

**Title**

Epidermal growth factor as a biologic switch in hair growth cycle

**Author**

Kingston K. L. Mak and Siu Yuen Chan

Department of Paediatrics and Adolescent Medicine, The University of Hong Kong,

Faculty of Medicine Building, 21 Sassoon Road, Hong Kong

**Correspondence**

Dr. Siu Yuen Chan

Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Faculty

of Medicine Building, 21 Sassoon Road, Hong Kong

Tel: +852 28199352

Fax: +852 28166974

E-mail address: **[sychan@hkucc.hku.hk](mailto:sychan@hkucc.hku.hk)**

SUMMARY

The hair growth cycle consists of three stages known as the anagen (growing), catagen (involution) and telogen (resting) phases. This cyclical growth of hair is regulated by a diversity of growth factors. Whereas normal expression of both epidermal growth factor and its receptor (EGFR) in the outer root sheath is down-regulated with the completion of follicular growth, here we show that continuous expression of epidermal growth factor in hair follicles of transgenic mice arrested follicular development at the final stage of morphogenesis. Data from immunoprecipitation and immunoblotting showed that epidermal growth factor signals through EGFR/erbB2 heterodimers in skin. Furthermore, topical application of tyrophostin AG1478 or AG825, specific inhibitors of EGFR and erbB2 respectively, completely inhibited new hair growth in wild type mice but not in transgenic mice. When the transgenic mice were crossed with *waved-2* mice, which possess a lower kinase activity of EGFR, the hair phenotype was rescued in the offspring. Taken together, these data suggest that EGFR signaling is indispensable for the initiation of hair growth. On the other hand, continuous expression of epidermal growth factor prevents entry into the catagen phase. We propose that epidermal growth factor functions as a biologic switch which is turned on and off in hair follicles at the beginning and end of the anagen phase of the hair cycle, guarding the entry to and exit from the anagen phase.

## INTRODUCTION

The hair follicle is a regenerating system which undergoes cycles of renewal in three phases known as anagen, catagen, and telogen. Various signaling molecules are involved in different phases of hair growth (1). However, the mechanisms underlying the switch from phase to phase remain largely unknown. Among the many growth factors expressed in association with the development of hair follicles, epidermal growth factor (EGF<sup>1</sup>) is known for its effects on skin and hair development (2). Subcutaneous administration of EGF for 2 weeks into neonatal mice delayed the development of hair follicles and epidermis. Both the growth of hair bulbs and hair fiber production were retarded. The proliferative activity of the epidermis at birth was maintained for 8 days, resulting in a thicker epidermis compared to control mice. However, the delay in skin development was not prolonged, and the mitotic index and thickness of the epidermis declined to that of control values a few days later. By day 41, final hair length and diameter were not significantly reduced (3). EGF also had no effect on back skin when injected into mice with ages ranging from 12-20 days (2). The authors suggested that EGF was only active in skin during the neonatal period. In contrast, EGF induces follicle regression besides inhibiting hair fiber production and stimulating mitosis of the basal epidermal cells when infused into adult sheep (4). Subsequently, *in vitro* studies with isolated human hair follicles have shown that EGF stimulates DNA synthesis in the outer root sheath and hair follicle elongation but inhibits hair fiber production. DNA synthesis in the matrix cells is inhibited and they remain connected to the dermal papilla by a thin strand of epithelial cells, inducing an artificial catagen-like effect (5). Despite all these early studies, the physiological role of EGF on hair development and the signaling pathways

---

<sup>1</sup> The abbreviations used are: EGF, epidermal growth factor; TGF $\alpha$ , transforming growth factor  $\alpha$ ; EGFR, epidermal growth factor receptor; TBS, Tris-buffered saline; H&E, haematoxylin and eosin.

involved remain unclear. It has been shown that administration of anti-EGF serum can cause accelerated hair growth in new born mice (6). This suggests that endogenous EGF acts as an inhibitory molecule during follicle morphogenesis.

There are four members of the EGF receptor family namely EGFR or erbB1, erbB2, erbB3 and erbB4. EGF, transforming growth factor (TGF)  $\alpha$ , amphiregulin, and possibly epigen (7) bind EGFR which can subsequently form homodimers or heterodimers with any erbB receptors, whereas  $\beta$ -cellulin, epiregulin and heparin binding-EGF are ligands for both EGFR and erbB4 (8). A null mutation in *EGFR* is lethal during embryonic development. Only certain strains of mutant mice can survive up to three weeks after birth, with severe impairment in development of multiple organs including skin and failure of hair growth (9-11). Hair follicles in the neonatal skin cannot develop normally even when grafted to nude mice (12). In transgenic mice expressing a dominant negative mutant of EGFR in the epidermis and outer root sheath, the hair follicles cannot progress through follicle morphogenesis and eventually undergo necrosis (13). A loss of function mutation in *TGF $\alpha$*  (14, 15) or a specific point mutation in *EGFR* (16,17) results in a less severe phenotype of curly whiskers and pronounced waviness of first hair coat. Irregularly distributed hair follicles were found in the dermis and subcutis layers. Surprisingly, triple null mice lacking EGF, amphiregulin and TGF $\alpha$  demonstrate the same hair and skin phenotype as the TGF $\alpha$  null mice (18). This suggests that other members of the EGF family can compensate for the loss of the three EGFR ligands. Transgenic mice over-expressing the various EGFR ligands in skin are useful for addressing their specific roles.

Both amphiregulin and heparin binding-EGF are expressed in skin (19,20). Upon culture of keratinocytes, the expression of both molecules increases and they become the major autocrine factors for proliferation (21). Furthermore, trauma can lead to metalloproteinase-mediated cleavage of amphiregulin, heparin binding-EGF and TGF $\alpha$  from

### *EGF as a biologic switch in hair growth cycle*

their transmembrane precursors. The evidence suggests that ectodomain shedding of these growth factors is required for wound healing (22, 23). These three EGFR ligands are probably more important in wound healing and in skin pathology. Overexpression of amphiregulin in transgenic mice induces an epidermal hyperproliferation and inflammation which is similar to psoriasis, and is in agreement with the increased expression of this moiety in psoriasis patients. TGF $\alpha$  is expressed in the inner (14) and outer root sheath (24) in actively growing follicles. TGF $\alpha$  transgenic mice have been reported to exhibit a thicker epidermis and stunted hair growth but the epidermal scaliness disappeared and hair growth was partially restored at 5-6 weeks despite a persistent expression of the transgene. Benign skin papillomas were found in older animals. EGF is also expressed in the outer root sheath in the growing hair follicles (25) although the role of EGF in hair biology has not been clarified using the transgenic approach. For this purpose we have generated and analyzed a transgenic mouse line constitutively expressing EGF in skin and hair follicles. Our data suggest a specific role for EGF in the control of the hair cycle.

## EXPERIMENTAL PROCEDURES

### *Animals*

Mice were cared under the Laboratory Animal Unit, the University of Hong Kong. *Waved-2* mice bred with a mixed C57BL/6 and 129/sv background were a generous gift from Dr. Ashley Dunn (Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Australia). *Waved-2* mice have a hypomorphic allele of *EGFR* that contains a point mutation near the tyrosine kinase domain (16,17).

### *Generation of transgenic mice*

The full length mouse *Egf* cDNA of 4.6 kb was isolated from a C57BL/6 kidney cDNA library (Life Technologies, USA) and confirmed by sequencing. EGF transgenic mice were established using the promoter from cytomegalovirus to drive full length *Egf* cDNA joined to an SV40 polyA. The injection fragment of 6.5 kb was released and purified using the QIAEX gel extraction kit (Qiagen, USA), then passed through a spin-X column (Costar, USA). The purified DNA fragment was introduced at 6-8ng/ $\mu$ l into the pronucleus of FVB/N embryos by standard microinjection procedures (26). Transgenic mice were identified by PCR of tail DNA using primers for *Egf* cDNA and confirmed by Southern blotting (data not shown). The primer sequences (5' to 3') were gagaatgccgcctgcaccaacac and agggttctttggggggtttgatag, giving a PCR product of 435 bp.

### *Immunohistochemistry*

The locations of EGF expression on dorsal skin in heterozygous animals were determined at birth (Day 1), Day 4, 7, 10, 14, 17, 21, 28, 35, 40 and 50. After the first hair cycle, hair growth was asynchronised in wild type animals and therefore no further time points were examined. Skin samples were orientated and snapped frozen using the Hofmann

### *EGF as a biologic switch in hair growth cycle*

technique (27). Cryosections (10  $\mu\text{m}$ ) were stored at  $-70\text{ }^{\circ}\text{C}$  and equilibrated to room temperature before use. The sections were fixed in 4% paraformaldehyde for 10 min, washed and then treated with 3% hydrogen peroxide, washed thoroughly with distilled water and then washed again with Tris-buffered saline (TBS), pH7.4. After incubation with 10% normal goat serum, 3.33  $\mu\text{g}/\text{ml}$  rabbit anti-mouse EGF (Upstate, MA, USA) was applied overnight at  $4\text{ }^{\circ}\text{C}$ . Then the sections were washed with TBS and incubated with biotinylated goat anti-rabbit immunoglobulins (Dako, UK) diluted 1 in 100 for one hour. The sections were washed with TBS and incubated with streptavidin-biotinylated peroxidase complex (Dako) for 30 min. The sections were washed again and incubated for 5 min with a 1 mg/ml chromogen solution (Dako). After counterstaining with haematoxylin, the sections were dehydrated and mounted with Permount. As negative controls, the primary antibody was omitted or replaced with the same concentration of rabbit IgG (Dako). To confirm signal specificity, the primary antibody was blocked with 15  $\mu\text{g}/\text{ml}$  mouse EGF (Peprotec Inc., Israel) at  $4\text{ }^{\circ}\text{C}$  overnight before use.

### *TUNEL Assay*

The procedures recommended for the Apoptosis Detection kit (Promega Ltd, WI, USA) were followed on fixed frozen skin sections.

### *Measurement of skin thickness*

Haematoxylin and eosin (H&E) staining was performed on frozen sections (three heterozygous mice per time point, with control mice from nontransgenic littermates). Three representative sections for each sample were analyzed using a Leica DMRB microscope. The thickness of epidermal, dermal and subcutaneous layers was measured on digital images using the Stereo Investigator 4.0 software (Leica Ltd., Germany). A total of 50 data sets were

## *EGF as a biologic switch in hair growth cycle*

collected from the three samples at each time point. All data were reported as mean  $\pm$  SD and analyzed using the two-tailed unpaired *Student's t test*.

### *Protein preparation and immunoblotting*

Mice were sacrificed by cervical dislocation, and mid dorsal skin, liver and brain were immediately collected for protein preparation. Tissues were homogenized in 20 mM Hepes, pH7.4, 2 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 2  $\mu$ g/ml aprotinin. The homogenized samples were centrifuged at 4 °C for 5 min and the supernatants were collected. Samples (80  $\mu$ g aliquots as determined by the Coomassie Blue dye binding assay; Bio-Rad, USA) were separated on a 7.5% polyacrylamide gel and transferred to a Hybond-ECL membrane (Amersham Pharmacia Biotech, NJ, USA). For immunoblotting, the procedures in the chemiluminescent detection system (ECL Detection System; Amersham Pharmacia Biotech) were followed. The primary antibodies and working concentrations were: rabbit anti-mouse EGF (Upstate, 2  $\mu$ g/ml ); mouse anti-human EGFR (Transduction lab, UK, 1 $\mu$ g/ml); rabbit anti-mouse erbB2 (Santa Cruz, CA, USA, 0.2  $\mu$ g/ml); rabbit anti-mouse erbB3 (Santa Cruz, 0.2  $\mu$ g/ml); mouse anti-phosphotyrosine PY20 (Transduction lab, 1  $\mu$ g/ml). The secondary antibodies and working dilutions were: goat anti-mouse IgG (Transduction lab, 1:5000); goat anti-rabbit IgG (Amersham, Pharmacia Biotech, 1:4000).

### *Immunoprecipitation*

Specific antibody (1 $\mu$ g) was added to 500  $\mu$ g of crude protein samples and the mixture was made up to a final volume of 0.5 ml with IP buffer [1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, and 2  $\mu$ g/ml aprotinin]. Samples were



### *EGF as a biologic switch in hair growth cycle*

incubated at 4°C for 2 hours with gentle rocking. Protein A or G /agarose (40 µl) was then added and incubation was continued for 2 hours. The samples were centrifuged for 1 min at 4 °C and the supernatants discarded. The agarose beads were washed with 1 ml IP buffer at 4°C for 5 min. The samples were spun down and the washing procedures repeated 3 more times. Finally, 40 µl of sample buffer with 5% β-mercaptoethanol were added to the agarose beads and the samples used for immunoblotting.

### *Topical application of the erbB1 inhibitor AG1478 and erbB2 inhibitor AG825*

Heterozygous transgenic mice (Day 23, n=4) and nontransgenic littermates (n=4) were each divided into two groups of treatment and control animals. Tyrphostin AG1478 (10µg, Sigma, CA, USA) or Tyrphostin AG825 (60µg, Sigma) was dissolved in 1 ml DMSO and then mixed with 2 g of a neutral aqueous cream base (Orjon, Australia). An area starting midway along the back of the mice was shaved one day before application of the inhibitor or cream base. AG1478 or AG825 cream was applied topically every day for 15 consecutive days. In the case of the controls, only the cream base was administered. Each treatment was repeated on three litters (n=8). For large litters of more than 8 mice, 10µg AG1478 together with 60µg AG825 were added to 1ml DMSO, mixed with 2g cream and applied to the rest of the litter.

## RESULTS

### *Characterization of EGF transgenic mice*

While seven transgenic lines were generated with the CMV-Egf construct, only one line, designated 62.7, manifested phenotypic changes and the heterozygous animals were used for detailed analysis here. Line 62.7 exhibited thin hair, curly vibrissae and stunted growth (Fig 1a, b). Homozygous bred transgenic mice showed an even more severe

### *EGF as a biologic switch in hair growth cycle*

phenotype and appeared to be nearly nude. When bred into different genetic backgrounds, including C57BL/6, ICR, F1 hybrid of C57BL/6 and FVB, transgenic progeny showed similar hair phenotype as the FVB founder (not shown). From immunohistochemistry, EGF was expressed in the sebaceous glands, outer root sheath of hair follicles, stratum granulosum and basal keratinocytes of the epidermis during follicular morphogenesis in wild type mice (Fig 1c,d). The EGF expression in hair follicles was switched off once they had entered the telogen phase (Fig 1e). In contrast, transgenic mice demonstrated constitutive expression of EGF in the hair follicles (Fig 1f, g, h) which appeared to remain in stage 7 to 8 of follicular morphogenesis according to published guidelines by Paus (28). Results from RT-PCR and immunoblotting confirmed that EGF was strongly expressed in the skin of 62.7 mice with accumulation of the EGF precursor, which was in contrast to age-matched wild type mice (Fig.1 i). EGF protein could also be detected in the liver and brain of transgenic animals and this subtle phenotype is still under investigation. Only increased levels of EGF mRNA, but not of protein, were detected in other transgenic lines which correlated with the absence of abnormalities in the skin or other organs at the morphological or histological level.

### *Association of EGF expression with structural changes in the skin and hair cycling*

From H&E stained sections (Fig 2), wild type mice and transgenic mice had the same skin histology at birth (Day 1, Fig 2a, b). However, at Day 4, retardation of hair follicle and skin development was observed in the transgenic mice (Fig 2c, d). Although more than 90% of hair follicles had grown into the subcutis layer (stage 7 and 8), an increase in hair follicle size and total skin thickness was obvious in the wild type mice only. Furthermore, transgenic skin samples showed a thicker epidermis when compared to wild type animals at Day 4. There was an increase in the number of cell layers at the basal layer as well as the stratum corneum, which suggested epidermal hyperproliferation and a delay in epidermal

### *EGF as a biologic switch in hair growth cycle*

differentiation (Fig 2c, d insets). The hair follicles in wild type animals were in the dermis layer as the hair cycle proceeded to the catagen/telogen phase at Day 21 (Fig 2e, f). They were in the first anagen at Day 28 and already at telogen by Day 35. The duration of first anagen was much shorter than that reported for C57BL/6 (29). For transgenic animals, hair follicles were found in the subcutis layer when sampled at Day 7, 10, 14, 17, 21, 28, 35, 40 and no hair follicles of catagen or telogen phase were observed at any of these time points. Furthermore, no apoptotic cells were detected in either the lower bulb or the root sheath of transgenic follicles by TUNEL staining at any of the time points studied (not shown). This has been used to distinguish the late anagen follicles from those in early catagen (1). At Day 50, some telogen follicles could be occasionally found in certain transgenic animals. Skin thickness was measured using computer assisted morphometric software (Fig 3a). In general, total skin thickness in transgenic samples was less than in controls from newborn to around Day 14 after birth ( $p < 0.05$ ). However, as total skin thickness decreased in wild type mice when progressing through the hair cycle, transgenic skin became thicker than that of controls ( $p < 0.05$ ). The reduced skin thickness in transgenic mice in the first 14 days was mainly contributed by the subcutis (Fig 3d) ( $p < 0.05$ ). In contrast, the increase in thickness observed after Day 21 involved the epidermis, the dermis and the subcutis layers (Fig 3b-d). Furthermore, the hair shaft diameter of hair plugged from back skin was only half that of controls, although the percentage of various types of hair did not change (not shown).

### *EGFR signaling in hair development as revealed by immunoblotting*

Next, we investigated the pathway of EGF signaling in wild type and transgenic skin. EGFR was readily detected in crude skin lysates of both transgenic and wild type mice at Day 7 and Day 14 (Fig 4a). The receptors were strongly phosphorylated during this period which suggested that there was activation of downstream pathways for hair growth. However at

Day 21, when hair follicles were at the telogen phase in normal mice, the expression of EGFR was downregulated in both transgenic and wild type mice, although transgenic skin samples indicated a comparatively higher EGFR expression and stronger phosphorylation than samples from age-matched wild type animals. By Day 35, when all hair follicles were again at telogen phase in the wild type mice, the expression of EGFR was barely detectable in both wild type mice and transgenic mice. In order to determine the involvement of other EGFR family members, immunoblotting of erbB2 and erbB3 were performed. ErbB4 receptors were excluded since they were not detected in the skin by immunohistochemistry (30). For erbB2, expression was highest in both transgenic and wild type skin at Day 7. However, expression persisted at Day 35 in transgenic mice only and was not observed in wild type animals. Similar results were obtained for erbB3 expression.

After relating the expression of various erbB receptors to follicle morphogenesis and the hair cycle, we attempted to identify the dimerization partner(s) of erbB1 by immunoprecipitation using antibodies specific for either erbB2 or erbB3 (Fig 4b). For Day 14 samples, both erbB2 and erbB3 immunoprecipitates were phosphorylated at a comparable level in both transgenic and wild type skin samples. EGFR could be detected in both immunoprecipitates, implying EGFR/erbB2 and EGFR/erbB3 heterodimer formation in Day 14 skin samples. In contrast, for Day 35 skin, erbB2 activation was detected in transgenic but not in wild type mice and EGFR could be detected in erbB2 immunoprecipitates. Neither erbB3 activation nor EGFR/erbB3 heterodimerization was detected in transgenic or wild type samples at Day 35. Since erbB2 has no known ligand and requires a dimerization partner for phosphorylation, we conclude that erbB2 activation persisted in the transgenic skin through heterodimerization with EGFR.

*Topical application of erbB blockers*

Our data suggested that EGF signaling through EGFR/erbB2 and EGFR/erbB3 may be important in follicle growth and perhaps during the anagen phase of the hair cycle. We tested this hypothesis *in vivo* using control and transgenic mice. Either tyrphostin AG1478 or AG825, selective inhibitors of EGFR and erbB2 tyrosine kinase activity respectively, was administered to the skin of adult mice. An area on the dorsal side was shaved one day before the first inhibitor application (Fig 5a,b). Shaving could induce anagen (31) and allow easy assessment of hair growth. After 15 consecutive days of treatment, wild type mice treated with vehicle only had a new layer of hair (Fig 5e) whereas in wild type mice treated with either inhibitor, new hair growth was completely inhibited (Fig 5c). Skin histology after treatment showed that hair follicles were in the anagen phase of the hair cycle (not shown). In contrast, tyrphostin treated transgenic mice showed continuous hair growth (Fig 5d), although less dense hair was observed compared to the vehicle-treated transgenic mice (Fig 5f). Similar results were obtained when both inhibitors were applied simultaneously and there was a more obvious reduction in hair density. Furthermore, new hair growth in the transgenic mice treated with both inhibitors was two days slower than that of transgenic mice treated with a single inhibitor. Within the vehicle treated group, new hair growth in wild type mice was slower than in transgenic mice by two days. These data suggest that EGFR/erbB2 signaling is indispensable for hair growth.

*Rescue by breeding with waved-2 mice*

To further demonstrate that thin fur in line 62.7 was due to EGF overexpression and upregulated EGFR signaling, transgenic mice were mated with *waved-2* mice. Homozygous *waved-2* mutant mice (Egfr<sup>wa-2/wa-2</sup>, Fig 6a) were bred with homozygous 62.7 transgenic mice (CMV-Egf/CMV-Egf). All offspring were therefore double heterozygous for both genes

### *EGF as a biologic switch in hair growth cycle*

(*Egfr*<sup>wa-2/+</sup>, CMV-*Egf*/+). They all showed normal hair growth throughout the body (Fig 6b). Overexpression of EGF in double heterozygous mice was confirmed by immunoblotting. As expected, the level of EGF precursor detected in the skin of such mice was similar to that of heterozygous 62.7 mice (not shown). When heterozygous *waved-2* mice were bred with homozygous 62.7 transgenic mice, 17 out of 37 of the offspring had thin fur, indicating that the genetic rescue was due to the *waved-2* mutation instead of other differences in the genetic background.

Histological sections taken from the back skin showed that the hair follicles of double heterozygous mice were very similar to that of wild type mice at Day 7 (Fig 6c,e). More importantly, at Day 21 the hair follicles in the double heterozygous mice were in the dermis layer and the subcutis layer had reduced in thickness, in contrast to that of 62.7 transgenic mice (Fig 6f-h). This indicated that hair follicles in the double heterozygous mice had already entered the telogen phase.

## DISCUSSION

Based on the earlier studies of EGF in relation to skin and hair development and given that the EGFR family and ligands play a pivotal role in skin development, we have investigated the role of EGF and its signaling mechanism with the aid of a transgenic mouse line constitutively expressing EGF in skin and hair follicles. To our surprise we have found that constitutive expression of EGF inhibits entry into the catagen phase. Furthermore, EGFR/erbB2 signaling appears to be indispensable for hair growth.

In mice, follicular morphogenesis began at day 14.5 of embryonic development and lasted until around 3 weeks after birth (32). The present study showed that even when EGF was overexpressed, the skin histology was normal at birth. By Day 4, there was an increase in epidermal thickness but a decrease in both overall skin thickness and hair follicle diameter, consistent with earlier studies which involved the injection of EGF into newborn animals and showed an inhibitory role of EGF in skin development (2,3). It has also been shown that whereas EGFR is expressed continuously in the epidermis, expression in the hair follicles started a few days after birth. This correlates with the onset of disorganized hair follicle phenotype in loss-of function mouse mutants of EGFR generated by homologous recombination or expression of a dominant negative *EGFR* transgene (12,13). Some hair follicles even penetrate the underlying muscle layer a few days later (13). These data underscore the importance of EGFR signaling in controlling hair follicle orientation and elongation. The novel finding here is that continuous expression of EGF leads to arrest of the hair follicles at stage 8 of follicular morphogenesis. This conclusion appears to be in contrast to previous reports of catagen-promoting activity in sheep (4, 21) but this effect may be an artifact due to the high doses of EGF used. At lower doses of EGF, there was a reduction in follicle bulb size and a reduction in wool fiber diameter, similar to that observed in our transgenic mice. At higher doses, EGF induces wool follicle regression (4). This is likely to

be due to the excessive inhibition of EGF in bulb cell division which may subsequently lead to the observed increase in apoptosis (33). Also, in cultured human hair follicles, the stimulation of hair follicle elongation and outer root sheath proliferation by EGF but inhibition on the proliferation of matrix cells could lead to an artificial catagen. The matrix cells remained connected to the dermal papilla by a thin strand of epithelial cells (5) and this epithelial strand is also seen in follicles at certain stages of catagen (29). In summary, all reports point to a stimulatory effect of EGF on the proliferation of basal keratinocytes and outer root sheath cells but inhibition on proliferation of bulb cells.

Is EGF the physiologic ligand in controlling hair cycling? Various transgenic mouse lines have been produced to address the specific roles of EGF family ligands in skin development with the aid of the promoter from *keratin 14* to drive transgene expression in the basal epidermis and outer root sheath. Amphiregulin, heparin binding-EGF and TGF $\alpha$  produce different effects when over-expressed in skin (see Introduction). Arrest in hair cycle progression has not been reported in these mice. Furthermore, our EGF transgenic mice were not prone to develop papillomas after skin lesions or during aging as reported for TGF $\alpha$  mice (data not shown). From our immunoblot data it appears that EGF is likely to act through EGFR/erbB2 dimerization. Furthermore, switching EGF off is associated with progression of follicle morphogenesis to the catagen phase.

The present data using tyrophostins on mice at day 23 point to the indispensable role of EGFR/erbB2 signaling in the initiation of hair growth in the hair cycle. In wild type mice treated with the specific EGFR inhibitor, no new hair growth was seen. The same result was obtained using the specific inhibitor for erbB2, indicating that EGFR/EGFR signaling is inadequate for hair growth. Since erbB2 does not have its own ligand but signals through heterodimerization (8), our results indicate that EGFR/erbB2 signaling is involved. In contrast, tyrophostin treated transgenic mice continued to generate new hair, but at a lower



density. It is possible that with continuous overexpression of EGF in the transgenic mice, the inhibition of EGF signaling with either tyrphostin is incomplete. Furthermore, in vehicle treated mice, the transgenic mice were found to exhibit new hair growth much sooner than the wild type mice suggesting that EGF is important for the initiation of hair growth. It is interesting to note that in TGF $\alpha$  null mutant mice, hair regrowth after plucking is faster than that in wildtype mice (15). One intriguing possibility is that EGF is more important than other EGFR ligands in stimulating hair regrowth, and that TGF $\alpha$  competes with EGF for available EGFR. One of the important downstream signaling molecules may be Stat3. Although hair follicles in *Stat3*-disrupted mice can progress through follicle morphogenesis to telogen, they cannot progress to anagen. Importantly, keratinocytes isolated from these mice lack the normal response of migration to EGF (34). These data clearly indicate that the molecular control of hair growth during morphogenesis, although often referred as the ‘first anagen’, is different from anagen in subsequent hair cycles. We believe that EGF expression in the outer root sheath of hair follicles is important for downward growth of the hair follicle. EGF expression needs to be turned off before progression of the hair follicles to telogen. In subsequent hair cycles, EGF is again important for downward growth of the hair follicles and production of the hair shaft. Although it remains to be determined whether continuous EGF can lead to anagen arrest in the hair cycle, we would like to propose that EGF acts as a biologic switch guarding entry to and exit from the anagen phase of the hair cycle, as depicted in Fig. 7. The expression patterns of both EGF and its receptor are consistent with such a role. EGF, together with various other signaling molecules, forms a complex network for the precise control of the hair cycle. For example, fibroblast growth factor 5 is required for the transition to catagen. When the gene is mutated in mice, hair follicles fail to progress to the catagen phase and this gives rise to an abnormal long hair phenotype (35).

*EGF as a biologic switch in hair growth cycle*

The findings of this study may be relevant to new therapeutic approaches for the treatment of abnormally thin hair caused by abnormal EGFR signaling. On the other hand, prevention of unwanted hair growth can be achieved by topical application of EGFR/erbB2 inhibitors. For example, such a strategy may be of benefit in the treatment of women suffering from hirsutism or for cosmetic purposes.

ACKNOWLEDGEMENTS

We thank Ashley Dunn and Tracy Helman (LICR, Australia) for the kind gift of *wa-2* mice, Malcolm Turner (UCL, UK) and JD Huang (HKU) for comments on the manuscript, Anthony Chan and Priscilla Mak (HKU) for their interest and technical support. This study was funded by the University Block Grant, HKU.

REFERENCES

1. Stenn, K.S., and Paus, R. (2001). *Physiol. Reviews* **81**,449-494.
2. Cohen, S. (1986). *Biosci.i Rep.* **6**,1017-1028.
3. Moore, G.P.M., Panaretto, B.A., and Robertson, D. (1983). *Ana. Record* **205**,47-55.
4. Moore, G.P., Panaretto, B.A., Carter, N.B. (1985). *J. Invest. Dermatol.* **84**,172-175.
5. Philpott, M.P. and Kealey, T. (1994). *J. Invest. Dermatol.* **102**,186-191.
6. Zschiesche, W., Eckert, K. (1988). *Experientia* **44**,249-251.
7. Strachan, L., Murison, J.G., Prestidge, R.L., Sleeman, M.A., Watson J.D., and Kumble, K.D. (2001). *J Bio. Chem.* **276**,18265-18271.
8. Yarden, Y., and Sliwkowski, M.X. (2001). *Nat. Rev. Mol. Cell. Biol.* **2**,127-137.
9. Miettinen, P.J., Berger, J.E., Meneses, J., Phung, Y., Pedersen, R.A., Werb, Z., Derynck R. (1995). *Nature* **376**,337-341.
10. Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., et. al. (1995). *Science*, **269**, 230-234.
11. Sibilias, M., Wagner, E.F. (1995). *Science* **269**,234-238.
12. Hansen L.A., Alexander N., Hogan M.E., Sundberg J.P., Dlugosz A., Threadgill D.W., Magnuson T. , Yuspa S.H. (1997) *Am. J. Pathol.* **150**,1959-1975.
13. Murillas, R., Larcher F., Conti C.J., Santos M., Ullrich A., Jorcano J.L. (1995) *EMBO J.* **14**,5216-5223.
14. Luetkeke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O. and Lee, D.C. (1993). *Cell* **73**,263-278.
15. Mann, G.B., Fowler, K.J., Gabriel, A., Nice, E.C., Williams, R. L. and Dunn, A.R. (1993). *Cell* **73**,249-261.
16. Fowler, K.J., Walker, F., Alexander, W., Hibbs, M.L., Nice, E.C., Bohmer, R.M., Mann, G.B., Thumwood, C., Maglitto, R., Danks, J.A., Chetty, R., Burgess, A.W., Dunn, A.R. (1995). *Proc. Natl. Acad. Sci. USA* **92**,1465-1469.

17. Luetteke, N.C., Phillips, H.K., Qiu, T.H., Copeland, N.G., Earap, H.S., Jenkins, N.A. and Lee, D.C. (1994). *Genes Dev.* **8**,399-413.
18. Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A. and Lee, D.C. (1999). *Development.* **126**,2739-2750.
19. Piepkorn M., Underwood R.A., Henneman C., Smith L.T. (1995) *J. Invest. Dermatol.* **105**,802-809.
20. Downing M.T., Brigstock D.R., Luquette M.H., Crissman-Combs, M., Besner G.E. (1997) *Histochem. J.* **29**,735-744.
21. Piepkorn, M., Pittelkow, M.R., Cook P.W. (1998) *J. Invest. Dermatol.* **111**,715-721.
22. Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J.I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y., Matsuzawa, Y., Hashimoto, K., Taniguchi, N. (2000). *J. Cell Biol.* **151**, 209-219.
23. Sunnarborg, S.W., Hinkle, C.L., Stevenson, M., Russell, W.E., Raska, C.S., Peschon, J.J., Castner, B.J., Gerhart, M.J., Paxton, R.J., Black, R.A., Lee, D.C. *J. Biol. Chem.* **277**, 12838-12845.
24. Nixon, A.J., Broad L., Saywell, D.P. and Pearson, A.J. (1996) *J. Histochem. Cytochem.* **44**,337-387.
25. du Cros, D.L., Isaacs, K., Moore, G.P.M. (1992) *J. Invest. Dermatol.* **98**,109-115.
26. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo* , pp. 232-248, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Paus, R., Hofmann, U., Eichmüller, S., Czarnetzki, B.M. (1994). *Br. J. Dermatol.* **130**, 281-289.
28. Paus, R., Müller-Röver, S., van der Veen, C., Maurer, M., Eichmüller, S., Ling, G., Hofmann, U., Foitzik, K., Mecklenburg, L., Handjiski, B. (1999). *J. Invest. Dermatol.* **113**, 523-532.

29. Müller-Röver, S., Handjiski, B., van der Veen, C., Eichmüller, S., Foitzik, K., McKay, I.A., Steen, K.S., Paus, R. (2001). *J. Invest. Dermatol.* **117**, 3-15.
30. Xian, W., Rosenberg, M.P., DiGiovanni, J. (1997). *Oncogene* **14**,1435-1444.
31. Ogawa, H., Hattori, M. (1983). *Curr. Probl. Dermatol.* **11**, 159-170.
32. Hardy, M.H. (1992). *Trends Genet.* **8**, 55-61.
33. Hollis, D.E., Chapman, R.E. (1987). *J. Invest. Dermatol.* **88**,455-458.
34. Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., Yoshikawa, K., Akira, S., Takeda, J. (1999). *EMBO J.* **18**,4657-4668.
35. Hebert, J.M., Rosenquist, T., Gotz, J., Martin, G.R. (1994). *Cell* **78**, 1017-1025.

FIGURE LEGENDS

Figure 1. Hair phenotype in EGF transgenic mice. (a) A representative transgenic mouse at Day 21 with thin fur throughout the whole body as compared to the wild type littermate. (b) Vibrissae were curled anteriorly and significantly shorter than normal. (c-h) Immunohistochemistry of EGF in skin. (c) Endogenous EGF expression in wild type skin at Day 4 and (d) close-up of a hair follicle showing EGF expression in the outer root sheath. (e) EGF was not detected in hair follicles of wild type mice at Day 21. (f) Increase in EGF expression in Day 4 transgenic skin and (g) close-up of a hair follicle showing strong EGF expression in the outer root sheath. (h) EGF expression in hair follicles was still detectable at Day 21 in transgenic skin. (i) Transgene expression in seven transgenic lines. Only line 62.7 showed markedly increased expression of *EGF* in skin in both RT-PCR and immunoblots. Scale bar = 200  $\mu$ m.

Figure 2. Microscopic examination of H&E stained skin sections. Left panel: wild type; right panel: transgenic. (a&b) Dorsal skin sections of control and transgenic mice at Day 1, showing no obvious difference. (c&d) At Day 4, hair follicles had entered the subcutis layer. The skin thickness and hair follicle size in control mice had increased significantly. An increase in thickness of the epidermis was observed in the transgenic mice as a result of an increase in the number of cell layers in basal layers and stratum corneum (arrows and inset). (e&f) Gross structural difference of transgenic mouse skin compared with wild type mice at Day 21. In wild type mice, hair follicles had moved up to the dermis layer and proceeded to telogen phase. Hair follicles of transgenic mice were still in the subcutis layer. (g&h) At Day 50, wild type hair follicles remained at telogen and transgenic hair follicles remained in the subcutis. Scale bar = 200  $\mu$ m.

Figure 3. Analysis of skin thickness throughout the hair cycle. The thickness at each stage was measured for (a) total skin, (b) epidermis, (c) dermis and (d) subcutis. A reduced total skin thickness was observed for transgenic mice from birth to Day 14 ( $p < 0.05$ ). This phenomenon reversed from Day 21 onwards with thicker skin in the transgenic mice ( $p < 0.05$ ). The reduction in skin thickness in the first 14 days was contributed mainly by the subcutis layer ( $p < 0.05$ ). The increase in skin thickness of transgenic mice after Day 21 was contributed by all three layers ( $p < 0.05$ ). The asterisk represents a significant difference using *Student t-test*. The thickness of skin samples from normal mice oscillated according to the different phases of the hair growth. In contrast, transgenic skin only showed small changes in skin thickness. Values shown are mean  $\pm$  1 SD and n= 50.

Figure 4. Immunoblots of crude skin samples from specific time points. (a) On both Day 7 and Day 14, expression and phosphorylation of EGFR were detected in both wild type and transgenic mice. On Day 21 and Day 35, normally the telogen phase, both the expression level and phosphorylation level of EGFR were intensively down-regulated. However, samples from transgenic animals had comparatively higher levels of EGFR expression and phosphorylation than samples from wild type animals. Complete down-regulation of erbB2 and erbB3 expression was observed on Day 35 in wild type but not in transgenic skin. Comparable amounts of the mature form of EGF were detected at each stage in transgenic and wild type samples. (b) Immunoprecipitation of erbB2 and erbB3. On Day 14, no observable difference between wild type and transgenic skin was detected with respect to phosphorylation of erbB2 and EGFR/erbB2 heterodimerization. Similar results were obtained for erbB3. On Day 35 (telogen), only transgenic skin showed EGFR/erbB2 heterodimerization and phosphorylation.



Figure 5. Topical application of specific tyrosinase inhibitor. (a&b) An area on the dorsal side of the mouse was shaved one day before the first inhibitor application in (a) wild type and (b) transgenic littermates. (c&d) After 15 consecutive days of treatment with AG1478 in a neutral cream base, transgenic mice showed a new layer of hair growth. The same result was obtained with application of AG825 (not shown). However, less dense hair growth was observed compared to transgenic mice treated with cream base. Hair growth in wild type mice was completely inhibited by AG1478 treatment. (e&f) A new layer of hair growth was observed in both (e) wild type and (f) transgenic mice treated with cream base only in the same experiment.

Figure 6. Rescue of thin fur phenotype by breeding 62.7 transgenic mice with  $Egfr^{wa-2/wa-2}$  mice. (a) A homozygous *waved-2* mouse at 3 months old. Only the first hair coat is waved and older animals such as the one shown had a normal coat phenotype but curly whiskers. (b) Offspring of a homozygous *waved-2* mutant intercrossed with a homozygous transgenic mouse from line 62.7. Hair density appeared normal throughout the whole body but whiskers remained curly. (c, f) Representative H&E stained skin sections of wild type mice at Day 7 and 21. (d, g) Skin sections of heterozygous transgenic mice at Day 7 and 21. (e, h) Skin sections of double heterozygous mice at Day 7 and 21. The skin morphology of double heterozygous mice was similar to that of wild type mice, with the hair follicles in the dermis layer at Day 21 indicating that they were in the telogen phase. Scale bar = 200  $\mu$ m.

Figure 7. Model for EGF as a switch in hair growth. Follicular morphogenesis begins at 14.5 days post coitum (dpc). EGF is switched on in the hair follicles a few days after birth and switched off as hair follicles enter catagen. EGF is again turned on in anagen and turned off

*EGF as a biologic switch in hair growth cycle*

in catagen in subsequent hair cycles. In both situations, EGF signals through EGFR/erbB2 heterodimers.

*EGF as a biologic switch in hair growth cycle*

Figure 1

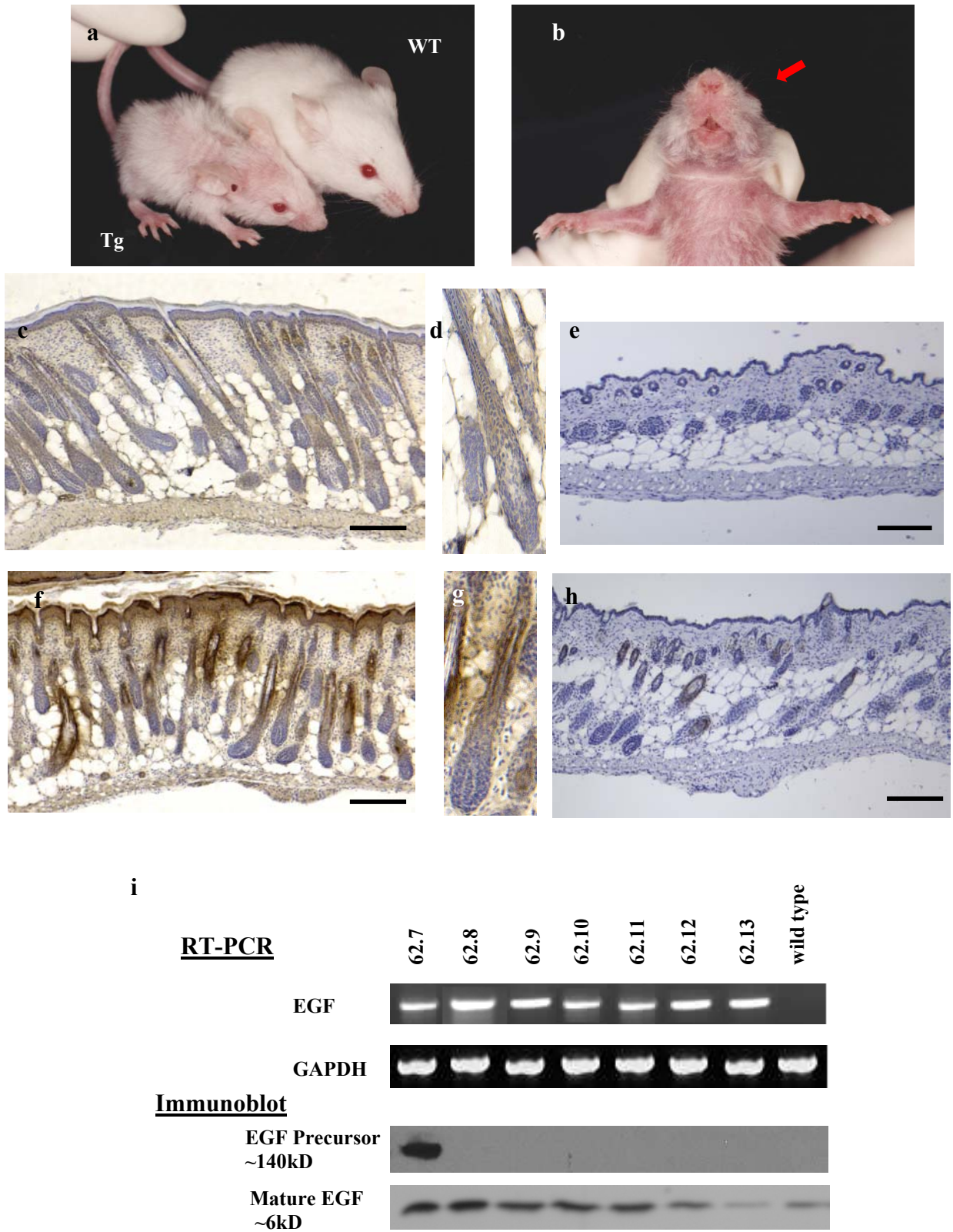


Figure 2

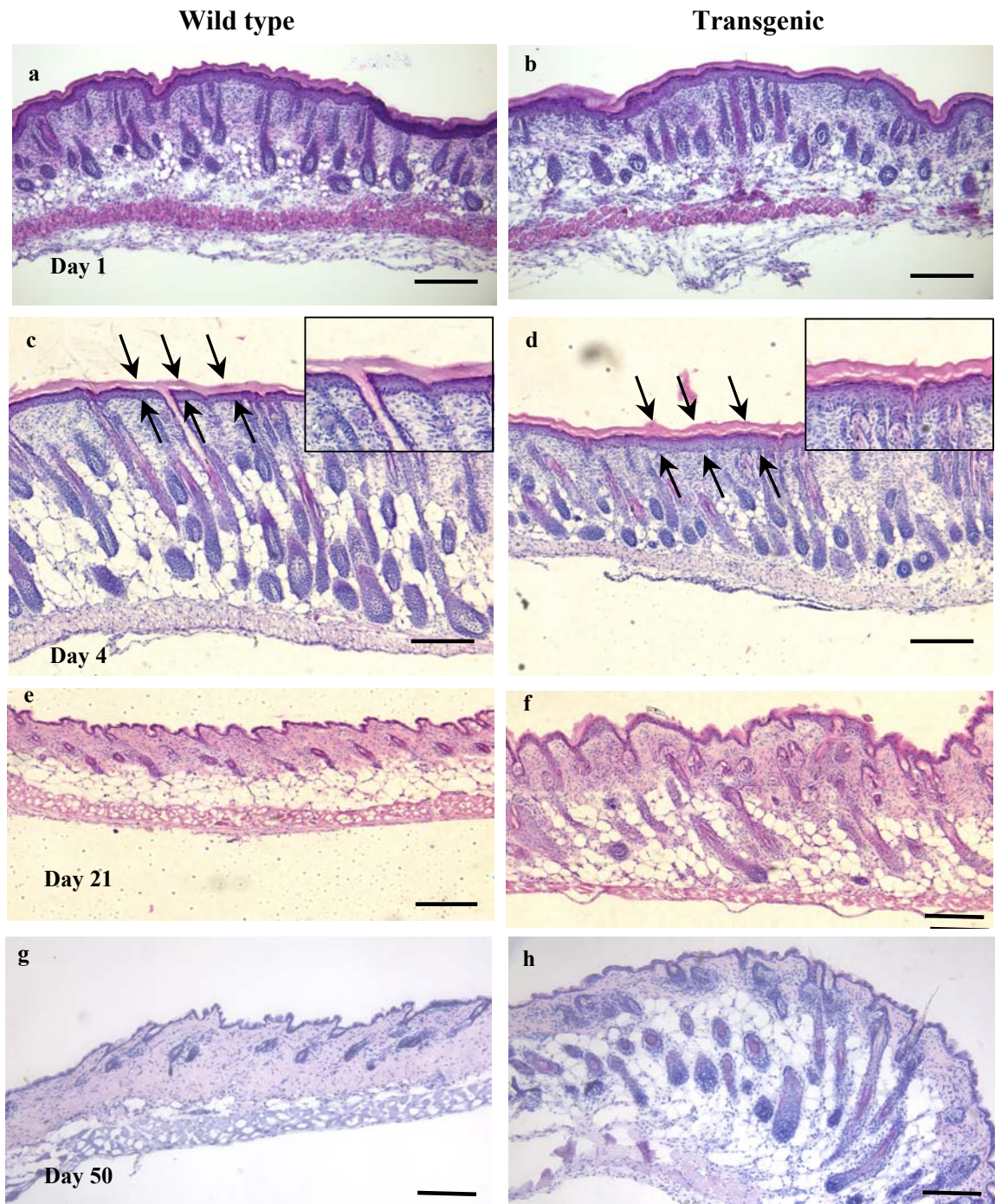
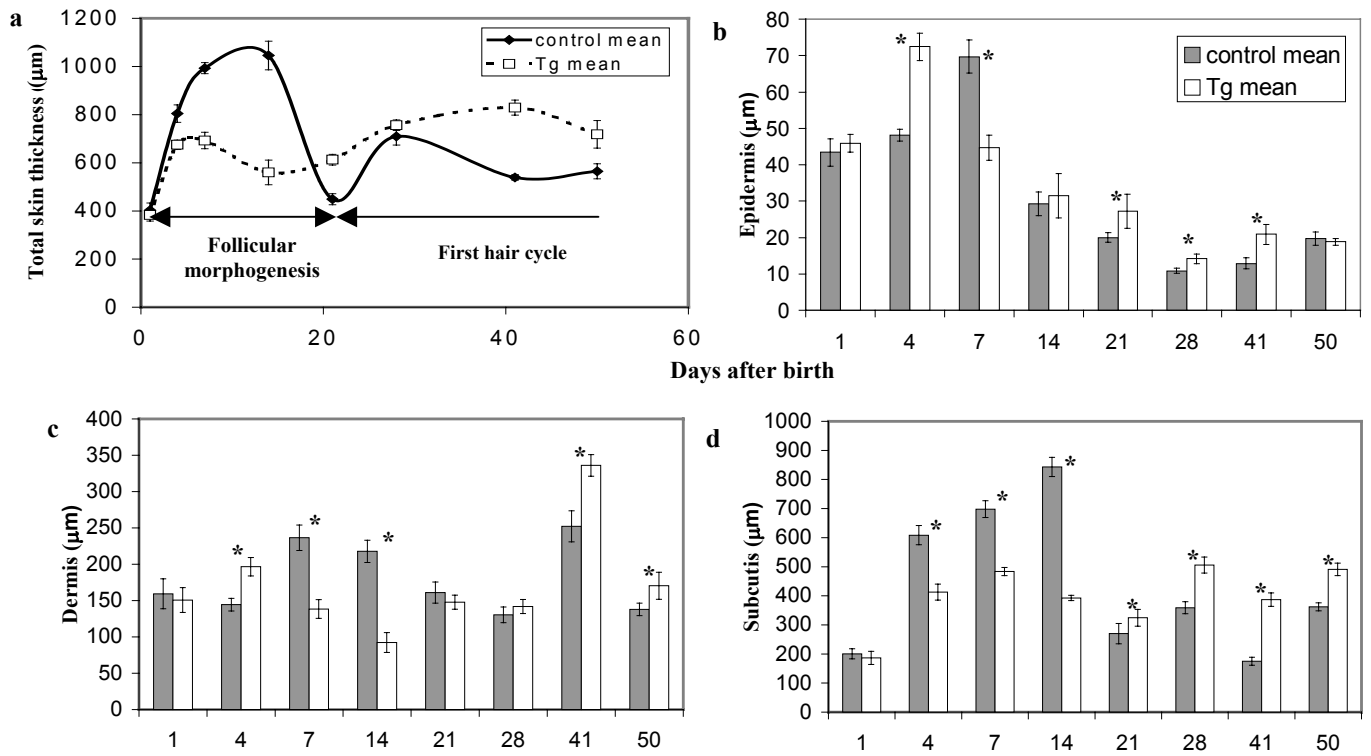


Figure 3



*EGF as a biologic switch in hair growth cycle*

Figure 4

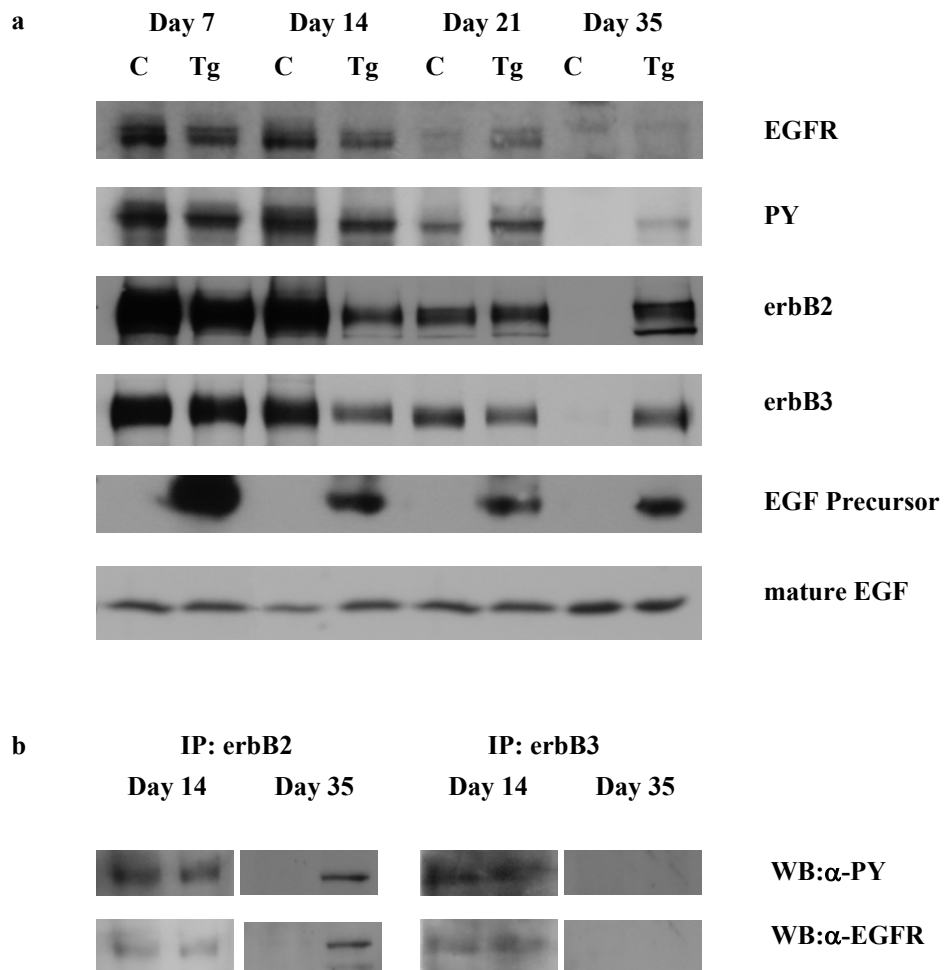


Figure 5

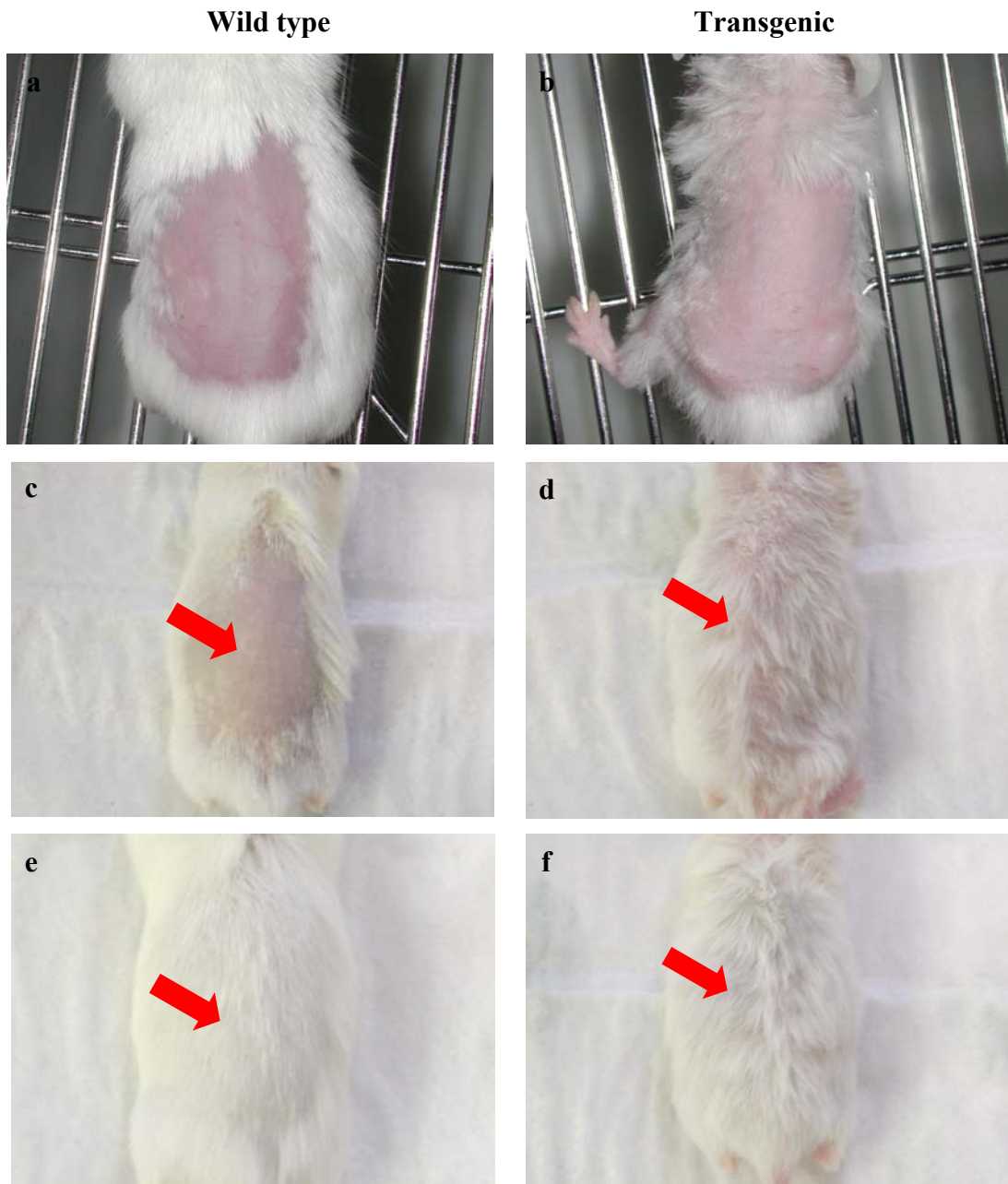


Figure 6

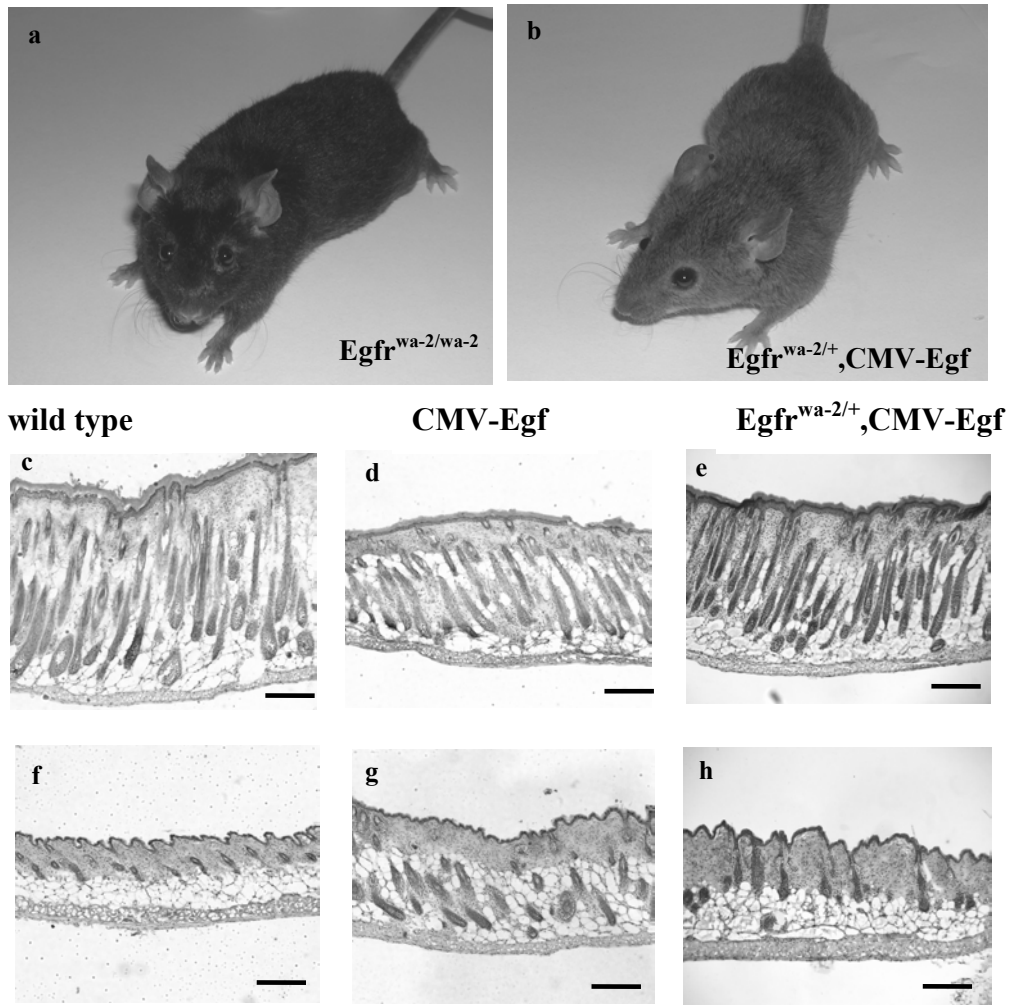




Figure 7

