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Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation

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In this study we used a dominant-negative FGF receptor mutant to block FGF function in a specific tissue of transgenic mice. The mutant receptor, which is known to block signal transduction in cells when co-expressed with wild-type receptors, was targeted to suprabasal keratinocytes using a keratin 10 promoter. The transgene was expressed specifically in the skin and highest expression levels were found in the tail. Expression of the mutant receptor disrupted the organization of epidermal keratinocytes, induced epidermal hyperthickening and resulted in an aberrant expression of keratin 6. This suggests that FGF is essential for the morphogenesis of suprabasal keratinocytes and for the establishment of the normal program of keratinocyte differentiation. Our study demonstrates that dominant-negative growth factor receptors can be used to block selectively the action of a growth factor in specific tissues of transgenic mice.

Key words: dermis/epidermis/FGF/FGF receptor/keratin

Introduction

Fibroblast growth factors (FGFs) comprise a family of polypeptide mitogens which include acidic FGF (aFGF), basic FGF (bFGF), the products of the oncogenes *int-2* (FGF-3), *hst* (FGF-4), FGF-5, FGF-6 as well as keratinocyte growth factor (KGF). They act as potent mitogens for a large variety of cell types but they also stimulate differentiation processes *in vitro* and *in vivo* (reviewed by Thomas, 1987; Burgess and Maciag, 1989; Klagsbrun, 1989).

Four different FGF receptors (FGFR1, FGFR2, FGFR3 and FGFR4) have recently been identified (Lee *et al.*, 1989; Dionne *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991). All FGF receptor cDNAs encode transmembrane protein tyrosine kinases with either two or three immunoglobulin-like (Ig-like) domains and a highly acidic region in the extracellular part of the receptor. Furthermore, several splice variants of FGFR1 and FGFR2 have been identified (reviewed by Johnson and Williams, 1993). Splice variants that differ in the second half of the third Ig-like domain are of particular importance, since they have been shown to have different ligand-binding specificities and are expressed in different cells and tissues (Miki *et al.*, 1992; Werner *et al.*, 1992a).

We have chosen the skin as a model system to study the role of FGFs within a specific tissue. Evidence for a function of FGFs in the skin has come mainly from a series of wound healing studies in which externally applied bFGF was shown to have a beneficial effect on dermal and epidermal wound healing (Greenhalgh *et al.*, 1990; Hebda *et al.*, 1990; Tsuboi and Rifkin, 1990). Furthermore, we have recently demonstrated the induction of expression of endogenous FGFs, particularly that of KGF, during the healing of full-thickness wounds (Werner *et al.*, 1992b).

The function of FGF in normal skin is presently unknown. Recently we found a remarkably localized expression of FGFR2 in the basal and suprabasal layers of embryonic and adult epidermis (Peters *et al.*, 1992; Werner *et al.*, 1992b). Since a splice variant of this receptor form is known to bind KGF (Miki *et al.*, 1991), this finding suggests that dermally derived KGF (Finch *et al.*, 1989) stimulates the epidermal FGFR2 in a paracrine manner. In addition, another member of the FGF family might stimulate FGFR2 in the suprabasal layers of the epidermis.

Given the localized expression of FGFR2 in the epidermis, we used a new strategy for selectively blocking FGF receptor signal transduction in this compartment of the skin and thereby to determine whether there was a biological role for FGF in the non-proliferating, suprabasal cells of the epidermis. Recent studies in *Xenopus* oocytes have shown that the signal transduction through FGF receptors and other tyrosine kinase receptors can be blocked by co-expression of dominant-negative receptor mutants (Amaya *et al.*, 1991; Ueno *et al.*, 1991, 1992). These dominant-negative mutants are characterized by the lack of a functional tyrosine kinase domain (Honegger *et al.*, 1990; Ueno *et al.*, 1991). Upon ligand binding, the mutant receptors form non-functional heterodimers with the full-length wild-type receptors, thereby blocking signal transduction (Honegger *et al.*, 1990; Kashles *et al.*, 1991; Ueno *et al.*, 1991). Most importantly, the dominant-negative action is specific for each growth factor receptor; for example a truncated FGF receptor only blocks signal transduction through FGF receptors but not through the epidermal growth factor receptor or platelet-derived growth factor receptor. Dominant-negative receptors should therefore be useful to block selectively the action of a specific growth factor *in vitro* and *in vivo*.

In this study we used a suprabasal keratin promoter [bovine keratin 10 (K10) promoter; Rieger *et al.*, 1985; Blessing *et al.*, 1987] to express a dominant-negative FGF receptor mutant in the differentiating cells of the epidermis of transgenic mice. Here we demonstrate that the expression of a dominant-negative FGF receptor mutant in transgenic mice under the control of this promoter induced phenotypic abnormalities in the epidermis. This finding provides the first

evidence that FGF is involved in the differentiation and morphogenesis of epidermal keratinocytes *in vivo*. Furthermore, our results demonstrate that dominant-negative growth factor receptors can be used to block selectively the action of a growth factor in specific tissues of transgenic mice.

Results

Expression of FGFs and FGF receptors in the dermis and epidermis

To determine potential sites of action of FGFs in the skin, we first investigated the endogenous expression of all known FGFs and their receptors in the dermis and epidermis of mouse tails using RNase protection assays. We found expression of bFGF exclusively in the dermis, whereas aFGF mRNA was detected in the dermis as well as in the epidermis (Figure 1). Consistent with recent data from other investigators (Finch *et al.*, 1989) we also found expression of KGF exclusively in the dermis. Since KGF is a secreted mitogen, it might be transported from the dermis to the epidermis. To assess the localization of KGF protein within the skin, we have performed Western blot experiments on heparin Sepharose purified homogenates from dermis and epidermis of mouse tail skin. We detected KGF protein in the dermis but not in the epidermis (data not shown). No expression of FGF-3 (int-2), FGF-4 (hst), FGF-5 or FGF-6 was found in mouse tail skin.

Examining the distribution of FGF receptors in the skin, we found FGFR1 expressed at high levels in the dermis but only at low levels in the epidermis (Figure 2A). Recent studies have shown that the major FGFR1 splice variant in the skin (FGFR1-IIIc; for nomenclature see Johnson *et al.*, 1991) binds aFGF and bFGF with high affinity (Werner *et al.*, 1992a). Other FGFR1 splice variants which have different ligand binding specificities comprise $<5\%$ of all FGFR1 forms in the skin (Werner *et al.*, 1992a).

In contrast to the pattern of expression of FGFR1, we found expression of FGFR2 and FGFR3 mRNA in the epidermis as well as in the dermis (Figure 2B and C). This is consistent with recent results of *in situ* hybridizations, where FGFR2 mRNA was detected in the basal and suprabasal layers of the epidermis as well as in the hair follicles (Peters *et al.*, 1992; Werner *et al.*, 1992b). As shown in Figure 2B and C, two different forms of FGFR2 and FGFR3 were found in the skin. The FGFR2-IIIc variant, which has been shown to bind aFGF and bFGF with high affinity but does not bind KGF (Dionne *et al.*, 1990; Miki *et al.*, 1992), was exclusively detected in the dermis. Other splice variants of FGFR2 that differ in the exon encoding the third Ig-like domain were found primarily in the

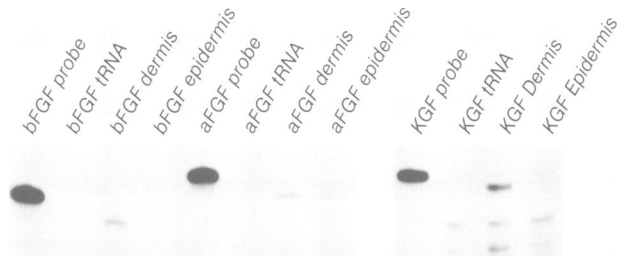

Fig. 1. Expression of FGF mRNAs in the dermis and in the epidermis of mouse tails. Mouse tail skin was incubated for 20 min at 37°C in a solution of 2 M NaBr. Epidermis was subsequently separated from the underlying dermis and immediately frozen in liquid nitrogen and used for RNA isolation. 50 μg RNA from dermis and epidermis were subsequently analyzed by RNase protection assay for the expression of aFGF, bFGF and KGF mRNA. 1000 c.p.m. of the hybridization probe were used as size markers. 50 μg tRNA was used as a negative control.

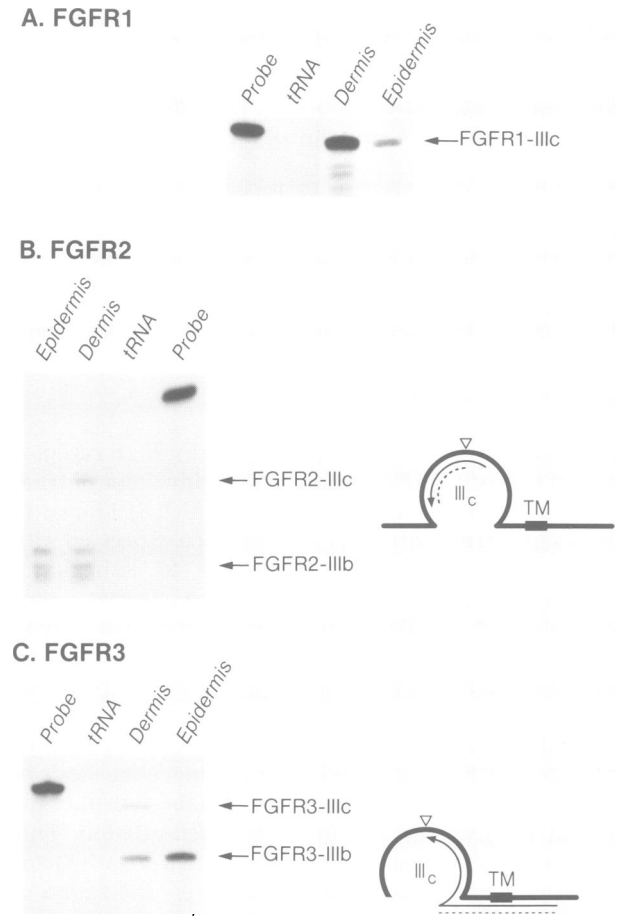

Fig. 2. mRNA expression of FGF receptors in the dermis and in the epidermis of mouse tail skin. 50 μg RNA from dermis and epidermis were analyzed by RNase protection assay for the expression of FGFR1, FGFR2 and FGFR3. A 325 nt hybridization probe which is complementary to the transmembrane region and the juxtamembrane regions of murine FGFR1 was used to detect FGFR1 transcripts. This probe does not distinguish between different FGFR1 splice variants. For the detection of FGFR2 mRNA, we used a 161 nt hybridization probe which is complementary to part of the third Ig-like domain of FGFR2 including exon IIIc. The probe is indicated with an arrow in the diagram in panel **B**. A 161 bp protected fragment corresponding to the complete hybridization probe is expected for FGFR2 transcripts containing exon IIIc. The 100 bp fragment which is protected by FGFR2 transcripts that are differentially spliced within the third Ig-like domain is indicated with a dotted line. The arrow indicates the point of sequence divergence between the FGFR2-IIIc splice variant and other splice variants. The transmembrane region is labelled TM. For the detection of FGFR3 transcripts we used a 430 nt probe which includes sequences from the second half of the third Ig-like domain of murine FGFR3 (exon IIIc) to the intracellular juxtamembrane region (indicated with an arrow in the diagram in panel **C**). A 430 bp protected fragment corresponding to the complete hybridization probe is expected for transcripts containing exon IIIc. The 395 bp protected fragment which is indicated by a dotted line in the same diagram is generated by transcripts that are differentially spliced within the third Ig-like domain.

epidermis and to a lesser extent in the dermis. Using a probe which is specific for the FGFR2-IIIb exon, we demonstrated that these splice variants include the IIIb exon (data not shown). The encoded receptor has been shown to bind KGF and aFGF with high affinity and bFGF only with low affinity (Miki *et al.*, 1991, 1992). The FGFR2 transcripts which we detected in the epidermis therefore represent receptors for KGF and aFGF.

Surprisingly we also detected two different fragments that were protected by FGFR3 mRNA. The larger fragment protects mRNA which encodes the published FGFR3 variant (Ornitz and Leder, 1992). The smaller fragment is generated by a novel FGFR3-IIIb variant (S.Werner, unpublished data). Whereas the published FGFR3-IIIc variant binds aFGF with higher affinity than bFGF (Ornitz and Leder, 1992), the binding specificity of the new FGFR3 variant has yet to be determined.

In summary these results show that a large variety of different FGF receptor forms are differentially expressed in the dermis and in the epidermis, suggesting specific functions of each FGF in different compartments of the skin.

Transgenic mice express a dominant-negative FGF receptor mutant under the control of a keratin 10 promoter

To assess the biological role of FGF in the epidermis, we expressed a dominant-negative FGF receptor mutant under the control of the promoter of a bovine homologue of the human K10 gene in transgenic mice. This promoter has been shown to target the expression of transgenes to the differentiating cells of the epidermis (Bailleul *et al.*, 1990). A truncated form of FGFR1-IIIc that contains only 26 amino acids of the intracellular juxtamembrane region but lacks the complete tyrosine kinase domain was used as a transgene. This truncated FGFR1 (FGFR1tr) has been shown to block signalling through the FGFR1, FGFR2 (KGFR splice variant) and FGFR3 proteins in *Xenopus* oocytes (Ueno *et al.*, 1992). The blocking response depends on the endogenous and truncated receptors binding the same ligand. Since the truncated FGFR1-IIIc binds aFGF and bFGF but not KGF, it should block the action of aFGF and bFGF but not that of KGF. Furthermore, the signal transduction through other growth factor receptors is unaffected by this truncated FGFR1 mutant (Amaya *et al.*, 1991; Ueno *et al.*, 1991; H.Ueno and L.T.Williams, unpublished data). A schematic representation of the DNA fragment which was used for injections is shown in Figure 3A.

Fertilized eggs were injected with purified insert DNA. The transgene was found in seven out of 19 pups, as judged by Southern blot analysis of their tail DNAs (data not shown). Three founder mice expressed high levels of the transgene as assessed by RNase protection assay of tail skin RNA (Figure 3C) using the probe shown in Figure 3B. These mice were bred to generate transgenic lines for use in subsequent analysis.

The transgene is specifically expressed in the skin

Expression of the transgene mRNA was detected exclusively in the skin and was found at highest levels in ear and tail skin (Figure 4A). This mRNA expression pattern correlates with the expression of FGFR1tr protein. As shown in Figure 4B, a 60 kDa FGFR1-specific band was detected in homogenates of tail and ear skin but not in back skin. Furthermore, this protein was not found in skin homogenates of control animals. A 120 kDa protein which was also recognized by FGFR1 antibodies and was found in tail and ear skin lysates of transgenic mice, most likely represented a dimer of the truncated receptor, since the levels of this protein could be reduced by addition of higher DTT concentrations (0.1 M) to the sample buffer (data not shown). The existence of dimeric forms of FGFR1 is consistent with recent data, where dimers of soluble receptors were detected under denaturing conditions, even in the absence of ligand (Duan *et al.*, 1992; Werner *et al.*, 1992a).

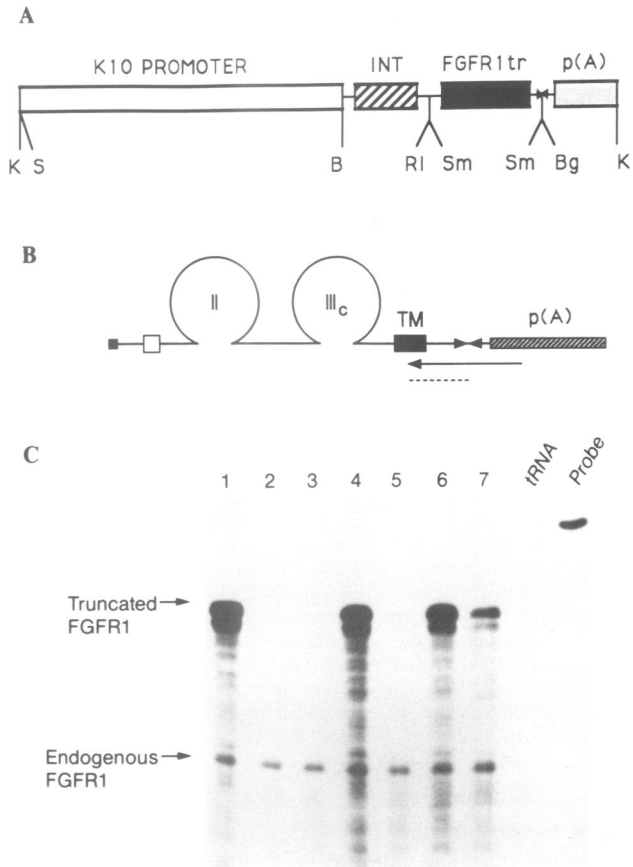

Fig. 3. A diagram of the transgene construct is shown in (**A**). Functional elements include the promoter of the bovine K10 gene (open box), the rabbit β-globin intron (diagonally striped box), the coding sequence of the truncated FGFR1 cDNA including the ATG translation initiation codon (black box), an oligonucleotide linker containing three stop codons in all reading frames (indicated with a star) and the human growth hormone poly(A) (dotted box). Restriction sites: B: *Bam*HI, Bg: *Bgl*II, E: *Eco*RI, K: *Kpn*I, S: *Sma*I. The mRNA of the transgene is shown in (**B**). Functional elements include the ATG translation initiation codon (small black box on the left), the highly acidic region of FGFR1 (open box), Ig-like domains II and IIIc (II and IIIc), the transmembrane region of FGFR1 (TM), the stop codon (double arrow) and the human growth hormone poly(A) (dashed box). The hybridization probe that was used to detect the transgene mRNA is indicated with an arrow. A protected fragment which corresponds to the complete hybridization probe is expected for the transgene mRNA, whereas a shorter protected fragment is expected for transcripts encoding endogenous FGFR1 (indicated with a dotted line). (**C**) 50 μg total cellular RNA from mouse tail skin was analyzed by RNase protection assay for the expression of the transgene and the endogenous FGFR1 using the probe described in (**B**). The protected fragments obtained by mRNA encoding the truncated FGFR1 and by endogenous FGFR1 are indicated. 1000 c.p.m. of the hybridization probe were loaded in the lane labelled 'probe'. 50 μg tRNA were used as a negative control. The numbers 1–7 indicate transgenic founder mice nos 1–7.

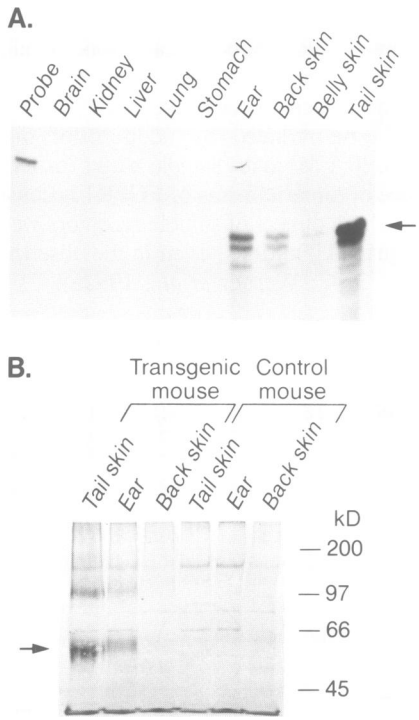

Fig. 4. The mRNA expression of the truncated FGF receptor in various mouse tissues is shown in (**A**). 50 μg total cellular RNA from different tissues and organs of transgenic mice was analyzed by RNase protection assay for the expression of the FGFR1tr transgene, using the probe described in Figure 3B. 1000 c.p.m. of the hybridization probe were loaded as a size marker in the lane labelled 'probe'. The major protected fragment is indicated with an arrow. The protein expression of the truncated FGFR1 in mouse skin is shown in (**B**). The truncated FGFR1 protein was partially purified from lysate of 15 mg total skin using wheatgerm agglutinin–Sepharose beads. Proteins bound to the Sepharose were eluted with Laemmli sample buffer and analyzed by Western blotting using the polyclonal antiserum Ab15 which is directed against the extracellular region of FGFR1. The truncated FGFR1 protein is indicated with an arrow.

Transgenic mice exhibit abnormalities in the epidermis of the tail and the ear

Transgenic mice that expressed high levels of the transgene were further examined for phenotypic abnormalities in the skin. None of the transgenic mice had obvious macroscopic abnormalities, but a histological analysis of the skin revealed a significant difference in the epidermis of the tails of transgenic mice from three independent lines when compared with control mice. Characteristic features of transgenic mice tail epidermis included hyperthickening, the presence of several layers of nucleated cells with basal cell-like morphology in the suprabasal layers, a high degree of disorganization in the basal and lower suprabasal layers, and most often an irregular border between dermis and epidermis (Figure 5B and C). The extent of these abnormalities correlated with the expression level of the truncated FGFR1 and was particularly dramatic in homozygous animals in which we observed at least a 50-fold overexpression of the truncated receptor compared to the wild-type receptor (Figure 5C).

To assess whether the transgene is indeed expressed in those areas which reveal significant abnormalities, we performed *in situ* hybridizations on tail skin sections using a hybridization probe complementary to the human growth hormone polyadenylation sequence which is part of the

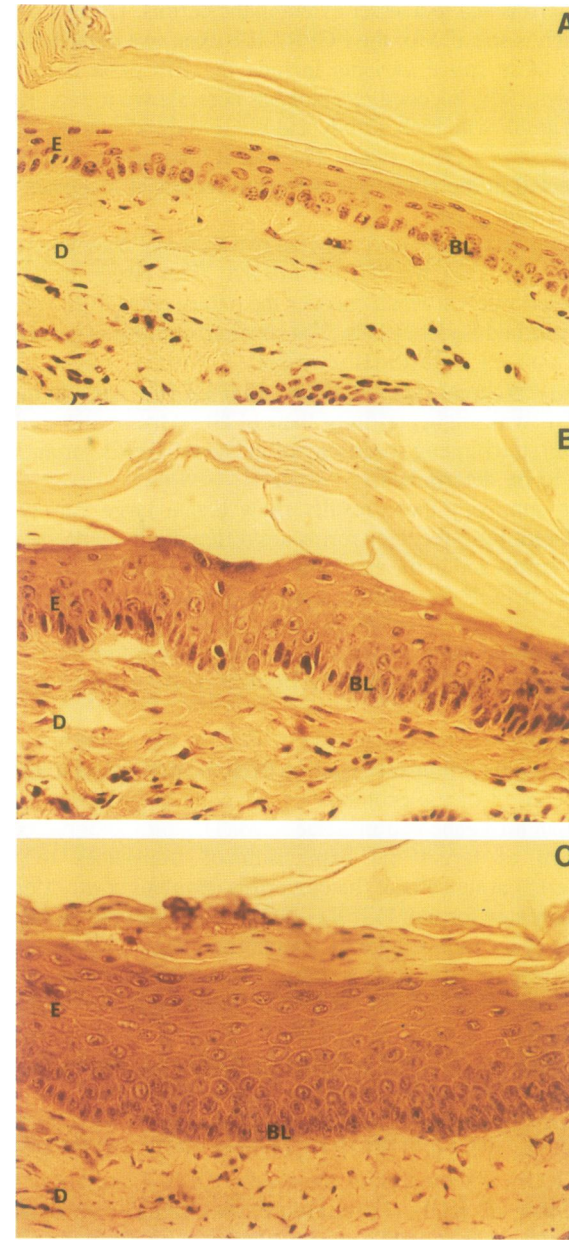

Fig. 5. Histology of tail skin from transgenic mice and control mice. Tail skin from a nontransgenic control mouse (**A**), a heterozygous FGFR1tr transgenic mouse (**B**) and a homozygous FGFR1tr transgenic mouse (**C**) (all 2 months old) were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (6 μm). Sections were stained with hematoxylin and eosin. D, dermis; E, epidermis; BL, basal layer. Note the presence of several layers of nucleated cells in the suprabasal layers of transgenic mice and their high degree of disorganization.

transgene mRNA. As shown in Figure 6A and B, high expression levels of the transgene mRNA were detected in the suprabasal layers of transgenic mouse skin. Most importantly, transgene expression was also found in those suprabasal cells which morphologically resembled basal cells. Expression of the transgene was found in all areas of the epidermis but was highest in the infundibular regions of the hair follicles (labelled with arrows in Figure 6A and B). These regions revealed the most intense phenotype. In addition to the suprabasal expression, transgene mRNA was also detected in very few basal cells which is consistent with the observation of K10 expression in basal cells which become committed to terminal differentiation (Moll *et al.*,

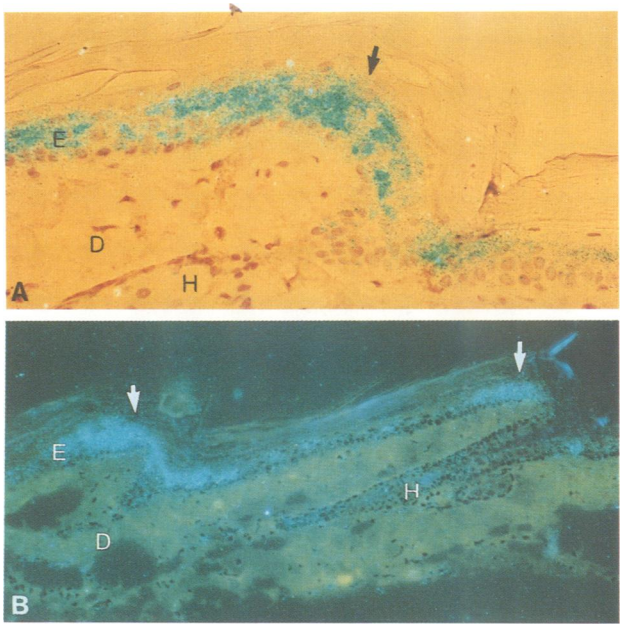

Fig. 6. *In situ* localization of transgene FGFR1tr mRNA in tail skin from a 4 month-old heterozygous transgenic mouse. Tail skin sections of a heterozygous transgenic mouse were hybridized with a ^{35}S-labelled riboprobe complementary to the human growth hormone polyadenylation sequence which is part of the transgene mRNA. They were coated with NBT nuclear emulsion, exposed for 2 weeks and counterstained with hematoxylin–eosin. Silver grains were visualized by epifluorescence and appear as green dots. The infundibular regions of the hair follicles where highest levels of transgene expression were found are labelled with arrows. D, dermis; E, epidermis; H, hair follicle.

1982; Schweizer *et al.*, 1984; Huitfeldt *et al.*, 1991).

In addition to the tail, the ear skin also had distinct, but much more subtle abnormalities (data not shown). The lack of abnormalities in the skin of the back and the belly correlated with the low expression levels of the transgene in these regions. This finding is consistent with recent *in vitro* data showing that at least a 10-fold excess of the truncated receptor is necessary to block FGF receptor signalling by the wild-type receptor (Ueno *et al.*, 1992). The high correlation of the intensity of the phenotypic abnormalities with the levels of transgene expression provides evidence that the observed phenotype in the epidermis is due to the expression of the truncated FGF receptor and therefore to the inhibition of FGF receptor signalling. These results provide the first evidence that FGF is involved in epidermal differentiation and morphogenesis.

Biochemical analysis of transgenic mouse skin

To determine whether keratinocytes in transgenic mouse epidermis regulate specific marker gene expression according to the normal differentiation program, we analyzed the expression of keratins in control and transgenic mouse tail epidermis by immunofluorescence. In normal epidermis, keratins 14 and 5 (K14 and K5) are expressed at high levels in the basal layer and only at low levels in the suprabasal layers (Fuchs and Green, 1980; Nelson and Sun, 1983; Roop *et al.*, 1983). When basal cells become committed to terminal differentiation and move to the suprabasal layer, they synthesize a new pair of keratins, K1 and K10, which are the major differentiation products of mature epidermis (Fuchs and Green, 1980).

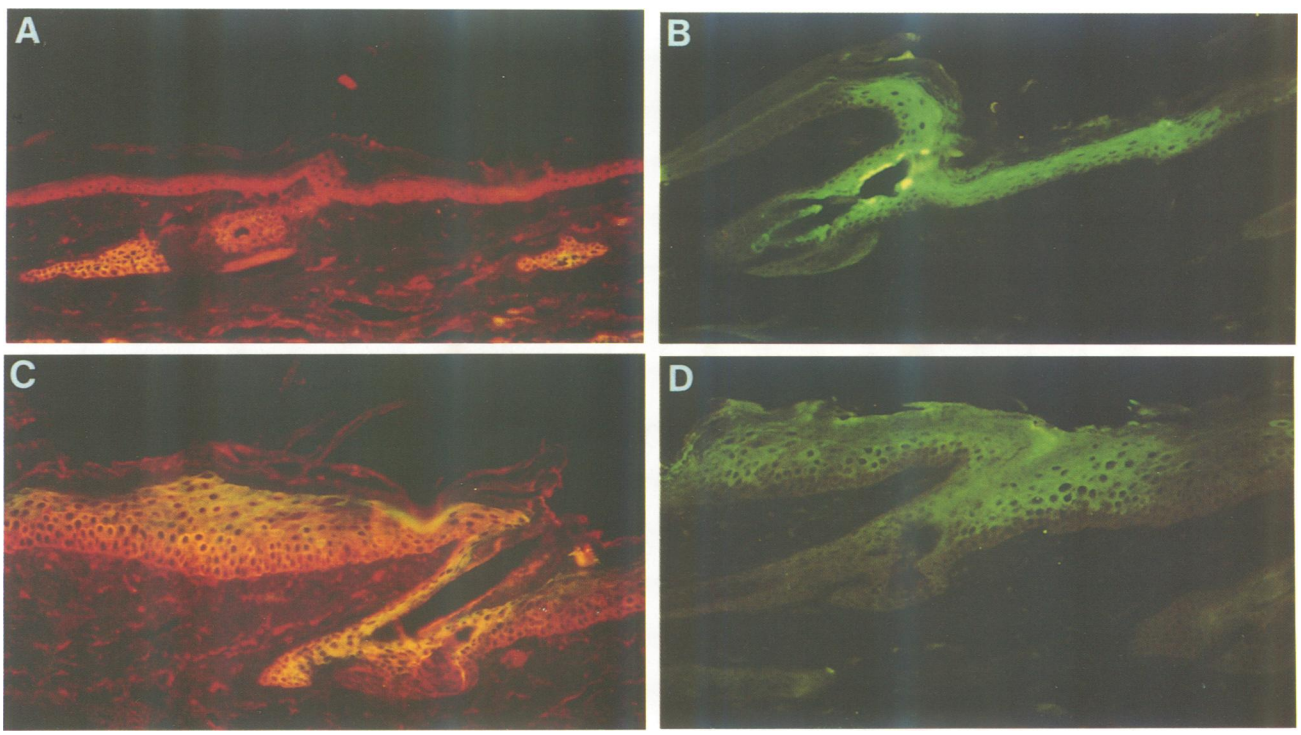

Fig. 7. Keratin expression in tail epidermis of homozygous transgenic (C and D) and control mice (A and B). (**A** and **C**) Sections were incubated with antibodies to K6 (green) and K14 (red). Cells co-expressing K6 and K14 appear yellow in this double exposure. Cells expressing exclusively K14 appear red, cells expressing exclusively K6 appear green. Note the increased number of cell layers expressing K14 in transgenic mouse epidermis and the abnormal expression of K6 in interfollicular epidermis of transgenic mice. (**B** and **D**) Sections were incubated with antibodies to K10 (green). Expression of K10 was restricted to suprabasal layers in transgenic and control mice.

As shown in Figure 7A and C, the number of cell layers expressing high levels of the basal cell keratin K14 was increased in transgenic tail skin. A similar expanded expression was observed for K5 (data not shown).

The expression of differentiation specific keratins K1 and K10 differs between regions of control skin. While expressed uniformly in suprabasal cells of corpus and limb skin, expression in ear and tail epidermis was high in the infundibular region of the hair follicle but absent in interfollicular epidermis (Figure 7B for K10). Similar to control mice, expression of K10 (Figure 7D) and also K1 (not shown) was restricted to suprabasal layers of transgenic mouse epidermis. In spite of their undifferentiated morphology and their high expression of the basal keratins K5 and K14, most suprabasal cells expressed the differentiation-specific keratins K1 and K10. Therefore the cells that constitute the expanded epidermis in the transgenic mice expressed both basal cell keratins and keratins normally characteristic of differentiated keratinocytes.

Consistent with the expression of endogenous K10, expression of the transgene driven by the K10 promoter was highest in the infundibular regions of the hair follicles (see Figure 6B). However, the transgene was also expressed in keratinocytes of interfollicular epidermis, which normally do not express K10 (Figure 6B). It is possible that in normal skin endogenous K10 is not translated in these areas.

In normal skin, K6 expression is restricted to the hair follicles and is only transiently expressed in the proliferating epidermis of wounds. However, it is aberrantly expressed in the suprabasal epidermal layers in hyperplastic, neoplastic and psoriatic skin (Moll *et al.*, 1982; Weiss *et al.*, 1984; Stoler *et al.*, 1988). In tail skin of control mice, K6 expression was restricted to the hair follicles (Figure 7A) and only occasional cells of the interfollicular epidermis expressed this keratin, presumably in response to tail biting. In contrast, K6 was widely expressed in interfollicular epidermis of the transgenic mice in suprabasal and also in some basal cells (Figure 7C). This expanded pattern of K6 expression correlated with the expression level of the transgene and was most dramatic in homozygous mice.

In summary, the keratin expression pattern in transgenic mice shows that inhibition of FGF function in the epidermis changed the regular expression pattern of these proteins and therefore disrupted the normal program of keratinocyte differentiation.

Suprabasal transgene expression stimulates basal cell proliferation

To assess the proliferation status of the keratinocytes in transgenic mouse tail epidermis, we labelled the proliferating cells with 5-bromodeoxyuridine (BrdU) *in vivo* and stained the labelled cells with an antibody against BrdU. As in control mice, proliferation was restricted to the basal cell layer in transgenic mouse epidermis (Figure 8). However, the basal cell proliferation was significantly higher in transgenic mouse epidermis than in control tail skin. Whereas only 2−5% of the basal cell nuclei were labelled in control tail epidermis (Figure 8A), up to 25% of the basal cells of the transgenic mice incorporated BrdU (Figure 8B). The finding that no proliferation was observed in the suprabasal cells that expressed the transgene implies that inhibition of FGF receptor function in these cells had no direct effect on epidermal cell proliferation. In contrast, the basal cell compartment is stimulated as a consequence of the action of the transgene in the suprabasal compartment. The observed phenotype of transgenic mouse epidermis is therefore generated by a primary effect of the transgene on the suprabasal cells. The abnormal suprabasal cells in turn affected the basal cells which underwent hyperproliferation.

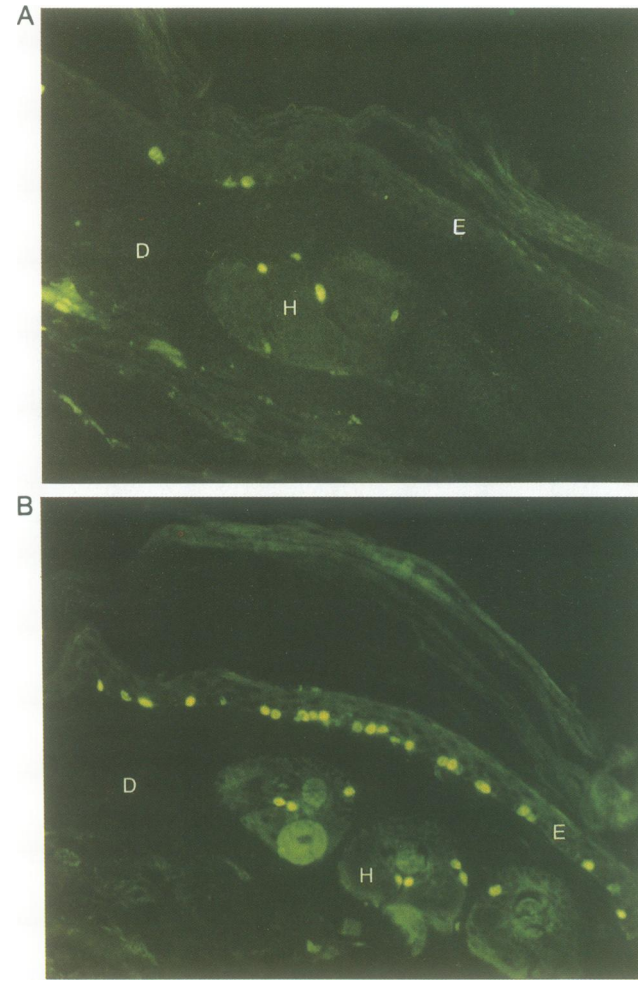

Fig. 8. Detection of proliferating keratinocytes using 5′-bromodeoxyuridine labelling. Mice were sacrificed 2 h after injection (i.p.) with BrdU. Tail skin was fixed in 70% ethanol, embedded in paraffin and sectioned. Sections were stained with an FITC-conjugated antibody against BrdU. Due to the fixation and slide treatment procedure, the morphology of the epidermis is distorted. (**A**) Control tail skin; (**B**) tail skin from a heterozygous transgenic mouse. E, epidermis; H, hair follicle. Stained nuclei are restricted to the basal layer of the epidermis as well as to the hair follicles.

Discussion

We have investigated the biological functions of FGFs in the epidermis by targeting expression of a dominant-negative FGF receptor mutant to the differentiating, non-proliferating keratinocytes. As previously shown, the signal transduction by wild-type FGF receptors is blocked in cells expressing the mutant receptors at significantly higher levels compared with the wild-type receptors (Amaya *et al.*, 1991; Ueno *et al.*, 1992). High expression levels of the dominant-negative receptor were achieved in this study by use of a strong keratin promoter. Transgenic mice expressing high levels of the mutant receptor revealed significant phenotypic abnormalities in the epidermis of the tail, presumably because

the endogenous FGF receptors were blocked. The undifferentiated morphology of the keratinocytes in the suprabasal layers and their aberrant expression of keratins suggests that FGF is essential for a correct differentiation pattern of epidermal keratinocytes. Our results provide the first evidence for a role of FGF in these processes and demonstrate that dominant-negative growth factor receptors provide a powerful tool for the study of growth factor action within a specific tissue in transgenic mice.

A remarkable variety of FGF ligands and receptors is expressed in the skin. aFGF mRNA was found in the dermis and in the epidermis, whereas bFGF and KGF mRNA and also KGF protein were exclusively detected in the dermis. We found expression of the IIIb variant of FGFR2 and also the newly discovered IIIb variant of FGFR3 predominantly in the epidermis. Since the IIIb variants of FGFR1 and FGFR2 bind aFGF with much higher affinity than bFGF, it seems likely that the IIIb variant of FGFR3 also preferentially binds aFGF. This suggests that aFGF rather than bFGF is a natural ligand for the FGF receptors on the epidermis. This hypothesis is further supported by our finding that aFGF is the only FGF that is synthesized in this compartment of the skin. Since aFGF is not a secreted mitogen, its mechanism of action is presently unclear. However, one might speculate that it is released upon cell death by terminally differentiated keratinocytes. In this case it might stimulate particularly the FGF receptors on the suprabasal keratinocytes.

Studies on the FGF and PDGF receptors have suggested that a truncated receptor will inhibit the response of a co-expressed wild-type receptor to a specific ligand, if both receptors are capable of binding the ligand (Ueno *et al.*, 1992; H.Ueno and L.T.Williams, unpublished data). The isoform of FGFR1 that we used (IIIc) binds aFGF and bFGF with high affinity but does not bind KGF and will therefore inhibit the response of full-length FGFR1, FGFR2 and FGFR3 to aFGF and bFGF but not to KGF. This is consistent with our hypothesis that aFGF is likely to be the form of FGF that is blocked in the transgenic mice in these studies, since aFGF is in the epidermis but bFGF is not.

In order to act as a dominant-negative mutant, the truncated receptor must be expressed at much higher levels than the endogenous wild-type receptor. For its use in transgenic experiments it was therefore essential to drive the expression of the truncated receptor by a particularly strong promoter. Furthermore, it was important to express the transgene exclusively in the skin, since an inhibition of FGF function in other parts of the body might have severe side effects or might even be lethal for the embryo. We therefore decided to use a keratin promoter, since keratins are (i) highly expressed in epithelial cells and (ii) regulated in a tissue- and differentiation-specific fashion (Moll *et al.*, 1982). Since we were particularly interested in the role of FGF for the differentiating, non-proliferating cells of the epidermis, we used the bovine K10 promoter to target the dominant-negative FGF receptor mutant to the suprabasal cells.

Using this approach, we obtained three independent transgenic mouse lines which all revealed the same phenotypic abnormalities in the epidermis of the tail and to a lesser extent also in the ear. The observation of the same phenotype in different mouse lines shows that the observed abnormalities do not result from an insertional mutagenesis of a cellular gene but presumably from the inhibition of FGF receptor signalling. In general, the severity of the phenotype showed a high correlation with the level of transgene expression. However, we observed variations within different mice which expressed similar levels of the truncated receptor and even within the skin of a single mouse. Our *in situ* hybridizations demonstrated a patchy expression pattern of the transgene; the intensity of the phenotype correlated with the expression level of the truncated FGF receptor. The reason for the variation within different animals with similar levels of transgene expression is presently unclear. However, one might speculate that minor differences in the levels of endogenous receptors could influence the phenotype.

In normal epidermis, K5 and K14 are expressed mainly in the basal layer (Nelson and Sun, 1983; Roop *et al.*, 1983) and K1 and K10 are expressed in suprabasal layers (Fuchs and Green, 1980). In a variety of hyperproliferative diseases, expression of the basal keratins extends to the suprabasal layers and a new pair of keratins (K6 and K16) are induced in the differentiating cells (Moll *et al.*, 1982; Weiss *et al.*, 1984; Stoler *et al.*, 1988). In this study we demonstrated that blocking action of FGFs induces K6 expression and enhances the number of cell layers that express high levels of the basal cell keratins K5 and K14. These results are surprising, since they suggest that FGFs act as modulators of differentiation and morphogenesis in the suprabasal layers. In contrast, a series of *in vitro* and also *in vivo* studies support a mitogenic function of FGF for non-differentiated keratinocytes (Ristow and Messmer, 1988; Finch *et al.*, 1989; Shipley *et al.*, 1989; Hebda *et al.*, 1990; Greenhalgh *et al.*, 1990; Tsuboi and Rifkin, 1990). It is possible that basal cells *in vivo* respond mitogenically to FGFs, particularly to KGF. However, when basal cells become committed to terminal differentiation and move into the suprabasal layer, they might respond differently to aFGF. If this were the case, one might expect a very different phenotype upon expression of a truncated FGF receptor in the basal cells. These studies are currently in progress.

Interestingly, we also detected phenotypic abnormalities in the basal cells, although they did not express the transgene. In addition, these cells were found to be hyperproliferative. These results imply that the phenotypic changes in the suprabasal cells which are caused by the transgene stimulate the basal cell compartment. In fact, basal cell hyperproliferation as a consequence of a defect in the suprabasal cells has been shown by other authors (Fuchs *et al.*, 1992), suggesting that suprabasal cells normally exert a growth inhibitory effect on their immediate precursors. These studies demonstrate that interactions within different layers of the epidermis, either by paracrine mechanisms or cell–cell contacts are important for the maintenance of this tissue. The exact mechanisms of these interactions have yet to be elucidated.

In contrast to the basal and suprabasal layers, no obvious abnormalities were detected in the granular and spinous layer of transgenic mouse epidermis. The expression pattern of filaggrin, a marker protein of the granular layer, was similar in normal and transgenic epidermis (data not shown). However, we cannot exclude the existence of less obvious abnormalities in these layers which have not been detected.

In summary, our data show that the targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice creates a phenotype which is characterized by disruption of keratinocyte organization and aberrant

keratin expression in the basal and suprabasal layers. The strong levels of K10 expression in transgenic mouse tails show that the phenotype is not due to loss of endogenous K10 expression due to competition of the transgene for transcription factors. Furthermore, we can exclude the possibility that expression of any gene under this promoter unspecifically creates the same phenotype, since expression of a *ras* oncogene under the control of the bovine K10 promoter created a very different phenotype which was characterized by papilloma formation (Bailleul *et al.*, 1990). In contrast, it seems likely that the observed phenotype is indeed generated by inhibition of FGF receptor function. This is supported by recent studies with *Xenopus* embryos, where the phenotype generated by a dominant-negative FGF receptor could be rescued by injection of wild-type receptors (Amaya *et al.*, 1991).

The data presented provide the first example for an important role of FGF in keratinocyte organization and differentiation. This is an exciting example of the pleiotropic effects of FGFs which can act as growth factors or as differentiation factors, depending on the cell type and the environment. Finally our study is the first example of a successful inhibition of growth factor function in transgenic mice, using targeted expression of dominant-negative growth factor receptors. This approach should also be useful to study the function of FGF receptors and other tyrosine kinase receptors in various other tissues, provided that a strong, tissue-specific promoter is available.

Materials and methods

Plasmid construction

A truncated FGFR1 cDNA was cloned by PCR from cDNA of Swiss 3T3 fibroblasts. The cloned cDNA contains the complete extracellular region of murine FGFR1-IIIc, including two Ig-like domains, the transmembrane region and 26 amino acids of the intracellular juxtamembrane region. The truncated FGFR1 fragment (1.1 kb) was cloned into the vector pBluescript KS+ (Stratagene). A 0.65 kb β-globin intron fragment (O'Hare *et al.*, 1981) was inserted at the 5'-end of this construct. The 0.63 kb poly(A) fragment of the human growth hormone gene was inserted at the 3'-end of the truncated FGF receptor using an oligonucleotide linker which has stop codons in all three reading frames. Finally, a 5.5 kb fragment containing the bovine K10 promoter (described as bovine keratin VI in Bailleul *et al.*, 1990) was inserted 5' of this cassette. The complete insert was excised with *Kpn*I and prepared for microinjection into fertilized eggs.

Generation of transgenic mice

Standard procedures were followed in order to generate transgenic mice (Hogan *et al.*, 1986). Fertilized eggs were obtained following superovulation and mating of CBYB6F1/J females (Jackson Laboratories). The purified 7.9 kb insert DNA was injected into the pronuclei of one-cell stage embryos at a concentration of 1.5 μg/ml (~400 copies of DNA per embryo). A total of 122 microinjected eggs were transferred at the 2-cell stage into the oviducts of eight pseudopregnant recipient females on day one of gestation.

Identification of transgenic mice

Mouse tail DNA was analyzed for integration of the transgene by Southern blot or dot blot analysis. The 0.65 kb β-globin intron fragment was used as a probe.

RNA isolation and RNase protection assay

For RNA isolation of dermal and epidermal RNA, mouse tail skin was incubated for 20 min at 37°C in a 2 M NaBr solution. Epidermis and dermis were separated and immediately frozen in liquid nitrogen. RNA isolation and RNase protection assays were performed as recently described (Werner *et al.*, 1992a).

The DNA templates used to detect different FGFs as well as FGFR3 are described elsewhere (Werner *et al.*, 1992b). Other templates were (i) the 325 bp murine FGFR1 fragment (Peters *et al.*, 1992); (ii) the 161 bp fragment corresponding to nucleotides 1493–1654 of the *bek* variant of murine FGFR2 (Raz *et al.*, 1991; it was cloned by PCR and encodes part of the third Ig-like domain of FGFR2, including the IIIc exon); (iii) the 271 bp fragment corresponding to nucleotides 831–1102 of the KGF receptor variant of FGFR2 (Miki *et al.*, 1991; it was cloned by PCR and encodes the complete third Ig-like domain of FGFR2, including exon IIIb); and (iv) a 403 bp fragment containing the coding sequences of the transmembrane region and part of the intracellular juxtamembrane region of FGFR1 at the 5'-end and part of the human growth hormone poly(A) at the 3'-end (see Figure 3B; this fragment was generated by PCR from the transgene plasmid).

In situ hybridization

In situ hybridization of paraffin sections from mouse tail skin was carried out according to Wilkinson *et al.* (1987). A 600 nt cRNA complementary to the human growth hormone polyadenylation sequence was used as a probe.

Western blot analysis of the truncated FGF receptor in the skin

Back skin, tail skin and whole ears were frozen in liquid nitrogen. 100 mg tissue were homogenized in 3 ml 2×lysis buffer [1×lysis buffer: 1% Triton X-100, 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM PMSF, 1% aprotinin (0.15 U/ml), 15 μg/ml leupeptin, 10 μg/ml pepstatin]. The tissue extract was cleared by centrifugation and the supernatant diluted 1:1 with water. 1 ml of each diluted sample was cleared by centrifugation. FGF receptor proteins were enriched by binding to wheatgerm agglutinin–Sepharose beads (Lee *et al.*, 1989) and detected by Western immunoblotting using the polyclonal antiserum Ab15 which is directed against the extracellular region of FGFR1 (Werner *et al.*, 1992a). The antiserum detects FGFR1, FGFR2 and to a lesser extent also FGFR3.

Histology

Skin sections were fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Sections (6 μm) were stained with hematoxylin–eosin.

Indirect immunofluorescence of skin sections

Double-label immunofluorescence analysis was carried out using monospecific polyclonal antibodies to keratins as previously described (Roop *et al.*, 1984, 1985, 1987). Carnoy's fixed skin biopsy specimens were incubated overnight with affinity purified rabbit antiserum to keratins K1, K10, K5, K14 or K6 (1:500) and antiserum raised in guinea-pig to K14 (1:1000). Sections were then incubated for 30 min at room temperature with normal goat serum (1:200, Kirkegaard and Perry, Gaithersburg, MD) and biotinylated goat anti guinea-pig IgG (1:100, Vector Laboratories, Burlingame, CA). They were subsequently incubated with FITC-conjugated swine anti-rabbit IgG (1:40, Dako Corp., Santa Barbara, CA) and streptavidin–Texas red (1:400, Bethesda Research Laboratories, Gaithersburg, MD) for 30 min at room temperature. All reagents were diluted in PBS containing 12% bovine serum albumin. Slides were examined under a Nikon Labophot microscope with an epifluorescence attachment.

Detection of proliferating cells by labelling with 5'-bromodeoxyuridine

Transgenic mice and control mice were injected (i.p.) with BrdU (Sigma, 250 mg/kg in 0.9% NaCl). Mice were sacrificed 2 h after BrdU injection. Tail skin was fixed in cold 70% ethanol and paraffin embedded. Sections were stained with an FITC-conjugated monoclonal antibody against BrdU (Boehringer Mannheim Biochemicals) as described by the manufacturer.

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