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Transgenic expression of human amphiregulin in mouse skin: inflammatory epidermal hyperplasia and enlarged sebaceous glands

Yong Li¹, Stefan W. Stoll¹, Sahil Sekhon², Caroline Talsma¹, Maya I. Camhi², Jennifer L. Jones², Sylviane Lambert¹, Hue Marley¹, Laure Rittié¹, Marina Grachtchouk¹, Yi Fritz², Nicole L. Ward², and James T. Elder^{1,3}

¹Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI, USA

²Department of Dermatology, Case Western Reserve, University, Cleveland, OH, USA

³Ann Arbor VA Hospital, Ann Arbor, MI, USA

Abstract

To explore the role of amphiregulin in inflammatory epidermal hyperplasia, we overexpressed human AREG (hAREG) in FVB/N mice using a bovine K5 promoter. A construct containing AREG coding sequences flanked by 5' and 3' untranslated region sequences (AREG-UTR) led to a >10-fold increase in hAREG expression compared to an otherwise-identical construct containing only the coding region (AREG-CDR). AREG-UTR mice developed tousled, greasy fur as well as elongated nails and thickened, erythematous tail skin. No such phenotype was evident in AREG-CDR mice. Histologically, AREG-UTR mice presented with marked epidermal hyperplasia of tail skin (2.1-fold increase in epidermal thickness with a 9.5-fold increase in Ki-67⁺ cells) accompanied by significantly increased CD4⁺ T-cell infiltration. Dorsal skin of AREG-UTR mice manifested lesser but still significant increases in epidermal thickness and keratinocyte hyperplasia. AREG-UTR mice also developed marked and significant sebaceous gland enlargement, with corresponding increases in Ki-67⁺ cells. To determine the response of AREG-UTR animals to a pro-inflammatory skin challenge, topical imiquimod (IMQ) or vehicle cream was applied to dorsal and tail skin. IMQ increased dorsal skin thickness similarly in both AREG-UTR and wild type mice (1.7- and 2.2-fold vs vehicle, $P < 0.001$ each), but had no such effect on tail skin. These results confirm that keratinocyte expression of hAREG elicits inflammatory epidermal hyperplasia, and are consistent with prior reports of tail epidermal hyperplasia and increased sebaceous gland size in mice expressing human epigen.

Keywords

amphiregulin; EGF receptor; psoriasis; sebaceous glands; skin inflammation

Correspondence: James T. Elder, University of Michigan, 7412 Medical Sciences Building 1, 1301 E. Catherine, Ann Arbor, MI 48109-5675, USA, Tel.: +734 647 8070, Fax: +734 615 7277, jelder@umich.edu.

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Supporting Information: Additional supporting data may be found in the supplementary information of this article.

Introduction

Psoriasis is a common inflammatory and hyperplastic skin disease with substantial comorbidities and major economic impact (1). In recent years, a variety of mouse models have been advanced in an effort to better understand the pathogenesis of psoriasis (2). An emerging consensus supports the concept that mouse models provide many useful avenues for dissecting the complex pathogenesis of psoriasis, but no individual model can completely recapitulate the pathogenesis of this uniquely human disease (2,3).

We and others have observed that transforming growth factor- α and other members of the epidermal growth factor receptor (EGFR) ligand family were overexpressed in psoriatic lesions relative to normal skin (4–10). Mice engineered to express a genomic copy of human *AREG* in basal keratinocytes (KC) under the control of a human keratin 14 (K14) promoter (11), or a cDNA copy of human *AREG* mRNA in suprabasal KC under the control of a human involucrin promoter (12), develop inflammatory hyperplasia of the skin with some similarities to psoriasis. Moreover, antibody blockade of human AREG (hAREG) has been reported to improve psoriasis in a psoriatic skin xenograft model (13).

Epidermal growth factor (EGF) and all other EGFR ligands can increase the proliferation of cultured human KC (14–16). AREG is by far the most abundant EGFR ligand expressed by human KC (17) and supports the proliferation of KC under autocrine growth conditions (18,19). However, AREG mRNA levels are much lower in normal and psoriatic lesional human skin *in vivo* than they are in cultured KC *in vitro* (9,17,20). While biological blockade of inflammatory signals including TNF, IL-23 and IL-17 markedly improves psoriasis (3), case reports of responses of psoriasis to EGFR inhibitors (EGFRIs) in patients with cancer have been sparse and have described both improvement and exacerbation (21,22). Moreover, antibodies and kinase inhibitors targeting the EGFR (EGFRIs) produce a papular and pustular eruption known as the PRIDE syndrome (papulopustules and/or paronychia, regulatory abnormalities of hair growth, itching and dryness due to EGFRIs) (23), which has proven to be the major side effect limiting the use of EGFRIs in cancer therapy (24). We have recently shown that EGFRIs elicit these rashes in an IL-1-dependent manner in human skin (25). Taken together, these observations raise the question of whether the growth-promoting properties of AREG for KC that are observed *in vitro* are relevant to the pathogenesis of psoriasis.

Previous hAREG-overexpressing mice were reported to develop such severe skin inflammation that they were not able to breed (11,12). In an effort to overcome this experimental limitation, we expressed hAREG under the control of a widely used bovine keratin 5 (K5) promoter (26), with or without flanking untranslated region (UTR) sequences normally present in human *AREG* mRNA. Here, we characterize several gross and microscopic phenotypes of these mice, including outcomes following topical application of imiquimod (IMQ), a toll-like receptor 7 agonist known to produce inflammatory epidermal hyperplasia when applied to mouse skin (27). Our results demonstrate that mice bearing AREG-UTR sequences in addition to the K5 promoter are viable and develop hAREG-induced inflammatory hyperplasia, particularly of the tail skin. These mice also develop

sebaceous gland enlargement and hyperplasia reminiscent of mice with skin-directed expression of the related EGFR ligand, epigen (28,29).

Materials and methods

AREG transgenic expression constructs

We assembled two AREG expression constructs within the pBK5 vector, which is based on the pBluescript cloning vector (Life Technologies, Carlsbad, CA USA) and contains a 5.2 kb DNA fragment with bovine K5 regulatory sequences, a beta-globin intron, a Kozak sequence, a polylinker cloning site and two polyadenylation sequences (26) (Fig. S1). Additional construct details are presented in the Supporting Information. Constructs were prepared for pronuclear microinjection into FVB/NCrl oocytes according to the standard protocol of the University of Michigan Transgenic Animal Core (www.med.umich.edu/tamc/). Oocytes from FVB/NCrl mice (Charles River Laboratories, Malvern, PA, USA) were used to match the host strain used by Cook *et al.* (11,12).

Measurement of AREG expression

Tail skin was removed from underlying bone and cartilage by means of a ventral longitudinal incision and blunt dissection, followed by immediate snap freezing of the harvested skin in liquid nitrogen. For assessment of transcript expression, the snap-frozen skin tissue was rapidly pulverized using a hammer and further homogenized by vortexing with glass beads (G-8772; Sigma, St. Louis, MO, USA) in RNeasy lysis buffer, followed by RNA purification using the RNeasy kit (Qiagen, Chatsworth, CA, USA). RNA quality was assessed and RNA concentrations were determined using an Agilent RNA 6000 nanochip (Agilent Technologies, Palo Alto, CA, USA). cDNA was prepared by reverse transcription of 0.5 μ g of RNA template using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), followed by qPCR using an AB 7900HT Fast-Real Time PCR System with TaqMan gene expression assays (Applied Biosystems) for human *AREG* (Hs00950669_m1) and mouse *Rplp0* (Mm00725448_s1). For assessment of hAREG protein levels, snap-frozen mouse tail skin tissues mechanically disrupted as described above were lysed in radioimmunoprecipitation assay buffer, cleared by brief microcentrifugation and measured by sandwich ELISA (R&D Systems, Minneapolis, MN USA) as previously described (18,19).

Quantitation of histology and immunohistochemistry

For assessment of epidermal thickness, Ki-67 staining and immune cell infiltration, dorsal or tail skin was fresh-frozen in tissue freezing medium (TFM; General Data, Cincinnati, OH, USA), cryosectioned and stained to detect Ki-67-positive cells as well as various immune cell subsets as well as epidermal thickness, which were then enumerated as described (30,31). For assessment of sebaceous gland area and density, samples of dorsal and/or tail skin from a different set of mice were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μ m thickness and stained with H&E. Sections were photographed using a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY, USA) equipped with a 1.4 megapixel Jenoptik ProgRes CF^{cool} digital camera (Jenoptik, Jena, Germany). Additional details are presented in the Supporting Information.

Oil Red O staining of WT and AREG-UTR mice

Four AREG-UTR and four age-matched WT littermate controls were analysed for dorsal and tail skin. TFM-embedded tissue samples were cut at 10 μ m thickness and fixed for 20 min in 2% paraformaldehyde. Oil Red O staining was performed for 15 min with freshly prepared Oil Red O staining solution [obtained by diluting a 0.5% stock solution of Oil Red O (Sigma Aldrich, St. Louis, MO, USA) in isopropanol with distilled water (3:5, v/v), allowed to stand for 10 min and filtered]. Slides were then thoroughly rinsed in 60% isopropanol, mounted with 90% glycerol (Thermo Fisher Scientific, Waltham, MA, USA) and sealed with clear nail polish. Sections were examined and photographed on a Zeiss Axioskop microscope.

Methods and morphometry for IMQ treatment of mouse skin

Dorsal skin of AREG-UTR or wild-type littermates was shaved using electric clippers 1 day prior to IMQ treatment. IMQ 5% cream (Nycomed, Melville, NY, USA) (62.5 mg daily) or an equivalent amount of Lanorcream vehicle (Gallipot, St. Paul, MN, USA) was applied either to the preshaved dorsal skin or to unshaved tail skin daily for five consecutive days, as described (27). IMQ was applied either to dorsal skin or tail skin; vehicle was applied to both sites in the control animals. On day 6 (24 h after the last treatment), mice were sacrificed, and dorsal and tail skin was formalin-fixed, embedded in paraffin, sectioned at 5 μ m and stained with H&E. Photomicrographs were then quantitated as described above, with the area of the epidermis being measured between the basement membrane and the top of the stratum granulosum, excluding the stratum corneum. The epidermal area was then divided by the length of basement membrane to determine the epidermal thickness.

Statistical analysis

Data were analysed for statistical significance utilizing GraphPad Prism (version 6 for Mac OSX; GraphPad Software, La Jolla, CA, USA). Specific statistical tests used for each experiment were determined by initial assessment of the normality of the data distribution and whether variances were equal or unequal. Analyses used for each experiment differed as determined by experimental design and pretests for normality and are indicated in the individual figure legends. In all figures, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

Results

Two expression constructs were generated in the vector pBK5 (26) (Fig. S1; see Materials and Methods). One construct (here termed AREG-CDR) contained only *AREG* cDNA sequences from the 5' ATG to the 3' stop codon. The second construct (AREG-UTR) contained the *AREG* coding region sequences flanked by 5' and 3' UTR sequences defined by restriction sites originally used by Cook *et al.* (12) to express human *AREG* cDNA in mice under the control of an involucrin promoter. After microinjection into FVB/NCrI mouse eggs and implantation, eight founder lines were generated from the AREG-UTR construct and two from the AREG-CDR construct. Seven of the eight AREG-UTR founder lines developed visible skin phenotypes, whereas neither of the AREG-CDR founder lines did. At 3 weeks of age, five of the eight AREG-UTR founder mice appeared runted (founders 544, 545, 664, 668 and 695), four had incomplete eyelid opening (founders 531,

544, 545 and 695), five had varying degrees of alopecia (founders 544, 545, 668, 695 and 664), and two had scruffy fur (founders 531 and 551). The clinical phenotype of founder 545 is presented in the top panel of Fig. 1a. One AREG-UTR founder appeared normal at 3 weeks and remained phenotypically normal for over 1 year (data not shown). Two of the most severely runted AREG-UTR founders had to be euthanized for ethical reasons.

Because of the runting observed in several founders, and because the K5 promoter drives expression in all stratified and pseudostratified epithelia (32), we examined the gross and microscopic morphology of the oesophageal and stomach epithelia in F2 offspring of two AREG-UTR founders and their wild type (WT) littermates, in addition to the skin. However, no abnormalities could be detected (data not shown). Consistent with the clinical phenotype of restricted palpebral aperture observed in the AREG-UTR mice (Fig. 1b), we did observe histological abnormalities in the eyes of these two transgenic (TG) mice, consisting of inflammation and scarring in the palpebral conjunctivae (data not shown).

By 10–11 months of age, all five of the surviving AREG-UTR founders continued to manifest one or more of a similar set of phenotypic abnormalities (founder 531 – reduced palpebral aperture, alopecia of the head; founder 545 – total alopecia, masses along the distant tail, reduced palpebral aperture; founder 551 – scruffy fur, immobility; founder 668 – scruffy fur, immobility; and founder 695 – scruffy fur, reduced palpebral aperture). In contrast, none of the AREG-CDR founders developed phenotypic abnormalities as they aged (data not shown).

The five surviving AREG-UTR founders with phenotypic abnormalities were backcrossed as young adults to FVB/NCrl mice, and multiple litters of offspring were produced through successive matings. Suggestive of germline mosaicism, TG F1 mice were not recovered in expected Mendelian ratios, as only five living TG pups were recovered out of 175 known offspring of the five backcrossed founders (the total number of pups could be higher due to cannibalism). F1 mice from three AREG-UTR founders (531, 545 and 683) were further backcrossed with naive FVB/NCrl animals; all three of these F1 mice produced TG offspring at expected Mendelian ratios in the F2 generation (23/44 live, 33/64 total for founder 531, 12/25 live, 18/36 total for founder 545 and 5/8 live for founder 683). Photographs of F1 and F2 offspring of founder 545 are shown in the lower panels of Fig. 1a.

F2 mice from AREG-UTR founders 531 and 545 were back-crossed two additional times onto the FVB/NCrl background and investigated in further detail. F3 and F4 offspring were born at expected Mendelian ratios for both lines (data not shown). Generally, F1 and higher descendants of founders 531 and 545 lacked the severe alopecia and runting seen in the founder animals, but continued to manifest similar findings in each passage, dominated by restricted eye apertures and scruffy, greasy-appearing fur which persisted for at least 1 year (Fig. 1b). In addition, AREG-UTR mice frequently developed elongated and curved nails, a phenotype never seen in WT littermates (Fig. 1c). AREG-UTR mice also developed erythema of their tails, accompanied by increased tail diameter and more prominent plate markings (Fig. 1d). Some, but not all of the AREG-UTR mice developed scaling and crusting of tail skin. As they aged, AREG-UTR mice in both founder lines also developed

protuberant growths on the tail. Often, but not always, these growths presented at the site of tail snipping (Fig. 1d).

Two AREG-CDR founders were also backcrossed to FVB/NCrl mice, ultimately producing nine litters, six of which contained viable offspring, with 13 of 31 living pups and 12 of 26 dead pups being transgenic [25 of 57 (44%) overall]. However, none of these F1 offspring manifested a phenotype. In an effort to understand the phenotypic differences between AREG-UTR and AREG-CDR mice, we measured human *AREG* mRNA and hAREG protein levels in tail skin of F2 mice by quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2a, human *AREG* transcript levels were much higher in AREG-UTR than in AREG-CDR mice. As assessed by ELISA of tail skin extracts, AREG-UTR mice also strongly over-expressed hAREG, whereas AREG-CDR mice did not (Fig. 2b). Based on these findings and the lack of a visible phenotype, AREG-CDR mice were not studied further.

The clinically apparent thickening of AREG-UTR tail skin was evident at the microscopic level as marked acanthosis (Fig. 3a). Additionally, the aforementioned protuberant growths appearing at sites of trauma were papillomatous in nature (Fig. 3b). Morphometric quantitation of non-papillomatous dorsal tail epidermis revealed a marked and significant increase in epidermal thickness (2.1-fold), accompanied by a 9.5-fold increase in keratinocyte proliferation as assessed by Ki-67 staining (Fig. 4a). We also found a lesser yet significant 1.25-fold increase in epidermal thickness in the dorsal skin of AREG-UTR mice relative to littermate controls, which was accompanied by a significant 1.65-fold increase in the density of Ki-67⁺ cells (Fig. 4a).

Accompanying these increases in epidermal thickness and keratinocyte hyperplasia, we found a significant, 6.6-fold increase in skin-infiltrating CD4⁺ T cells in the tail skin of AREG-UTR mice compared to WT littermates (Fig. 4b). While skin-infiltrating CD8⁺ T cells also appeared to be elevated, this trend failed to reach statistical significance in our sample. We also observed a trend towards increased numbers of CD4⁺ and CD8⁺ T cells in dorsal skin; however, these differences did not reach statistical significance (Fig. 4b). No significant differences in F4/80⁺ macrophages or in CD11b⁺ or CD11c⁺ myeloid cells were detected between WT and TG animals (Fig. S2).

Consistent with the clinical appearance of tousled, greasy fur (Fig. 1), haematoxylin and eosin (H&E)-stained sections of dorsal skin revealed enlarged sebaceous glands in AREG-UTR mice, compared to littermate controls (Fig. 3c). Morphometric analysis confirmed this increase in size to be statistically significant, although no differences in sebaceous gland density were observed (Fig. 4c). Indicative of increased proliferation, AREG-UTR mice also manifested a significant increase in the number of Ki-67⁺ sebocytes in both dorsal and tail skin (Fig. 4d). Finally, as determined by Oil Red O staining, sebum production was much greater in dorsal skin of AREG-UTR mice compared to WT controls, with deposition of large amounts of sebaceous material in the sebaceous glands, within the follicular infundibulum and onto the skin surface (Fig. 3d).

Because epidermal hyperplasia was accompanied by increased inflammatory cell infiltration in AREG-UTR mice, we hypothesized that overexpression of human *AREG* might potentiate or amplify cutaneous responses to inflammatory stimuli. To test this hypothesis, we applied IMQ cream onto the dorsal and tail skin of AREG-UTR and WT littermates daily for a period of 5 days as previously described (27). IMQ application significantly increased epidermal thickness of dorsal skin of both AREG-UTR mice and WT littermates, compared to vehicle alone (Fig. S3); however, no significant difference in IMQ response was observed between WT and AREG-UTR mice. Confirming the results shown in Fig. 4a, tail epidermal thickness was greater in AREG-UTR than WT animals. However, topical IMQ failed to elicit further increases in tail skin epidermal thickness in either AREG-UTR or WT mice (Fig. S3).

Discussion

The mechanisms responsible for the marked inflammatory epidermal hyperplasia that is characteristic of psoriasis remain unclear. Genetic and pharmacological evidence points to interconnected roles for TNF/NF κ B, IL-23, interferon and IL-17-mediated signalling pathways in the pathogenesis of psoriasis (3,33). On the other hand, considerable functional evidence also exists for the importance of the EGFR ligand AREG in promoting the proliferation and survival of human KC *in vitro* (17,19,34,35). Elsewhere, we have presented evidence that the related EGFR ligand heparinbinding EGF-like growth factor regulates KC motility and invasiveness (35). Both of these ligands are known to be overexpressed in psoriasis lesions (4–10). However, to date, no significant genetic signals for psoriasis have mapped to the EGFR or its ligands, and biological and pharmacological EGFRIs have by and large failed to improve psoriasis (21,22). Nevertheless, the striking inflammatory and hyperplastic skin phenotypes manifested by two strains of AREG TG mice (11,12), coupled with the marked overexpression of AREG and other EGFR ligands in psoriatic lesions, prompted us to further explore the biological outcomes following KC-specific overexpression of hAREG in TG mice.

Noting the pronounced (indeed, lethal) inflammatory skin phenotypes manifested by two prior strains of AREG TG mice (11,12), we initiated an effort to generate TG mice overexpressing AREG in the skin, taking advantage of the well-characterized bovine K5 promoter to drive KC-specific gene expression (26). In an effort to modulate hAREG expression, in the AREG-UTR construct, we included 5'- and 3'-flanking untranslated sequences that are normally present in human *AREG* mRNA (Fig. S1), mimicking the untranslated human *AREG*-UTR sequences present in *INV* promoter-driven human *AREG*-expressing construct generated by Cook *et al.* (12). In contrast, only AREG protein coding sequences were present in the *AREG*-CDR construct. Compared to AREG-UTR mice, the AREG-CDR mice expressed much lower levels of human *AREG* mRNA and hAREG protein (Figure 2) and had no detectable phenotype (data not shown). These results suggest that UTR sequences present in full-length human *AREG* mRNA contribute to the physiological impact of the transgene in an otherwise-isogenic mouse environment.

AREG-UTR mice developed a milder phenotype compared to mice carrying a genomic copy of AREG driven by a K14 promoter (K14-ARGE) mice (11) and mice expressing an

involucrin-driven hAREG cDNA (INV-AR mice) (12). It is possible that the bovine K5 promoter yielded lower levels of AREG expression compared to the constructs employed by Cook and colleagues, thereby leading to a milder phenotype in our animals more compatible with viability and breeding. However, there were notable similarities between the various lines. Like AREG-UTR mice (Fig. 1), both K14-ARGE and INV-AR founder mice displayed erythematous skin with patches of alopecia. These findings were largely but not completely confined to the tail skin in AREG-UTR mice, whereas they involved truncal, ear and tail skin in the K14-ARGE and INV-AR mice (11,12). It is possible that the greater epidermal hyperplasia that we observed in tail versus dorsal skin of AREG-UTR mice might be due to high levels of bovine K5 promoter activity in tail epidermis [see Fig. 2 of Ref. (36)]. All three strains of mice developed areas of epidermal hyperplasia that were associated with lymphocytic infiltration, as documented by CD3 staining in K14-ARGE and INV-AR mice (11,12), and in our case by increased CD4+ T-cell staining (Fig. 4b). Finally, both AREG-UTR K14-ARGE mice exhibited papillomatous tail growths [Fig. 3b and (11), respectively].

AREG-UTR mice clearly had a prominent skin phenotype of tousled, greasy fur (Fig. 1) accompanied by hyperplastic sebaceous glands (Figs 3c and 4c and d) producing large amounts of sebum (Fig. 3d). These findings were not reported for either K14-ARGE or INV-AR mice (11,12). However, a very similar phenotype, consisting of increased tail epidermal thickness and dorsal skin sebaceous gland enlargement, has been reported in mice expressing human epigen (*EPGN*) under control of a chicken beta-actin promoter paired with a CMV enhancer (*EPGN*-tg mice) (29). Interestingly, AREG and *EPGN* are both EGFR ligands with distinctive features, including persistent and potent biological actions in spite of low receptor binding affinity (37,38). Moreover, AREG and *EPGN* are similar in structure and reside within ~200 kb of each other in the human genome. Histological analysis of *EPGN*-tg founders revealed increased sebocyte proliferation in these mice, but the widespread expression of the transgene in this construct resulted in infertility (29). More recently, a conditionally expressed *EPGN* transgene was generated by crossing mice carrying a tetracycline response element-driven *EPGN* cDNA to a K14 reverse tetracycline transactivator driver mouse, allowing doxycycline (Dox)-inducible expression of *EPGN*. By controlling the timing of Dox addition, it was determined that *EPGN* expression was critical during embryonic development in order for mice to manifest sebaceous gland enlargement (28). Additional studies strongly implicated the EGFR in this process (28,39). Dox-treated mice developed epidermal thickening of tail epidermis accompanied by prominent hyperplasia of sebaceous glands in dorsal skin (28). Notably, the tail epidermis of *EPGN*-tg mice was considerably more hyperplastic than was dorsal epidermis (28), just as we have observed for AREG-UTR mice (Fig. 4). Because the promoters utilized in these studies differed substantially, it is possible that these phenomena may relate to intrinsic biological properties of AREG and *EPGN* themselves, rather than differences in promoter strength (36).

We frequently observed elongation and curvature of the nails in AREG-UTR mice as they aged (Fig. 1c), a feature not thus far reported in K14-ARGE and INV-AR mice (11,12) or in

EPGN-tg mice (28,29). In the former case, this may have been due to the early demise of the TG animals. Additional studies will be needed to elucidate the role of AREG in nail biology.

Given the pro-inflammatory effect of hAREG that we observed in AREG-UTR mice (Fig. 4b), we asked whether K5-driven overexpression of hAREG might exacerbate TLR-elicited skin inflammation following topical IMQ application (27). Using epidermal thickness as a well-validated surrogate measure of skin inflammation in this model (27,40), we confirmed increased acanthosis following IMQ application to dorsal skin as expected, but failed to detect an additional effect in AREG-UTR mice (Fig. S3). Possibly, this could be due to low *AREG* transgene expression and/or effect in dorsal skin. Additionally, IMQ did not increase tail skin thickness in either AREG-UTR or WT mice, relative to vehicle controls (Fig. S3). This could be due to decreased penetration of IMQ through the thickened and compact stratum corneum of tail skin, relative to dorsal skin.

Regarding the relevance of these findings to human psoriasis, we would note that the enlargement and hyperactivity of sebaceous glands that is characteristic of AREG-UTR (Figs 3c and d and 4c and d) and EPGN-tg mice (28,29) is actually the opposite of the sebaceous gland atrophy that has been observed in psoriatic patients (41). Particularly when taken together with emerging genetic and pharmacological data implicating the TNF/NF κ B, IL23 and IL17 axes in the pathogenesis of psoriasis (3), our results fall short of supporting a central, keratinocyte-autonomous role for AREG-induced epidermal hyperplasia in the pathogenesis of psoriasis, which we had envisaged previously (4). Nevertheless, our results do indicate that like the closely related EGFR ligand EPGN, AREG has a multifaceted role in the mechanisms through which EGFR signalling regulates the biology of the epidermis and its appendages, involving coordinated actions on T cells as well as KC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AREG	amphiregulin
CMV	cytomegalovirus
Dox	doxycycline
ELISA	enzyme-linked immunosorbent assay
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRI	EGFR inhibitor
EPGN	epigen
H&E	haematoxylin and eosin
hAREG	human amphiregulin
IMQ	imiquimod
INV-AR	involucrin-driven AREG cDNA construct
K14-ARGE	K14-driven genomic AREG construct
qPCR	quantitative polymerase chain reaction
TFM	tissue freezing medium
TG	transgenic
UTR	untranslated region
WT	wild type

Figure 1.

Clinical phenotypes of AREG-UTR transgenic (TG) mice. (a) Founder, F1 and F2 mice. Photographs of AREG-UTR founder 545 and wild type (WT) in F1 and F2 backcrosses. Top panels show founder 545 and two WT littermates. Note alopecia and runting of founder 545. Lower panels show F1 and F2 FVB/N backcross offspring of founder 545, along with littermate controls. Note development of matted, greasy-appearing fur. (b) Phenotype of adult AREG-UTR mice. Upper photographs show WT mouse H634 (female, FVB/N backcross F4, age 54 weeks). Note smooth fur, normal eye opening, the presence of vibrissae and normal nails. Lower photographs show AREG-UTR TG littermate H630 (also age 54 weeks). Note greasy fur, incomplete eye opening, lack of vibrissae and elongated nails. (c) Nail phenotypes in WT versus AREG-UTR mice. Paws of a 14-month-old female WT mouse are shown on the left, and those of AREG-UTR TG littermates are shown on the right. Top panel, dorsal view, bottom panel, ventral view. Note elongated and curved nails. (d) Tail phenotypes in TG mouse skin. Upper panel shows thickening and erythema of an 8-month-old adult AREG-UTR (TG) mouse, compared to a WT littermate. Second panel shows scaling and crusting in another adult AREG-UTR mouse. The middle two panels compare rear paws and tail of 11-month-old adult WT versus an AREG-UTR TG littermate. Note nail overgrowth and tail erythema in TG compared to WT mice. The lower panels depict knob-like growths at the tip of the tail and a tail tumor.

Figure 2.

Expression of human *AREG* mRNA and hAREG protein in wild type (WT) versus AREG-CDR and AREG-UTR mice. (a) *AREG* mRNA levels in tail skin, as assessed by quantitative polymerase chain reaction (qPCR). Data are expressed as a per cent of 36B4 (*RPLP0*) expression. *** $P < 0.005$ vs. WT, by one-way ANOVA with Holm–Sidak test for multiple comparisons. (b) hAREG protein levels in tail skin, as assessed by enzyme-linked immunosorbent assay (ELISA). Offspring of AREG-UTR founders 531 and 545 are compared to AREG-CDR animals and WT littermates (WT littermate values pooled between experiments). *** $P < 0.005$ vs. WT, by one-way ANOVA with Holm–Sidak test for multiple comparisons.

Figure 3.

Histological phenotypes of AREG-UTR transgenic (TG) mice. (a) Acanthosis and epidermal hyperplasia in AREG-UTR mouse skin. Representative photomicrographs of wild type (WT) and AREG-UTR TG littermates. 10× objective (100× final magnification), scale bars = 250 μm . (b) Histology of tail growths from AREG-UTR mice. Upper panel, knob-like growth from tip of tail, at site of tail snip. Lower panel, tumor near base of tail. Note papillomatous growth patterns. 5× objective (509 final magnification); four microscopic fields were merged to generate the upper panel, and three fields were merged to generate the lower panel. Scale bars = 500 μm . (c) Enlarged sebaceous glands in AREG-UTR mice. Representative photomicrographs of H&E-stained dorsal skin from a 12-month-old male WT mouse and an AREG-UTR TG littermate sacrificed on the same day. Note enlarged sebaceous glands in AREG-UTR compared to WT mice (arrowheads). 5× objective (50× final magnification), scale bars = 500 μm . (d) Increased sebum elaboration in AREG-UTR mice relative to WT littermates. Dorsal skin from four AREG-UTR mice and four age-matched WT littermates was cryopreserved in tissue freezing medium (TFM), sectioned at 6 μm and stained with Oil Red O. Representative sections demonstrate increased staining in sebaceous glands as well as the follicular infundibulum and surface epidermis in TG versus WT mice. Scale bar = 100 μm .

Figure 4.

Quantitation of histological parameters in transgenic (TG) versus AREG-UTR wild type (WT) mice. (a) Quantitation of epidermal thickness (upper panel) and Ki-67-positive cells per mm basement membrane (lower panel), in tail and dorsal skin of WT versus AREG-UTR mice. $N = 6$ AREG-UTR mice and three age-matched WT littermates. $*P < 0.05$, and $***P < 0.005$ by two-tailed unpaired t -test. (b) Increased numbers of CD4+ T cells in tail skin of AREG-UTR mice (black bars, $n = 6$) compared to age-matched WT mice (open bars, $n = 3$). Upper panel, CD4+ T cells per 20 $\times$ objective field. Lower panel, CD8+ T cells per 20 $\times$ objective field. (*) Indicates $P < 0.05$ by two-tailed unpaired t -test. (c) Quantitation of sebaceous gland area and density in AREG-UTR (black bars, $n = 16$) compared to WT mice (open bars, $n = 8$). Quantitation was performed as described in Materials and Methods. Upper panel, cross-sectional area per sebaceous gland. Lower panel, sebaceous gland density in glands per unit length of epidermis. Bars indicate mean + SEM. $*P < 0.05$ by two-tailed unpaired t -test. (d) Quantitation of increased Ki-67-positive cells in sebaceous glands of AREG-UTR mice (black bars, $n = 6$) compared to age-matched WT controls (open bars, $n = 3$). Due to the multilobular nature of sebaceous glands, data are expressed as Ki-67+ sebocytes per follicular unit. $*P < 0.05$ and $**P < 0.01$ by two-tailed unpaired t -test.