,D; white arrow). To analyze the phenotype in more detail, we examined the expressions of two epidermal differentiation markers, loricrin and transglutaminase 1 (TGase 1). The expression of loricrin was reduced dramatically in the *Fgf10*$^{-/-}$ skin (Fig. 2F),

but TGase 1 was expressed in the upper spinous and granular layers of both the control and *Fgf10*$^{-/-}$ skins (Fig. 2G,H). Similarly, K14 protein was normally expressed in the basal layer and hair follicles of *Fgf10*$^{-/-}$ mice (Fig. 2E–H). To investigate cell proliferation rates, PCNA expression levels were assessed. A significant decrease (an average of 20% reduction was evaluated by several sections) in the number of proliferating cells was observed in the basal layer of *Fgf10*$^{-/-}$ mice (Fig. 2I,J).

3.3. Analysis of hair development in *Fgf10*$^{-/-}$ derived full-thickness skin grafts

Because *Fgf10*$^{-/-}$ mice die soon after birth due to a lack of lung development, we transplanted fetal skin to athymic nude mice to study postnatal hair development. While *Fgf10* is expressed in wild-type hair follicles (Fig. 1D), transplanted *Fgf10*$^{-/-}$ skin grafts generated abundant pigmented hairs, comparable to controls. The morphology of hair follicles from *Fgf10*$^{-/-}$ mice was indistinguishable from that of control follicles (Fig. 3A,B), and no major abnormalities were detected on the surface or inner structure of the hairs examined by SEM (Fig. 3C,D) and light microscopy (data not shown).

3.4. Other Fgf transcripts, Fgf7 and Fgf2, were detected in the *Fgf10*$^{-/-}$ skin

Fgf10$^{-/-}$ skin showed hypoplastic epidermal morphogenesis (Fig. 2), but no prominent abnormality during hair development (Fig. 3). Hence, we considered the possibility that other members of the Fgf family might compensate for the loss of *Fgf10*. Among the known *Fgf* family members, *Fgf10* is most similar in sequence and biological properties to *Fgf7*. *Fgf7* and *Fgf2* mRNAs were detected in wild-type hair follicles, as previously described [9,17,18]; *Fgf2* and *Fgf7* transcripts were expressed in the hair matrix and dermal papilla, respectively (Fig. 4A). Next, we examined the levels of expression of *Fgf7*, *Fgf2* and several other important regulators during skin morphogenesis, including *Skn-1a* [19], *sonic hedgehog* (*Shh*) [20,21], *TGF-β2* [22] and *PDGF-A* [23]. mRNA expression of *Fgf7*, *Fgf2* and other regulators was similar in skin samples from *Fgf10*$^{-/-}$ and control mice (Fig. 4B).

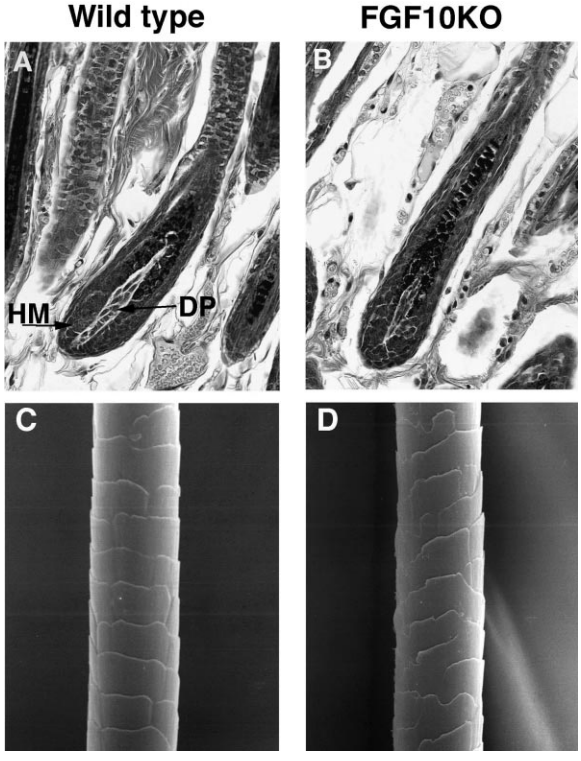

Fig. 3. Analysis of *Fgf10*$^{-/-}$ hair. H&E stained skin sections of wild-type (A) and mutant mice (B). SEM analysis showing the surface of the hairs of wild-type (C) and mutant mice (D). Magnifications: ×400 (A and B), ×1500 (C and D). DP, dermal papilla; HM, hair matrix.

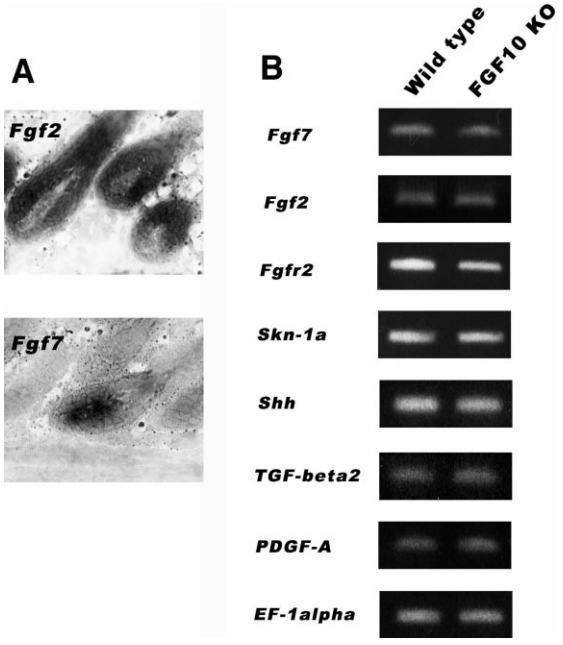

Fig. 4. In situ hybridization of *Fgf2* and *Fgf7* in wild-type hair follicles (A; magnifications: ×200). Detection of other *Fgf* and other regulatory factor transcripts in *Fgf10*$^{-/-}$ skin (B). mRNA was isolated from 18.5 dpc fetal mouse dorsal skin and subjected to RT-PCR as described in Section 2.

4. Discussion

During wound healing, administration of the FGF10 protein leads to significant stimulation of epithelial growth and granulated tissue formation [24], and significant increase in wound collagen content [25]. However, the physiological role of *Fgf10* during skin morphogenesis has not been well established.

FGF10 binds to FGFR2 with an affinity comparable to that of FGF7. FGFR2 has two receptor isoforms, IIIb and IIIc. *Fgfr2IIIb*, which is expressed mainly in the basal layer and hair follicles of the skin, is important for skin development as shown by a recent knockout experiment [26]. Furthermore, overexpression of truncated *Fgfr2* has been shown to induce severe skin abnormalities, including epidermal atrophy and hair follicle defects [27]. These results suggest that the FGFR2 ligands, FGF7 and FGF10, play important roles in hair and epidermal morphogenesis.

Gene knockout experiments for Fgf family members have revealed abnormalities in hair development. *Fgf5*$^{-/-}$ mice demonstrated an abnormal long hair phenotype [28] and the hair coat of *Fgf7*$^{-/-}$ mice has a greasy or matted appearance

[11]. In this study, *Fgf10* transcripts were detected in the dermal papilla and the hair matrix in hair follicles (Fig. 1D), but no abnormalities were found in hair development of *Fgf10*$^{-/-}$ mice, suggesting that other *Fgf*s expressed in hair follicles (Fig. 4B) may have redundant function with *Fgf10*.

Whereas *Fgf7* knockout mice have no prominent abnormalities in epidermal development [11], we demonstrated aberrant epidermal morphogenesis in *Fgf10*$^{-/-}$ newborn mice. Presumably, FGFR2 ligands differ in their ability to compensate for each other, thus yielding distinct phenotypes in knockout mice for the various FGFR2 ligands. Recognizing such possibilities, it was revealed that mRNAs encoding *Fgf7* and *Fgf2* were present in the skin samples of *Fgf10*$^{-/-}$ mice in levels similar to those in controls (Fig. 4B).

KHGs are the most conspicuous organelles in the granular cells of keratinized epidermis. In rodents, KHGs are composed of F-granules and L-granules. In the present study, the expression of filaggrin mRNA, a major component of F-granules, was similar in *Fgf10*$^{-/-}$ and control mice (data not shown). By contrast, loricrin, which is a major component of L-granules and coexpressed with filaggrin in terminally differentiating keratinocytes, was reduced dramatically in the *Fgf10*$^{-/-}$ skin (Fig. 2F). It is likely that *Fgf10* functions during L-granule formation, possibly by regulating the expression of loricrin during differentiation of keratinocytes.

It was recently shown that the inherited skin diseases Vohwinkel's syndrome (VS) and progressive symmetric erythrokeratoderma (PSEK) are caused by loricrin mutations [29]. Although loricrin defects are responsible for such diseases, the pathoetiologic mechanisms for the altered epidermal differentiation remain to be explored. Previously, it was reported that the expression of FGF7 and its receptor, FGFR2, was altered in psoriasis [30]. Therefore, it would be intriguing to examine the functions of *Fgf10* in a pathological and molecular cascade underlying such skin diseases.

It was previously suggested that the POU domain factors, *Skn-1a* and *Oct-6 (Tst-1)*, might have redundant functions in epidermal development [19]. Our data suggests that *Fgf*s may also carry out redundant function in hair and epidermal development. It will be necessary to analyze the possible cross-regulatory functions of these signaling molecules during skin development by utilizing double or compound knockout mice (*Fgf* compound knockout mice, etc.).

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References

[1] Yamasaki, M., Miyake, A., Tagashira, S. and Itoh, N. (1996) J. Biol. Chem. 271, 15918–15921.
[2] Beer, H.D., Florence, C., Dammeier, J., McGuire, L., Werner, S. and Duan, D.R. (1997) Oncogene 15, 2211–2218.
[3] Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N. and Noji, S. (1997) Development 124, 2235–2244.
[4] Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B.L. (1997) Development 124, 4867–4878.
[5] Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M. and Simonet, W.S. (1998) Genes Dev. 12, 3156–3161.
[6] Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. and Kato, S. (1999) Nat. Genet. 21, 138–141.
[7] Emoto, H., Tagashira, S., Mattei, M.G., Yamasaki, M., Hashimoto, G., Katsumata, T., Negoro, T., Nakatsuka, M., Birnbaum, D., Coulier, F. and Itoh, N. (1997) J. Biol. Chem. 272, 23191–23194.
[8] Mason, I.J., Fuller Pace, F., Smith, R. and Dickson, C. (1994) Mech. Dev. 45, 15–30.
[9] Rosenquist, T.A. and Martin, G.R. (1996) Dev. Dyn. 205, 379–386.
[10] Werner, S., Peters, K.G., Longaker, M.T., Fuller Pace, F., Banda, M.J. and Williams, L.T. (1992) Proc. Natl. Acad. Sci. USA 89, 6896–6900.
[11] Guo, L., Degenstein, L. and Fuchs, E. (1996) Genes Dev. 10, 165–175.
[12] Hamada, K., Ozawa, K., Itami, S. and Yoshikawa, K. (1999) Exp. Dermatol. 8, 347–349.
[13] Igarashi, M., Finch, P.W. and Aaronson, S.A. (1998) J. Biol. Chem. 273, 13230–13235.
[14] Tagashira, S., Harada, H., Katsumata, T., Itoh, N. and Nakatsuka, M. (1997) Gene 197, 399–404.
[15] Hiiragi, T., Sasaki, H., Nagafuchi, A., Sabe, H., Shen, S.C., Matsuki, M., Yamanishi, K. and Tsukita, S. (1999) J. Biol. Chem. 274, 34148–34154.
[16] Yamada, K., Matsuki, M., Morishima, Y., Ueda, E., Tabata, K., Yasuno, H., Suzuki, M. and Yamanishi, K. (1997) Hum. Mol. Genet. 6, 2223–2231.
[17] Gonzalez, A.M., Buscaglia, M., Ong, M. and Baird, A. (1990) J. Cell Biol. 110, 753–765.
[18] du Cros, D.L., Isaacs, K. and Moore, G.P. (1993) J. Cell Sci. 105, 667–674.
[19] Andersen, B., Weinberg, W.C., Rennekampff, O., McEvilly, R.J., Bermingham Jr., J.R., Hooshmand, F., Vasilyev, V., Hansbrough, J.F., Pittelkow, M.R., Yuspa, S.H. and Rosenfeld, M.G. (1997) Genes Dev. 11, 1873–1884.
[20] Chiang, C., Swan, R.Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E.K., Cooper, M.K., Gaffield, W., Westphal, H., Beachy, P.A. and Dlugosz, A.A. (1999) Dev. Biol. 205, 1–9.
[21] St. Jacques, B., Dassule, H.R., Karavanova, I., Botchkarev, V.A., Li, J., Danielian, P.S., McMahon, J.A., Lewis, P.M., Paus, R. and McMahon, A.P. (1998) Curr. Biol. 8, 1058–1068.
[22] Foitzik, K., Paus, R., Doetschman, T. and Dotto, G.P. (1999) Dev. Biol. 212, 278–289.
[23] Karlsson, L., Bondjers, C. and Betsholtz, C. (1999) Development 126, 2611–2621.
[24] Xia, Y.P., Zhao, Y., Marcus, J., Jimenez, P.A., Ruben, S.M., Moore, P.A., Khan, F. and Mustoe, T.A. (1999) J. Pathol. 188, 431–438.
[25] Jimenez, P.A. and Rampy, M.A. (1999) J. Surg. Res. 81, 238–242.
[26] De Moerlooze, L., Spencer Dene, B., Revest, J., Hajihosseini, M., Rosewell, I. and Dickson, C. (2000) Development 127, 483–492.
[27] Celli, G., LaRochelle, W.J., Mackem, S., Sharp, R. and Merlino, G. (1998) EMBO J. 17, 1642–1655.
[28] Hebert, J.M., Rosenquist, T., Gotz, J. and Martin, G.R. (1994) Cell 78, 1017–1025.
[29] Ishida Yamamoto, A., Takahashi, H. and Iizuka, H. (1998) Histol. Histopathol. 13, 819–826.
[30] Finch, P.W., Murphy, F., Cardinale, I. and Krueger, J.G. (1997) Am. J. Pathol. 151, 1619–1628.