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Genetically Null Mice Reveal a Central Role for Epidermal Growth Factor Receptor in the Differentiation of the Hair Follicle and Normal Hair Development

Laura A. Hansen,* Natalie Alexander,* Margaret E. Hogan,† John P. Sundberg,† Andrzej Dlugosz,* David W. Threadgill,‡ Terry Magnuson,‡ and Stuart H. Yuspa*

From the Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, The Jackson Laboratory,† Bar Harbor, Maine, and the Department of Genetics,‡ Case Western Reserve University, Cleveland, Ohio*

Mice harboring a targeted disruption of the epidermal growth factor receptor (EGFR) allele exhibit a severely disorganized hair follicle phenotype, fuzzy coat, and systemic disease resulting in death before 3 weeks. This skin phenotype was reproduced in whole skin grafts and in grafts of EGFR null hair follicle buds onto nude mice, providing a model to evaluate the natural evolution of skin lacking the EGFR. Hair follicles in grafts of null skin did not progress from anagen to telogen and scanning electron micrografts revealed wavy, flattened hair fibers with cuticular abnormalities. Many of the EGFR null hair follicles in the grafted skin were consumed by an inflammatory reaction resulting in complete hair loss in 67% of the grafts by 10 weeks. Localization of follicular differentiation markers including keratin 6, transglutaminase, and the hair keratins* mHa2 *and* hacl-1 *revealed a pattern of premature differentiation within the null hair follicles. In intact EGFR null mice, proliferation in the interfollicular epidermis, but not hair follicles, was greatly decreased in the absence of EGFR. In contrast, grafting of EGFR null skin resulted in a hyperplastic response in the epidermis that did not resolve even after 10 weeks, although the wound-induced hyperplasia in EGFR wild-type grafts had resolved within 3 to 4 weeks. Thus, epithelial expression of the EGFR has complex functions in the skin. It is important in delaying follicular differentiation, may serve to protect the hair follicle from immunological reactions, and modifies both normal and wound-induced epidermal proliferation but seems dispensable for follicular proliferation. (Am J Pathol 1997, 150:1959–1975)

Understanding the regulation of the development and differentiation of the hair follicle is a major challenge to skin biologists. Hair follicles are composed of multiple layers of highly specialized epithelial cells, some of which differentiate as they move toward the surface of the skin to form a terminally differentiated hair shaft. The complexity of this structure is increased by recurrent cycling between active hair-growing and resting stages. It has been difficult to dissect the roles of local and systemic factors in the regulation of hair follicle cycling and hair differentiation. The epidermal growth factor receptor (EGFR), which is expressed in both the epidermis and the hair follicle,[1–4] has been implicated in hair follicle development.[3,5,6] In general, the EGFR is localized to the proliferating epithelial components of the skin but is paradoxically absent from the apex of the downgrowing anagen hair follicle.[3,7] Administration of epidermal growth factor (EGF) to newborn mice delays hair follicle development,[5,6] decreases the rate of hair growth,[5] and reduces hair diameter.[5] In addition, exogenous EGF results in thickening,[8] hyperkeratinization,[8] and hyperproliferation[8,9] in the epidermis. EGF and other EGFR ligands are potent mitogens for cultured keratinocytes[10–12] and promote cell migration.[13]

Mouse mutants with partially abrogated signaling through the EGFR have been characterized with defects in normal hair development. These include

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Address reprint requests to Dr. Stuart H. Yuspa, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Building 37, Room 3B25, MSC 4255, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Bethesda, MD 20892-0001.

waved-2, a spontaneous mouse mutation of the EGFR,[14] *waved-1*, a spontaneous mouse mutation of transforming growth factor-α (TGFα),[15] and genetically engineered TGF-α null mice resulting from the disruption of the TGF-α gene by homologous recombination.[16,17] All of these mutant mice exhibit a similar abnormal skin phenotype characterized by wavy hair, curly vibrissae, and follicular disorganization,[14–17] the severity of which decreases with increasing age.[16,17] Overexpression of TGF-α targeted to the skin, in contrast, results in hyperplasia and hyperkeratosis in the epidermis, which also decreases with increasing age of the mice.[18,19]

Recently, EGFR null mice[20–22] and mice with a dominant negative EGFR targeted to the skin via the bovine keratin 5 promoter[23] have been developed. All have an abnormal hair phenotype similar to but more severe than the natural mutants *waved-1* and *waved-2* or the TGF-α null genetically engineered mice. Survival of EGFR null mice varies dramatically depending on genetic background.[20,21] On a CD-1 background, approximately 50% of EGFR null embryos survive to birth, and of these, 10% survive to 18 days postnatally, although they exhibit many abnormalities including malformations of the kidneys, liver, colon, and brain.[20] EGFR null mice develop a delayed and fuzzy coat between 10 and 14 days postnatally, and the hair follicles are crowded and disoriented and occasionally appear to be inverted.[20,24] In contrast, the EGFR-deficient epidermis is hypoplastic.[20,24] The skin targeted dominant negative EGFR mouse displays a similar abnormal hair phenotype early in life with dramatic alopecia and dermal inflammation later, along with epidermal hyperproliferation.[23]

The profound influence of EGFR loss on normal hair development in EGFR null mice provides a unique model to examine the contributions of the EGFR to follicle cycling, follicle migration, hair differentiation, and epithelial-mesenchymal interactions. By grafting skin or skin cells from null and control genotypes to nude mouse hosts, we were able to directly examine the influence of the EGFR on the skin phenotype in the absence of systemic influences. This report implicates EGFR as an important regulator of the maintenance and differentiation of the hair follicle, specific to the epithelial compartment. It further suggests that EGFR in normal skin delays follicular differentiation without influencing follicular proliferation. In contrast, the EGFR modifies normal interfollicular epidermal proliferation without impacting on epidermal differentiation.

Materials and Methods

Animals

Homozygous or heterozygous EGFR null mice and wild-type siblings on a CD-1 background were developed using homologous recombination in 129/Sv-derived D3 embryonic stem cells.[20] Mice were genotyped using polymerase chain reaction as described elsewhere.[20] Pieces of full-thickness hairless skin from 2- to 5-day-old EGFR null or wild-type mice, approximately 2 cm in diameter, were grafted onto BALB/cByJ *HFh11^{nu}/Hfh11^{nu}* mice as described previously.[25] The donor skin was oriented in a reverse direction (ie, head to tail) on a wound created by removing a circular piece of full-thickness skin from the dorsum of the host animal. The junction of the graft and host skins was sealed with Superglue, and the grafts were held in place using VetWrap bandages (3M Corp., MN) for 1 week. This procedure produces a normal, haired donor skin.[25] At least five grafts of each genotype were removed at each time point from host mice between 2.5 and 10 weeks after grafting. Newborn mouse skin was floated overnight on 0.25% trypsin (Gibco, Grand Island, NY) and separated into dermal and epidermal fractions. Hair follicle buds were prepared from the epidermis using low-speed centrifugation and a Ficoll gradient.[26] Dermal cells were prepared from the dermis after 0.35% collagenase digestion.[26] Combination grafts of isolated cells consisting of hair follicle buds or dermal fibroblasts from null or wild-type genotypes were transplanted onto athymic nude mice as described elsewhere.[26] Mice were observed weekly and hair growth quantitated on a scale of 0 to 6. Full-thickness skin or isolated cell grafts were considered to be successful (takes) when hair was observed in the graft site. Approximately 70 and 75% of null and wild-type grafts, respectively, were successful. Unsuccessful grafts were excluded from further analysis.

Scanning Electron Microscopy (SEM)

A 1-cm^2 sample of mouse skin was removed from the graft site, avoiding the subcutaneous fat layer. The samples were placed, connective tissue side down, on dry nylon mesh and immersed in cold 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer. After overnight fixation at 4°C, samples were washed twice with 0.1 mol/L cacodylate buffer and post-fixed in 0.5% osmium tetroxide in 0.1 mol/L cacodylate buffer. Samples were subsequently dehydrated in a series of graded ethanols to 100%. After three changes in 100% ethanol, samples were critical point dried, attached to aluminum stubs with silver adhesive, and sputter coated with

15 nm of gold. Samples were examined in a JEOL 35C scanning electron microscope operated at 10 kV. Plucked hairs from skin grafts were directly mounted onto aluminum stubs with double-stick tape. Representative hairs from the four major truncal hair types,[27] if identifiable in mutants, were chosen. Samples were sputter coated with gold and examined as described above.

Bromodeoxyuridine (BrDU) Labeling and Immunohistochemistry

Dorsal skin from mice that were injected intraperitoneally with BrDU (250 μg/g) 1 hour before sacrifice was fixed in 70% ethanol, processed, embedded in paraffin or embedded in OCT (Miles, Elkhart, IN), and flash frozen for frozen sections. Sections were immunohistochemically stained for BrDU as described elsewhere[28] using an anti-BrDU antibody (Becton Dickinson, San Jose, CA), a biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA), horseradish-peroxidase-conjugated ABC reagent (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the substrate (Sigma Chemical Co., St. Louis, MO) and a hematoxylin counterstain. Interfollicular epidermal BrDU incorporation was quantitated by counting BrDU-labeled nuclei in 100 basal interfollicular epidermal cells from three randomly chosen areas in BrDU-stained sections of skin. Follicular proliferation was quantitated by counting BrDU-labeled and unlabeled nuclei in each of 10 hair follicle bulbs from longitudinal sections of skin.

Immunohistochemistry

For immunohistochemistry of differentiation markers, monospecific rabbit antisera produced against keratin 1, keratin 6, keratin 10, keratin 14, loricrin, or filaggrin[29] was incubated with paraffin-embedded skin sections, followed by incubation with anti-rabbit biotinylated secondary antibody (Vector), an ABC reagent (Vector), diaminobenzidine substrate (Sigma), and contrast green counterstain (Kierkegaard and Perry, Gaithersburg, MD). For immunohistochemistry using antibodies to EGFR (Life Technologies, Gaithersburg, MD) or SPR1 (obtained from T. Kartasova, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD), frozen sections were fixed in acetone and then incubated with primary antibody, followed by incubation with biotinylated anti-sheep (EGFR) or biotinylated anti-rabbit (SPR1) secondary antibody (Vector), ABC reagent (Vector), diaminobenzidine (Sigma), and contrast green counterstain (Kierkegaard and Perry).

Histochemical Localization of Transglutaminase Activity

Transglutaminase activity was detected using dansylcadaverine fluorescence in frozen sections of skin as described by Bernard et al.[30] Briefly, OCT-embedded frozen sections were hydrated in phosphate-buffered saline (PBS), incubated in 2 mmol/L dansylcadaverine solution containing 0.05 mol/L Tris, pH 8, 0.15 mol/L sodium chloride, 10 mmol/L calcium chloride, 2.5 mmol/L dithiothreitol, and 0.25% Triton X-100 for 1 hour at room temperature, washed three times in PBS followed by a final wash in water, and mounted using Aquamount and a coverslip. As a negative control, a serial section was treated in the same manner as described above except that 1 mmol/L EDTA was substituted for the calcium chloride.

Immunoblot Analysis

Homogenates from whole skin from EGFR homozygous null, heterozygous, and wild-type newborn, 5-day-old, and 18-day-old mice were prepared by 20 seconds of Polytron homogenization of skin in either Triton X-100 lysis buffer (for EGFR analysis) containing 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L phenylmethysulfonylfluoride, and 10 μg/ml aprotinin or sodium dodecyl sulfate (SDS) lysis buffer (for analysis of keratins and SPR1) containing 0.25 mol/L Tris/HCl (pH 6.8), 5% SDS, and 20% β-mercaptoethanol followed by centrifugation at 16,000 $\times$ *g* for 10 minutes. Equal amounts of homogenate protein were loaded onto SDS-PAGE gels for electrophoresis followed by transfer to nitrocellulose. Nitrocellulose blots were incubated with anti-EGFR or -SPR1 antibodies, followed by incubation with horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) and the signal detected using Renaissance chemiluminescence reagents (Dupont NEN, Boston, MA) and autoradiography film (Kodak, Rochester, NY).

In Situ *Hybridization*

Plasmids consisting of the Bluescript II KS+ or pGem3Z vector containing a 244- or 200-bp insert for *mHa2* or *hacl-1* were obtained from J. Schweizer (Institute of Applied Tumor Virology, Heidelberg, Germany) or T. Kuroki (University of Tokyo, Tokyo, Japan),

respectively. Radiolabeled sense and antisense probes were prepared using T7 and T3 RNA polymerases, respectively, to transcribe sequences in plasmid linearized with *Nar*I or *Sma*I, respectively, using uridine 5′-(α-[^{35}S]thio)triphosphate and a Riboprobe Gemini II kit (Promega, Madison, WI). Approximately equal incorporation of the radiolabel was found for the antisense and sense riboprobes. Ethanol-fixed, paraffin-embedded dorsal skin was probed as described elsewhere,[31] adapted from Newbold et al.[32]

Results

Timing of Follicular EGFR Expression in Wild-Type Mouse Skin Correlates with the Onset of the Hair Follicle Phenotype in EGFR Null Mice

In wild-type skin, EGFR was expressed in both the basal and suprabasal layers of the epidermis from newborn and 2-, 5-, 8-, 12-, and 18-day-old mice (Figure 1, A, C, and E, and data not shown) when examined with an antibody recognizing the cytoplasmic carboxyl-terminal region of the receptor. Consistent with previous reports in human skin,[1] developing wild-type anagen hair follicles exhibited little immunodetectable EGFR when invading the dermis in the first hair cycle (Figure 1A). By 5 days and thereafter, EGFR was detected in the outer root sheath of hair follicles as well as the epidermis of the wild-type skin (Figure 1C). These results suggest that EGFR-dependent signaling probably does not play a role in the earliest stages (before 5 days) of hair follicle development in the mouse, consistent with the lack of EGFR expression in the earliest stage of follicle development also reported in human, rat, and sheep skin.[7,33,34] Expression of EGFR at 5 days correlates with the onset of the abnormal, disorganized hair follicle phenotype in the null mice[20] and normal hair emergence in the wild-type animals. Similar immunostaining for EGFR was not detected in null mouse skin, although some regions demonstrated a faint signal, particularly suprabasally in the epidermis and in the bulb regions of the older follicles (Figure 1, B, D, and F). On immunoblots to detect EGFR in extracts of null skin, two faint bands can be seen, one with slightly higher mobility than the EGFR at 180 kd and another at approximately 150 kd (Figure 1G). The second band could represent the mRNA splicing variant lacking a portion of the extracellular domain described previously for this EGFR mutant.[20] However, EGFR catalytic activity is not detected in the skin cells of these animals (A. Dlugosz, L. Hansen, C. Cheng, N. Alexander, M. Denning, D. Threadgill, T. Magnuson, R. Coffey, and S. Yuspa, manuscript in preparation).

Delayed Hair Development, Decreased Graft Size, Increased Fragility of Hair Fiber, and Disorganized Hair Follicle Phenotype Found in EGFR-Deficient Skin Grafts

Because of the shortened life span and growth retardation of the EGFR null mouse, grafting of skin explants from 2- to 5-day-old null and wild-type mice onto athymic *HFh11^{nu}/Hfh11^{nu}* nude mouse hosts was performed to observe the effects of loss of EGFR in older skin without the compromising effects of systemic disease. In contrast to the hair fibers seen in the grafts of wild-type skin, which were mostly long and straight (Figure 2, A and B), EGFR null skin produced curly or wavy hair fibers that were much shorter at 2 and 3 weeks after grafting (Figure 2, A and C). The tips of these fibers appeared intact when magnified, suggesting that the shorter hair was not due to breakage or increased fragility. By 5 weeks, the hair on EGFR null skin grafts was much longer, similar to that of wild type, although it still appeared wavy or curly (Figure 2, D–F). These results are consistent with a delay or retardation in hair formation in grafted EGFR null skin analogous to the delayed coat formation in intact skin. Although explants of similar size were grafted onto athymic mice, after 10 weeks, EGFR null grafts were 35% smaller than wild-type grafts (Table 1). By 10 weeks after grafting, hair fibers on EGFR null but not wild-type skin were often very short and of irregular length (Figure 2, G–I), suggesting that breakage of the hair had occurred. Histologically, the disorganized hair follicle phenotype described in intact EGFR null mice[20] was reproduced in whole skin grafts (Figure 3B). EGFR null hair follicles exhibited striking disorganization, irregular placement, horizontal orientation, crowding, and multiple columns of out of register medullated cells within the hair shafts (Figure 3, B and G).

SEM Revealed Multiple Hair Shaft Abnormalities in EGFR Null Skin Grafts

Samples of skin were removed from EGFR null and wild-type grafts 2.5 or 3.5 weeks after grafting and processed for SEM. The 2.5-week specimens of wild-type skin consisted of an abundance of slightly curving hairs forming a dense mat with normal hair fibers exiting follicles (Figure 4A). The fibers had distinct, sharp cuticles with some debris

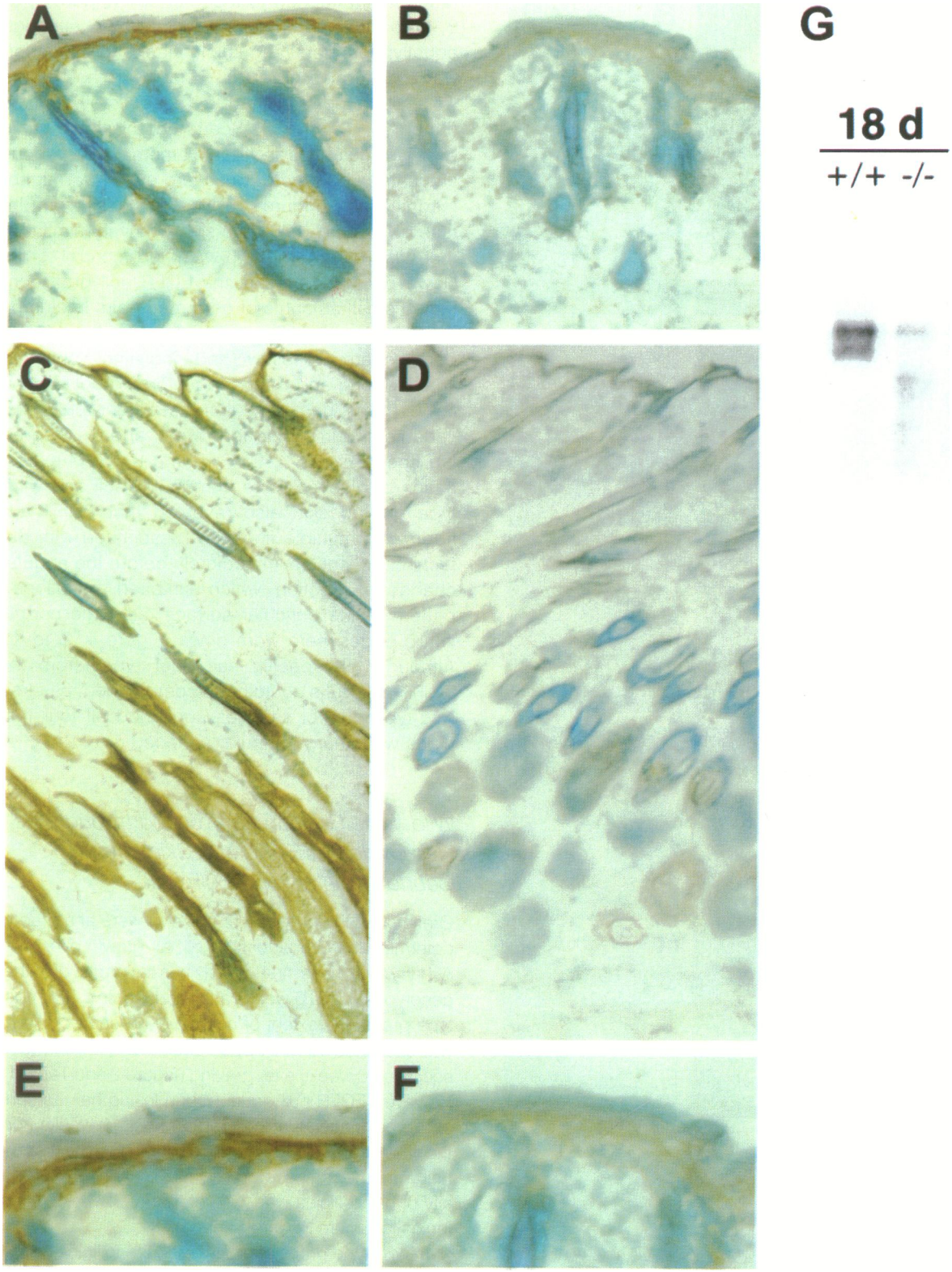

Figure 1. *EGFR expression in skin. Wild-type* (A, C, *and* E) *and homozygous EGFR null* (B, D, *and* F) *skin sections from 2-day-old* (A, B, E, *and* F) *and 12-day-old* (C *and* D) *mice were immunohistochemically stained for EGFR as described in Materials and Methods. Homogenate from 18-day-old EGFR null and wild-type skin was immunoblotted using an anti-EGFR antibody* (G). *Magnification,* ×*200* (A *and* B), ×*150* (C *and* D), *and* ×*400* (E *and* F).

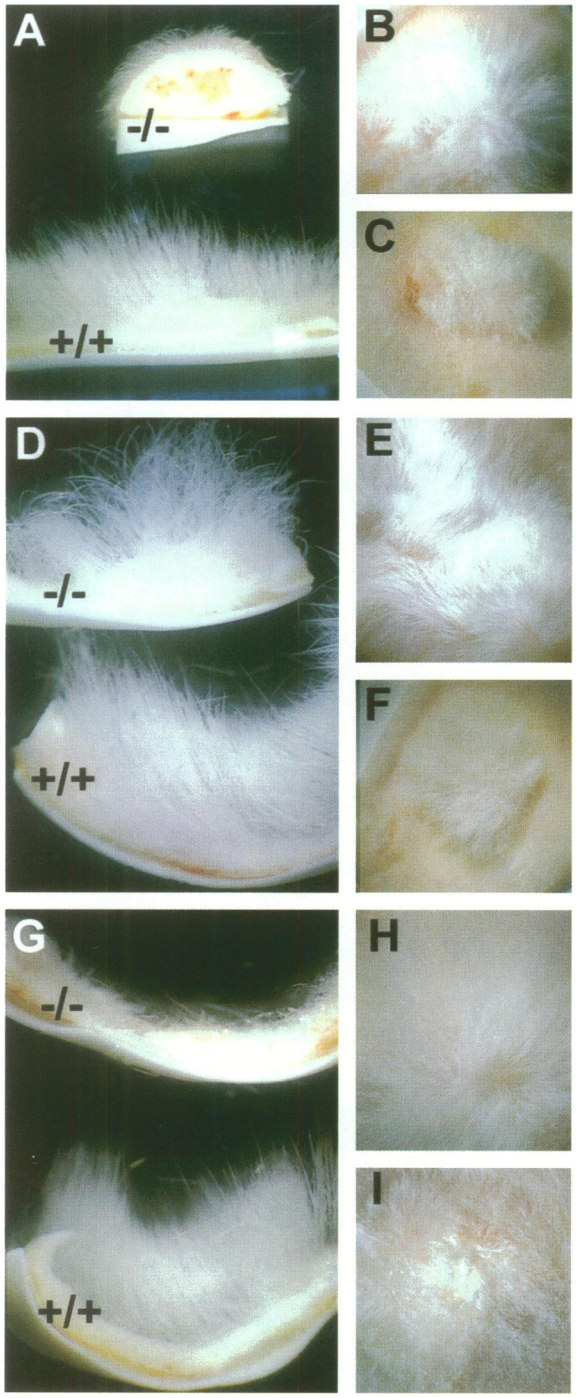

Figure 2. *Whole skin grafts of EGFR-deficient skin onto nude mice produced a shorter, curly hair and a smaller graft size. In representative grafts from EGFR wild-type* (A, B, D, E, G, *and* H) *and null* (A, C, D, F, G, *and* I) *skin onto athymic nude mice, some show a cross section of the grafted skin placed on Millipore filter paper* (A, D, *and* G). *Animals were euthanized 3* (A *to* C), *5* (D *to* F), *or 10* (G *to* I) *weeks after grafting. Magnification, approximately* ×7 (A, D, *and* G) *and* ×3 (B, C, E, F, H, *and* I). *Each photograph of a single graft is representative of at least four examined for each genotype at each time point.*

(squames) attached to their surface (Figure 4, C, E, and I). By contrast, the mutant hair, although equally abundant and thick, had a pronounced wavy pattern (Figure 4H). Emerging hair fibers were flattened and had a recognizable cuticle (Figure 4, D and F). Scattered missing cuticle cells, irregularities in fiber diameter, and various amounts of surface debris were evident at higher magnification (Figure 4F). By 3.5 weeks from the time of grafting, wild-type skin was very similar to that taken from the 2.5-week-old control graft. However, at 3.5 weeks, the EGFR null graft contained small amounts of deformed hairs in clumps with flattened hair fibers (Figure 4B). These fibers demonstrated shallow and indistinct cuticular scale, a long and deep longitudinal groove in the larger fibers, generally decreased fiber thickness, variable and irregular fiber width, and disintegrating fibers (Figure 4, D, F, J, and K). SEM examination of plucked hairs from EGFR null and wild-type grafted skin also revealed that EGFR null fibers had multiple fine kinks associated with defects in the cuticle immediately before the angular change in direction, which exhibited a scalloped pattern of the cuticular surface, as if the normal sharp edges were worn down (Figure 4, G to K). Although the specific hair fiber types from the EGFR null grafts could not be determined, plucked wild-type control samples contained all four hair types with sharp cuticular surface.

Wild-Type Skin Grafts Proceed through the Hair Cycle but EGFR Null Skin Grafts Cannot Maintain Hair Follicles

Grafts of EGFR wild-type or null skin were analyzed to determine the characteristics of hair growth over time. A hairy coat was first noted in skin grafts of either genotype by 2 to 3 weeks after grafting. In EGFR wild-type grafts, the extent of hair covering each graft site was sustained from its appearance at 2 to 3 weeks until sacrifice 10 weeks after grafting (Figure 2 and Table 1). However, EGFR null grafts began losing hair as early as 4 weeks after grafting (Table 1). By 10 weeks from the time of grafting, 67% of EGFR null grafts lost all the hair from the graft site grossly (Table 1), and the density of hair follicles was 17% of that at 3 weeks, histologically (Figure 3E and Table 1).

In wild-type grafts after 5 weeks, the follicles had, in general, entered telogen (Figure 3C). However, in EGFR null skin, pockets of inflammation associated with the hair follicles were observed histologically from 5 to 10 weeks, and no telogen hair follicles were de-

Table 1. *Loss of Hair Follicles and Decreased Graft Size in EGFR-Deficient Graft Skin*

Time (weeks)	Hair follicles/mm graft skin (microscopically)		Grafts with hair (grossly)		Graft size (cm)	
	−/−	+/+	−/−	+/+	−/−	+/+
3	9.0 ± 1.3 (n = 3)	9.0 ± 1.1 (n = 3)	6/6	10/10	ND	ND
5	4.1 ± 0.9 (n = 5)	7.3 ± 1.3 (n = 4)	4/6	10/10	ND	ND
10	1.5 ± 0.2* (n = 6)	5.3 ± 0.7 (n = 9)	2/6	10/10	0.79 ± 0.27 (n = 6)†	1.22 ± 0.27 (n = 8)

Hair follicles in at least three 1-mm long sections of H&E-stained skin sections from three to nine EGFR-deficient and wild-type skin grafts per time point were counted at ×100 using an ocular micrometer, and the mean ± SE is reported as hair follicle/mm graft skin. The presence or absence of graft skin hair was determined grossly and is reported as the proportion of grafts with hair relative to the number of successful grafts. Graft size was measured grossly 10 weeks after grafting using a ruler and is reported as the mean ± SE of six or eight grafts in EGFR-deficient and wild-type skins, respectively. ND, not done.

*Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.05$.

†Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.10$.

tected (Figure 3D). Coincident with hair loss in EGFR null skin was an inflammatory infiltrate particularly dense in the dermis surrounding a few remnants of hair follicles (Figure 3D). The inflammatory infiltrate contained plasma cells, giant cells, and macrophages, and fragments of hair were embedded in the thickened stratum corneum (data not shown). Eventually, all hair follicles disappeared from many of the graft sites (Figure 3E and Table 1). Thus, EGFR-deficient skin was unable to maintain hair follicle integrity or to cycle into telogen even when the host was in good health and deficient in cellular immunity.

EGFR-Deficient Skin Grafts Respond Aberrantly to the Wound Environment

EGFR-deficient intact skin was hypoplastic by day 18 but not by day 8[20] (Table 2). Although the hair follicle phenotype of intact EGFR null mouse skin was reproduced in the null grafts (Figure 3B), interfollicular epidermal hypoplasia was not detected as late as 10 weeks in grafted skin (Table 2 and Figure 3E). Between 19 and 32 days from the time of grafting, the epidermis in many of the grafts from both genotypes was hyperplastic (Table 2 and Figure 3,

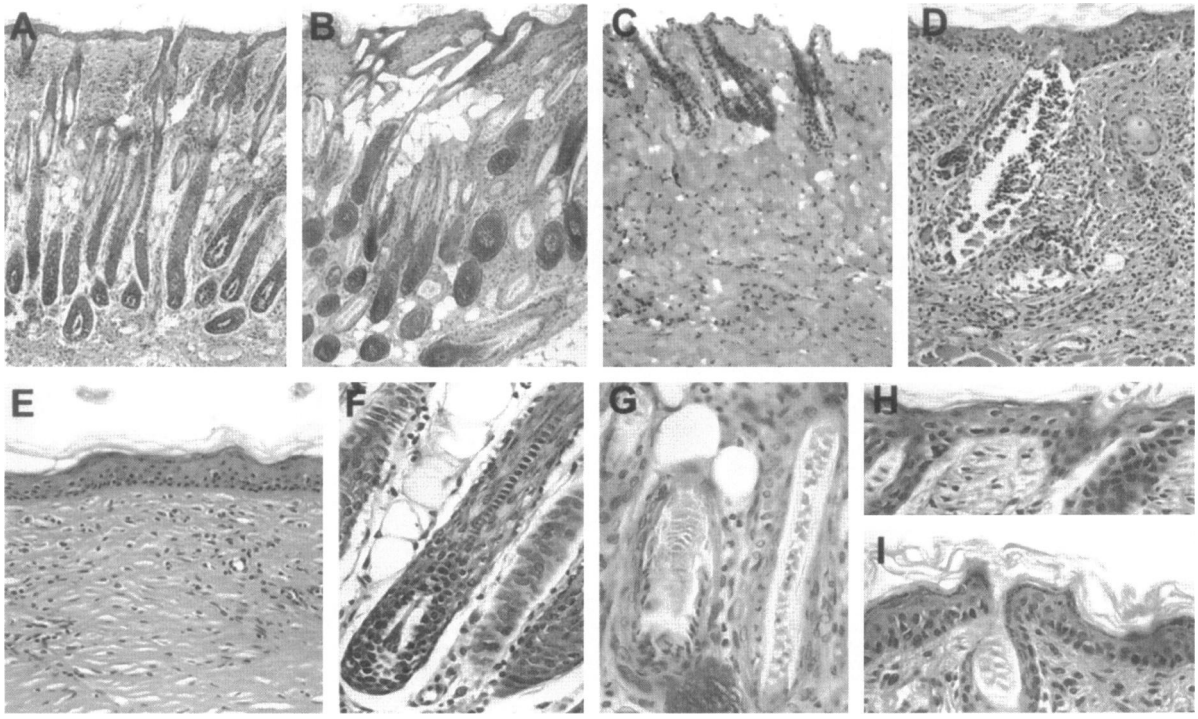

Figure 3. *Histological analysis of EGFR wild-type and null skin at various times after grafting to nude mice. H&E-stained sections of EGFR null* (B, D, E, G, *and* I) *or wild-type* (A, C, F, *and* H) *grafted skin 21* (A, B, F, G, H, *and* I), *35* (C *and* D), *or 70* (E) *days after grafting. Magnification,* ×*60* (A *to* E) *and* ×*400* (F *to* I). *Each section is characteristic of sections examined from at least four animals per time point.*

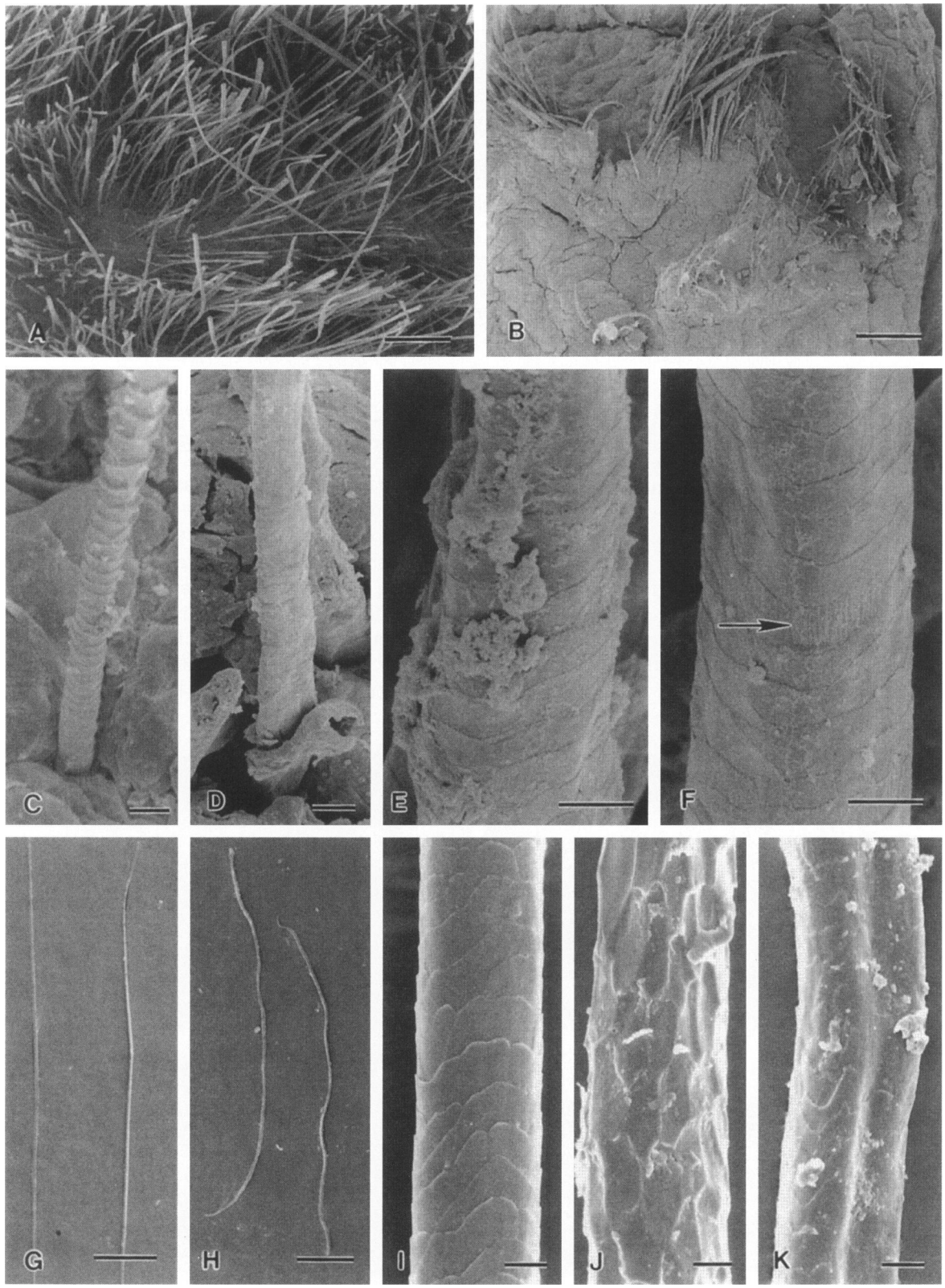
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Table 2. *Epidermis in EGFR-Deficient Skin Grafts, but Not Intact Skin, Are Hyperplastic in Comparison with EGFR Wild-Type Skin and Grafts*

Age (days)	Thickness (μm)		Number of cell layers	
	−/−	+/+	−/−	+/+
0 (intact)	28.5 ± 0.9	30.4 ± 2.0	5.5 ± 0.2	5.7 ± 0.2
2 (intact)	29.0 ± 3.7	29.0 ± 2.2	5.0 ± 0.4	5.6 ± 0.4
5 (intact)	24.6 ± 4.7	24.3 ± 1.5	4.9 ± 0.1	5.2 ± 0.4
8 (intact)	15.4 ± 0.0	18.6 ± 1.2	3.4 ± 0.4	3.4 ± 0.4
21 (graft)	35.4 ± 6.8†	22.3 ± 2.9	5.3 ± 0.5*	3.7 ± 0.5
35 (graft)	26.0 ± 1.9*	11.6 ± 1.1	4.2 ± 0.3*	2.1 ± 0.1
70 (graft)	28.6 ± 4.4*	10.4 ± 0.7	6.0 ± 0.7*	2.1 ± 0.1

Epidermal thickness (using an ocular and stage micrometer) and the number of nucleated cell layers were quantitated in H&E-stained skin sections at five randomly selected locations. Greater than or equal to two samples per time point for 0, 2, 5, and 8 days and greater than or equal to four samples for 21, 35, and 70 days were examined.

*Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.05$.

†Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.10$.

A, B, H, and I), but by 5 weeks, the hyperplasia had subsided in the wild-type but not EGFR-deficient graft skin. As seen in Table 3, epidermal BrDU labeling was decreased from 2 to 8 days of life in EGFR null newborns *in situ* compared with wild-type epidermis, but epidermal BrDU labeling was actually three times greater than controls in 10-week null grafts. Similarly, EGFR-deficient skin grafts remained hyperplastic with an average thickness of 28.6 μm and 6 nucleated cell layers whereas wild-type grafts had a relatively normal epidermal thickness of 10.4 μm and 2.1 cell layers (Table 2). Thus, grafted EGFR-deficient epidermis responded to activation by the wound-healing environment with a proliferative response much as did wild-type epidermis. However, this hyperplasia did not resolve even after 10 weeks. The hyperplasia that was sustained at 10 weeks in EGFR-deficient skin may be related to the inflammatory response involving the hair follicles, although there was not a direct correlation of focal inflammatory infiltrate and overlying hyperplastic epidermis and many of the skin grafts had been hairless for several weeks before the measurements were made (Table 1 and Figure 3E).

Table 3. *Decreased Epidermal Proliferation in EGFR-Deficient Intact, but Not Grafted, Mouse Skin*

Age (in days)	BrdU labeling (%)	
	+/+ EGFR genotype	−/− EGFR genotype
0 (intact)	11.0 ± 5.0 (n = 2)	8.0 ± 4.0 (n = 2)
2 (intact)	16.0 ± 0.5 (n = 2)	7.5 ± 1.5 (n = 2)*
5 (intact)	17.0 ± 2.0 (n = 2)	11.0 ± 1.0 (n = 2)
8 (intact)	17.0 ± 2.0 (n = 2)	5.5 ± 3.5 (n = 2)
70 (grafted skin)	4.6 ± 1.0 (n = 5)	15.5 ± 2.5 (n = 5)*

Proliferation was quantitated by counting BrDU-labeled cells, as described in Materials and Methods, in sections of skin from newborn (0 days) or 2-, 5-, or 8-day-old EGFR null and wild-type mice or in sections of grafts from EGFR null or wild-type skin onto athymic nude mice that were harvested after 70 days. One hour before sacrifice, mice were injected with BrDU, pieces of skin were fixed and processed for immunohistochemistry, and the number of BrDU-labeled basal cells counted and expressed as the percentage of total basal cells ± SE. n, number of skin samples analyzed.

*Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.05$.

Recombination Grafting Experiments Reveal that the Hair Follicle Defect Is in the Epithelium

To determine whether the aberrant hair phenotype depended on EGFR in the epithelial or mesenchymal population, recombinant grafts of primary keratinocytes or hair follicle buds with primary dermal fibroblast cell preparations from EGFR-deficient and wild-type mice were placed on *HFh11^{nu}/Hfh11^{nu}* athymic mice. When EGFR null keratinocytes or follicle buds were grafted together with dermal cells from either EGFR-deficient or wild-type mice, a shorter, wavy hair resulted at 2 to 3 weeks after grafting (Table 4) similar to that shown in Figure 2. In contrast to this, nude mice with wild-type keratinocytes or hair follicle buds grafted with either wild-type or EGFR-deficient dermal cells had grafts containing normal-appearing, longer hairs

Figure 4. *SEM of skin grafts and plucked hairs.* **A** *and* **B**: *Low magnification of control* (**A**) *and null* (**B**) *skin reveals the dense normal pelage* (**A**) *compared with the loss of hair fibers in 3.5-week-old null skin grafts* (**B**) *onto nude mice. Bar, 500 μm.* **C** *and* **D**: *Normal hair fibers emerge long and straight and with a sharp cuticular scale* (**C**). *By contrast, null hair fibers emerge longitudinally flattened with a slight wave from skin covered with prominent cornification and desquamation* (**D**). *Bar, 10 μm.* **E** *and* **F**: *Higher magnification of null hair fibers reveals longitudinal grooving, accumulation of debris, changes in fiber diameter, shallow cuticular scales, and striations on individual scales* (arrow). *Bar, 5 μm.* **G** *and* **H**: *Plucked hairs consisted of normal long and straight guard hairs* (**G**) *in the normal mice compared with short, irregularly waved, and broken EGFR null hairs* (**H**). *Bar, 500 μm.* **I** *to* **K**: *Higher magnification revealed normal straight hairs with sharp, prominent cuticular scales* (**I**) *compared with the null hairs that were wavy and had worn cuticular scales, surface debris, and a longitudinal groove with irregular sides* (**J** *and* **K**). *Bar, 10 μm. Two skins per genotype were analyzed.*

Table 4. *Recombination of EGFR Null and Wild-Type Hair Follicle Buds or Epidermal Keratinocytes with Dermal Cells Shows That the Epithelial EGFR Genotype Determines the Hair Follicle Phenotype*

Genotype*		Skin phenotype	
Keratinocytes	Dermal cells	Hair quality	Proportion with hair at 7 weeks
−/−	−/−	−/−	0/2
+/+	−/−	+/+	2/2
−/−	+/+	−/−	1/3
+/+	+/+	+/+	3/3
(BALB/c)	−/−	+/+	5/5
(BALB/c)	+/+	+/+	5/5

Epithelial cells and dermal cells prepared from EGFR null, wild-type, and BALB/c controls were combined as indicated above, grafted onto athymic nude hosts, and monitored weekly for the presence and quality of the graft hair. The phenotype of the graft skin was determined by hair quality, which was characterized as either wavy (−/−) or long and straight (+/+).

*All cells are of 129/SV/CD-1 strain unless identified as BALB/c. The −/− designates the EGFR null genotype and +/+ the EGFR wild-type genotype.

(Table 4). After 6 weeks, no visible hair or intact hair follicles remained on many grafts of EGFR-deficient epithelial cells regardless of the genotype of the dermal cells. Hair loss was associated with bits of hair, and hair follicles among numerous inflammatory cells were seen (Table 4). Thus, the loss of EGFR from the epithelial cell population of the skin alone is sufficient to reproduce the skin phenotype seen in the EGFR null mouse and the null skin grafts.

Aberrant Localization of Hair Follicle Markers Suggests Premature Differentiation of the Hair Follicle in EGFR Null Skin

Follicular cells differentiate vertically from the bulb to the surface of the skin coordinate with changes in gene expression related to hair formation. Using intact skin, we examined expression of several genes that have been localized to specific compartments of the differentiating hair follicle to detect defects in follicular differentiation in the EGFR null skin. Keratin 6 (K6) is a structural protein expressed in the innermost layer of the outer root sheath of hair follicles in normal mouse skin.[35,36] Beginning at 5 days and continuing through 18 days, K6 localized to the midportion of the hair follicle of wild-type skin (Figure 5, A and D) but was detected immediately adjacent and superior to the bulb in EGFR null hair follicles (Figure 5, B and C). In some sections of EGFR null mouse skin, K6 expression extended the length of the hair follicle and into the epidermis (Figure 5B).

Transglutaminase initiates cross-linking of proteins as part of the process of terminal differentiation involved in formation of both cornified envelopes in the epidermis and of hair in hair follicles. Transglutaminase activity was localized histochemically by the calcium-dependent cross-linking of dansylcadaverine in EGFR null and wild-type skin from mice 8, 12, or 18 days old. As shown in Figure 5, transglutaminase activity appeared immediately adjacent and superior to the hair follicle bulb in the EGFR null mice (Figure 5F), whereas in wild-type skin, activity began in the midportion of the follicles (Figure 5E).

Expression of SPR1, which is a substrate for transglutaminase and a constituent protein of the hair follicle and cornified envelope,[37–39] was assayed by immunostaining and immunoblotting experiments in skin or skin extracts from EGFR null and wild-type skin from 5- and 18-day-old mice. Cytosolic SPR1 was markedly decreased in EGFR null extracts at day 5 and 18 compared with controls (Figure 5G). SPR1 was detected equally in null and control skin by immunostaining at these time points (data not shown). This suggests that SPR1 is cross-linked early in EGFR null follicles where it can be detected *in situ* but is rapidly lost from the soluble protein pool.

Similar premature expression patterns were seen with other hair follicle markers. The hair keratin gene, *hacl-1*, is expressed in the keratogenous zone of the hair cortex in wild-type mouse skin,[40] as seen in samples from 18-day-old (but not in 0-, 2-, or 5-day-old) wild-type mice by *in situ* hybridization (Figure 6). In contrast, *hacl-1* expression in 18-day-old EGFR null mouse skin was reduced and localized to the bulb of the EGFR null hair follicles (Figure 6). *mHa2* is a hair keratin usually expressed in the hair cuticle beginning at 7 days,[41] as shown in 18-day-old wild-type hair follicles by *in situ* hybridization (Figure 6). As for *hacl-1, mHa2* expression was also reduced in the hair follicles from 18-day-old EGFR null mice and was localized to the hair bulb (Figure 6). These studies also demonstrate the premature formation of the hair shafts in null skin, which appears as brightly refringent in the dark-field analysis (Figure 6). In contrast to the marked alterations in differentiation caused by loss of EGFR in hair follicles, analysis of epidermal differentiation markers K1, K10, loricrin, and filaggrin by immunostaining indicated that the localization and intensity of the staining were similar in intact null and wild-type skin (Figure 7).

Decreased Interfollicular Epidermal Proliferation but Unaltered Follicular Proliferation in EGFR-Deficient Skin

Follicular proliferation was unaffected by loss of EGFR as determined by BrDU labeling in intact skin from EGFR null and wild-type mice (Figure 8, A and

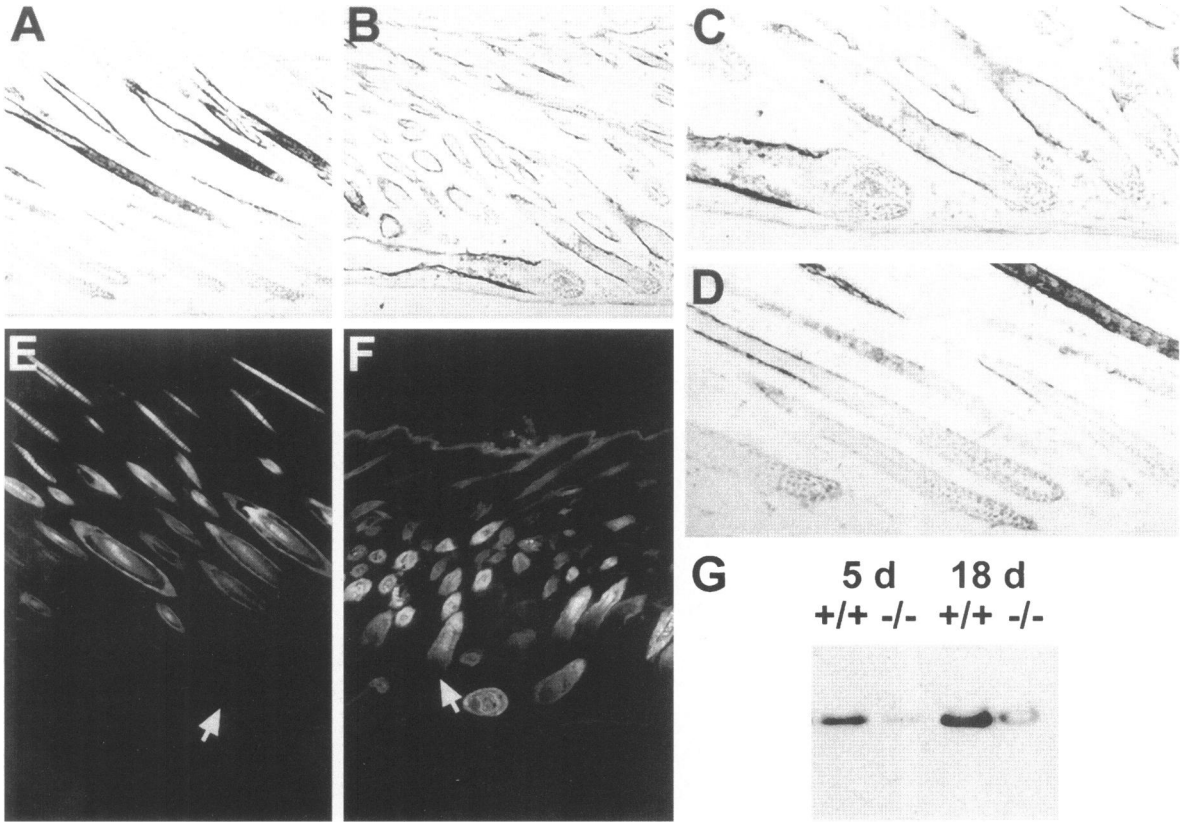

Figure 5. *Altered expression of markers of follicular differentiation in EGFR null skin. Wild-type* (A, D, *and* E) *and EGFR homozygous null* (B, C, *and* F) *skin sections from mice 8* (A *to* D) *or 12* (E *and* F) *days old were immunohistochemically stained for K6* (A *to* D) *or histochemically assayed for transglutaminase activity* (E *to* F) *as described in Materials and Methods. For* A *to* D, *n = 2; for* E *and* F, *n = 1 mouse examined. Magnification, ×60* (A, B, E, *and* F) *or ×300* (C *and* D). *Immunoblotting of whole skin lysates demonstrates decreased SPR1 levels in 5- and 18-day-old EGFR null skin* (G). Arrows *in* E *and* F *indicate bulb region.*

B, and Table 5). Substantial proliferation was detected in the bulb and infundibulum of follicles from EGFR-deficient intact skin (Figure 8B and Table 5). However, EGFR-deficient hair follicle bulbs had 32% fewer cells at 8 days compared with wild-type hair follicle bulbs (Table 5). This decreased bulb cellularity may be the result of premature differentiation and exit of cells from the bulb for more differentiated layers.

Discussion

Although the preponderance of the scientific literature documents an association of EGFR activation with the stimulation of cell proliferation, we have demonstrated here a role for EGFR in the regulation of differentiation of hair follicles in the skin. Our novel data exhibiting a pattern of premature differentiation involving multiple cell types including hair cortex, hair cuticle, and outer root sheath in EGFR null mice lead to a hypothesis that EGFR delays follicular differentiation. EGFR appeared to regulate, directly or indirectly, the expression of hair keratins important in hair formation, the expression of keratin 6, which may be related to differentiation of the outer root sheath, and the activity of the cross-linking enzyme transglutaminase and its substrate SPR1, which are important in the final stages of hair formation. The alterations in expression of K6, *mHa2*, and *hacl-1* reveal that the loss of EGFR results in dysregulated keratin gene expression within the hair follicle and also suggest that it results in premature maturation of the epithelial cells of the hair follicle. Premature differentiation of hair follicle bulb cells may result in early exit from this compartment and thus explain our finding of decreased bulb cellularity in the absence of decreased proliferation in EGFR null skin. Furthermore, cuticle defects and disintegration of hair fibers were found in EGFR null skin, which may be a result of altered gene expression and defective follicular differentiation. Hair abnormalities have also been observed in a skin-targeted dominant negative EGFR transgenic.[23] These results implicate EGFR in the coordination of the complex differentiation and mat-

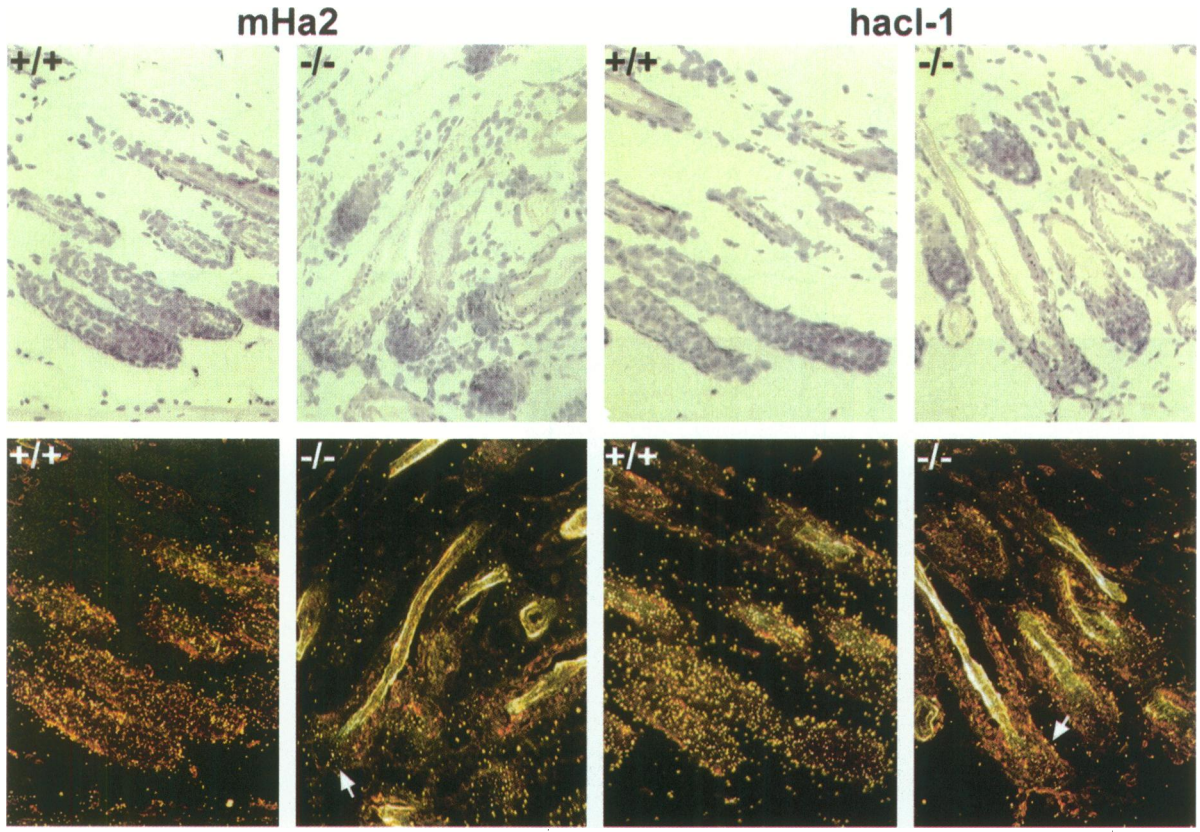

Figure 6. *Decreased expression of hair keratins* mHa2 *and* hacl-1 *mRNA in EGFR-deficient mouse skin. Expression of* mHa2 *and* hacl-1 *mRNA in 18-day-old EGFR null and wild-type skin was determined using* in situ *hybridization with antisense* and sense (data not shown) probes, which were photographed under bright-field (**top**) *or dark-field* (**bottom**) *conditions. Similar results for each genotype were obtained with 8-day-old skin. Magnification, ×300.* **Arrows** *indicate silver grains appearing in the bulb region.*

uration process of the hair follicle resulting in hair formation and hair growth. As EGFR is expressed in the outer root sheath of the wild-type mice, we further propose that the decreased hair keratin gene expression, hair fiber abnormalities, and increased fragility of the hair may be secondary to defects in the outer root sheath. Interestingly, EGFR appeared to be important in delaying or preventing differentiation within multiple compartments of the hair follicle such that inappropriately timed differentiation occurred in its absence.

Although EGFR was expressed from birth in mouse epidermis, its expression did not occur in the hair follicles until 5 days after birth, similar to the timing and localization of EGFR in previous reports.[1,3] Thus, the early stages of hair follicle development were independent of EGFR expression, and the onset of the disorganized hair follicle phenotype in EGFR null mice correlated with the beginning of EGFR expression in the hair follicle. In previous reports,[1,3] expression of EGFR has been localized to the outer root sheath of human and rat hair follicles but not in the earliest developing hair follicles, consistent with our findings in the mouse. This is consistent as well with our hypothesis that EGFR modifies the function of outer root sheath cells. The recombination grafting experiments demonstrated that EGFR was required only in the epithelial component of the skin for normal hair follicle development and maturation and that the requirement for EGFR is a local rather than a systemic phenomenon. This is consistent with the hair abnormalities seen in the transgenic dominant negative mouse where the transgene was targeted to the epithelial cell.[23]

The grafting experiments presented also demonstrate the importance of EGFR in maintaining the structure of the hair follicle over time. In EGFR null skin, hair follicles were consumed by inflammatory cells beginning as early as 2.5 weeks after grafting, which resulted in a hairless and graft skin devoid of hair follicles in most of the mice. The inflammatory infiltrate appeared to result in the degradation of the hair follicles specifically, leaving the epidermis intact but hyperplastic and hyperkeratotic. As the histology of age-matched EGFR wild-type graft skin revealed that many hair follicles had entered telogen at these

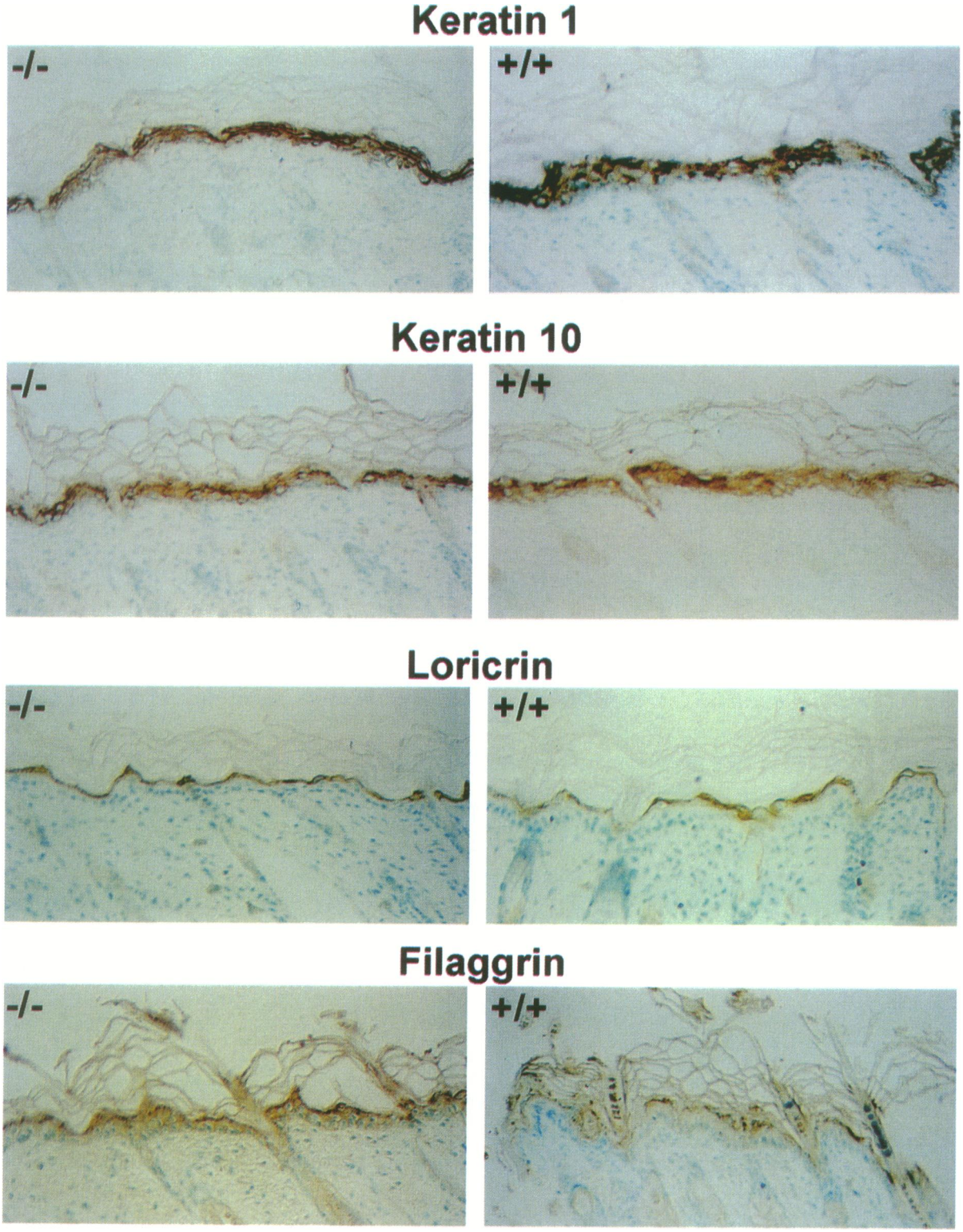

Figure 7. *Unaltered expression of markers of interfollicular epithelial differentiation in EGFR-deficient mouse skin. K1, K10, loricrin, and filaggrin were detected immunohistochemically in 5-day (K1 and K10; n = 2) and 8-day (loricrin and filaggrin; n = 2) skin from EGFR-deficient and wild-type skin as described in Materials and Methods. Similar results were obtained with skin from newborn and 2-, 12-, and 18-day-old mice. Magnification, ×250.*

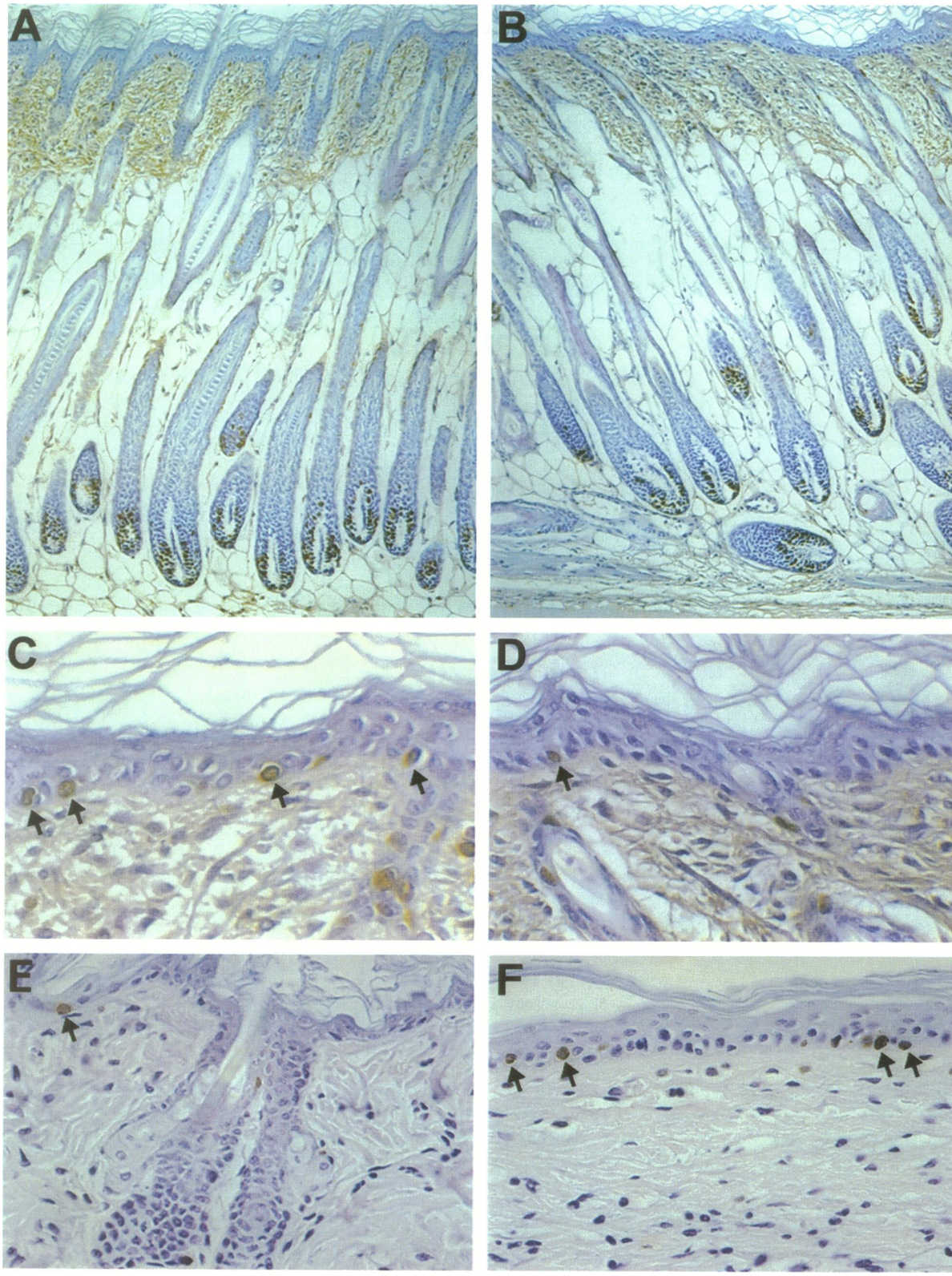

Figure 8. *Follicular and interfollicular epidermal proliferation in EGFR null and wild-type intact skin and whole skin grafts. Skin was removed from 8-day-old EGFR null* (B *and* D) *and wild-type* (A *and* C) *intact mice and from EGFR null* (F) *or wild-type* (E) *grafts after 10 weeks. One hour before sacrifice, mice were injected with BrDU. Paraffin-embedded sections were immunohistochemically stained with an anti-BrDU antibody as described in Materials and Methods. Magnification, ×100* (A *and* B), *×450* (C *and* D), *or ×300* (E *and* F).

Table 5. *Unaltered Follicular Proliferation in Intact EGFR-Deficient Skin*

	EGFR −/−		EGFR +/+	
Age (days)	% Labeled cells	Total cells/bulb	% Labeled cells	Total cells/bulb
2	39.3 ± 0.0 (n = 10)	64.6 ± 1.5* (n = 10)	39.2 ± 0.0 (n = 10)	97.5 ± 7.1 (n = 10)
5	29.5 ± 0.0 (n = 20)	89.8 ± 3.4* (n = 20)	28.8 ± 0.0 (n = 20)	120.8 ± 3.6 (n = 20)
8	21.0 ± 0.0 (n = 20)	115.8 ± 6.8* (n = 20)	23.3 ± 0.0 (n = 10)	169.7 ± 15.2 (n = 10)

Skin sections from EGFR-deficient and wild-type mice were immunohistochemically stained for BrDU and counterstained with hematoxylin as described in Materials and Methods. BrDU-labeled and unlabeled cells in at least 10 bulbs, in longitudinal sections of hair follicles, were counted per skin section and are reported as the percentage of bulb cells positively labeled for BrDU. The total number of cells per hair follicle bulb (mean ± SE) was also quantified.

*Mean is significantly different from the corresponding mean for the wild-type skin using a Student's *t*-test at $P \leq 0.05$.

time points, these results may suggest that a defect in the progression from anagen to catagen to telogen in EGFR null hair follicles may have triggered an immunological response that resulted in the destruction of the EGFR null hair follicles. Murillas et al[23] have previously reported such a defect in progression from anagen to catagen in skin-targeted EGFR dominant negative mice, which results in mostly hairless mice by several months of age.

The inflammation-associated loss of the hair follicles may be due to the role of EGFR in the resolution of inflammation in a wound-healing environment and only secondarily related to the hair cycle. During normal hair follicle cycling, the lower portions of the hair follicle exist in an immune privileged site and do not express major histocompatibility complex (MHC) class 1 antigens.[42,43] However, during the transition from anagen to catagen, MHC class 1 antigens are expressed in the lower follicle, macrophages infiltrate the area, and the lower portion of the hair follicle degenerates.[42,43] As EGFR null mice presumably lack EGF-induced suppression of oxygen radical production, which might be necessary for the resolution of inflammation,[44] expression of MHC class 1 antigens in early catagen could trigger the destruction not only of the lower follicle but also of the entire organoid. Lack of the ability to suppress the immune response might then be responsible for follicular destruction in EGFR null skin. Alternatively, EGFR may have a function in immune protection, either directly or by delaying maturation of hair follicle cells as hair itself is immunogenic.[43]

In contrast to the role of EGFR in hair follicles in the regulation of differentiation and hair follicle maintenance, we suggest that the primary function of EGFR in the epidermis is in regulating cell proliferation as a substantial decrease in epidermal proliferation occurred in intact EGFR null epidermis compared with wild-type epidermis whereas expression and localization of markers of epidermal differentiation were not affected by the loss of EGFR. These results differ from those of Miettenen et al,[22] who found decreased keratin expression in another EGFR null mouse model. The discrepancies between our results might be explained by strain difference, which can dramatically affect the health of the animal.[20,21] However, EGFR is not required for epidermal proliferation as epidermis of null grafts was hyperplastic. Therefore, we cannot exclude the possibility that the decreased epidermal proliferation in intact EGFR null skin may actually be the result of ill health in the mice.[20] Alternatively, growth-stimulatory cytokines produced in the graft site may have overcome the defect in proliferation in EGFR null skin through pathways that circumvent the EGFR, and null cells may be more sensitive to these alternative pathways for epidermal proliferation. For example, keratinocyte growth factor is produced during wound healing[45] and stimulates proliferation of EGFR null keratinocytes in culture (data not shown). It is unlikely that EGFR ligands in the wound site are responsible for the excessive proliferation as these ligands are incapable of stimulating growth of null keratinocytes *in vitro* (A. Dlugosz, L. Hansen, C. Cheng, N. Alexander, M. Denning, D. Threadgill, T. Magnuson, R. Coffey Jr., and S. Yuspa, manuscript in preparation).

Despite the robust proliferation in the epidermis of EGFR null explant grafts, these grafts were smaller than the wild-type controls. These data suggest a defect in cell migration in the EGFR null skin. EGF-mediated induction of integrins and promotion of cell migration have been reported previously,[46] and up-regulation and aberrant localization of $\alpha 3$ and $\alpha 6$ integrins were seen in the EGFR null skin.[20] Carroll et al[47] subsequently reported that ectopic expression of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins in transgenic mice results in follicular disorganization and increased epidermal proliferation. Thus, the disorganized hair follicle phenotype and smaller graft size in EGFR null skin grafts may be related to deregulated integrin

expression. Skin-targeted integrin transgenic mice also develop an inflammatory skin response, suggesting some overlap exists in the integrin and EGFR pathways.

In conclusion, we propose that EGFR functions to regulate and delay the differentiation of multiple cell types within the hair follicle, possibly directly in the cells of the outer root sheath, which may then impact secondarily on the maturation of the cells giving rise to the hair fiber. Thus, EGFR is intimately involved in the normal production of hair. We have also shown a complex role for EGFR in skin cell proliferation, in that it is dispensable for follicular cell proliferation but involved in interfollicular epidermal proliferation. The EGFR may also be necessary for the resolution of wound-induced hyperplasia within the epidermis. In addition, EGFR is necessary for maintenance of the integrity of the hair follicle and cycling of hair follicles from anagen into telogen. Thus, EGFR has a variety of functions within the skin that depend on both the cellular compartment in which it is expressed and the status of the skin.

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