Genetic deletion of amphiregulin restores the normal skin phenotype in a mouse model of the human skin disease tylosis

Vishnu Hosur*, Benjamin E. Low, Leonard D. Shultz and Michael V. Wiles

ABSTRACT
In humans, gain-of-function (GOF) mutations in RHBDF2 cause the skin disease tylosis. We generated a mouse model of human tylosis and show that GOF mutations in RHBDF2 cause tylosis by enhancing the amount of amphiregulin (AREG) secretion. Furthermore, we show that genetic disruption of AREG ameliorates skin pathology in mice carrying the human tylosis disease mutation. Collectively, our data suggest that RHBDF2 plays a critical role in regulating EGFR signaling and its downstream events, including development of tylosis, by facilitating enhanced secretion of AREG. Thus, targeting AREG could have therapeutic benefit in the treatment of tylosis.

KEY WORDS: CRISPR/Cas9, EGFR, RHBDF2, Amphiregulin, Tylosis

INTRODUCTION
The role of RHBDF2 in enhancing amphiregulin (AREG) secretion, and consequently activating the epidermal growth factor receptor (EGFR) pathway, has significance for the skin disease tylosis. In humans, autosomal dominant mutations in RHBDF2 cause tylosis (Blaydon et al., 2012; Saarinen et al., 2012), a form of focal nonepidermolytic palmoplantar keratoderma (PPK) that manifests on the skin of the palms and soles (Stevens et al., 1996). Tylosis patients also present oral leukokeratosis and follicular hyperkeratosis (Field et al., 1997; Hennies et al., 1995). Currently, there is no cure for tylosis. In a study to identify genetic variants underlying tylosis, Blaydon et al. used targeted capture array and next-generation sequencing and identified two heterozygous missense mutations in the RHBDF2 gene that underlie tylosis, p.Ile186Thr and p.Pro189Leu (Blaydon et al., 2012). Further, substantial evidence implicates the involvement of AREG-induced constitutive activation of the EGFR pathway in tylosis (Blaydon et al., 2012; Brooke et al., 2014; Hosur et al., 2014). In humans, we recently showed that similar to GOF mutations in the mouse Rhbdf2 gene (Rhbdf2P159L/P159L), GOF mutations in human RHBDF2 (AREG) increase AMPK in vivo, promote increased secretion of AREG in vitro (Hosur et al., 2014). To determine in vivo whether the Rhbdf2P159L/P159L mutation (Fig. 1A, loss of the cytosolic N-terminal domain of RHBDF2) phenocopies human tylosis, and whether the tylosis p.P189L mutation (Fig. 1B, loss of the cytosolic N-terminal domain of RHBDF2) in RHBDF2 increases AREG secretion, we generated mice carrying the human tylosis mutation p.P189L (p.P159L in mice) using CRISPR/Cas9-mediated targeting and homology-directed repair in C57BL/6J zygotes (Fig. 1C). We characterized the resulting Rhbdf2P159L/P159L mice and observed that similarly to Rhbdf2P159L/P159L mice (Hosur et al., 2014), mice carrying the human tylosis mutation present with complete hair loss (Fig. 1D). However, unlike Rhbdf2P159L/P159L mice (Hosur et al., 2014; Siggs et al., 2014), which present complete hair loss throughout their lives, Rhbdf2P159L/P159L mice developed a thin layer of curly hair coat that was noticeable in adult mice (Fig. 1E), indicating that the Rhbdf2P159L/P159L phenotype is more severe than that of Rhbdf2P159L/P159L mice. Nevertheless, Rhbdf2P159L/P159L mice elicited significantly accelerated wound healing, as determined by ear punch closure (Fig. 2A,B). Additionally, accelerated wound healing was associated with an increase in stimulated secretion of AREG in Rhbdf2P159L/P159L MEKs compared with Rhbdf2+/+ MEKs (Fig. 2C).

RESULTS AND DISCUSSION
The human tylosis disease mutation enhances AREG secretion
Autosomal dominant mutations in the human RHBDF2 gene cause tylosis (Blaydon et al., 2012; Saarinen et al., 2012). Significant evidence implicates the involvement of AREG-induced constitutive activation of the EGFR pathway (Blaydon et al., 2012; Brooke et al., 2014; Hosur et al., 2014) in human tylosis. We recently showed that GOF mutations in human RHBDF2 (AREG) enhance AMPK in vivo, promote increased secretion of AREG in vitro (Hosur et al., 2014). To determine in vivo whether the Rhbdf2P159L/P159L mutation (Fig. 1A, loss of the cytosolic N-terminal domain of RHBDF2) phenocopies human tylosis, and whether the tylosis p.P189L mutation (Fig. 1B, loss of the cytosolic N-terminal domain of RHBDF2) in RHBDF2 increases AREG secretion, we generated mice carrying the human tylosis mutation p.P189L (p.P159L in mice) using CRISPR/Cas9-mediated targeting and homology-directed repair in C57BL/6J zygotes (Fig. 1C). We characterized the resulting Rhbdf2P159L/P159L mice and observed that similarly to Rhbdf2P159L/P159L mice (Hosur et al., 2014), mice carrying the human tylosis mutation present with complete hair loss (Fig. 1D). However, unlike Rhbdf2P159L/P159L mice (Hosur et al., 2014; Siggs et al., 2014), which present complete hair loss throughout their lives, Rhbdf2P159L/P159L mice developed a thin layer of curly hair coat that was noticeable in adult mice (Fig. 1E), indicating that the Rhbdf2P159L/P159L phenotype is more severe than that of Rhbdf2P159L/P159L mice. Nevertheless, Rhbdf2P159L/P159L mice elicited significantly accelerated wound healing, as determined by ear punch closure (Fig. 2A,B). Additionally, accelerated wound healing was associated with an increase in stimulated secretion of AREG in Rhbdf2P159L/P159L MEKs compared with Rhbdf2+/+ MEKs (Fig. 2C).

Next, to examine whether mice carrying the human tylosis mutation phenocopy the Rhbdf2P159L/P159L mutation (Hosur et al., 2014), we performed histological analysis of skin sections from Rhbdf2+/+ and Rhbdf2P159L/P159L mice. Rhbdf2P159L/P159L mice presented with follicular dystrophy (F), enlarged sebaceous glands (S), hyperplasia (H), and hyperkeratosis (arrow) of skin (Fig. 2D). To assess enhanced EGFR signaling in the skin of Rhbdf2P159L/P159L mice, we performed immunohistochemical analyses of skin sections from Rhbdf2+/+ and Rhbdf2P159L/P159L mice. We observed a considerable increase in epidermal EGFR signaling pathway downstream effectors phospho-ERK1/2 and phospho-mTOR (Fig. 3A).

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Similarly, immunoblot analyses of mouse embryonic fibroblasts (MEFs) isolated from Rhbd2P159L/− mice and Rhbd2P159LP159L mice also revealed enhanced EGFR signaling as demonstrated by reduced EGFR protein levels (due to downregulation of sustained EGFR receptor activation), and by enhanced protein levels of phospho-AKT, phospho-ERK1/2 and phospho-S6 in Rhbd2P159LP159L MEFs compared with those of Rhbd2+/+ mice (Fig. 3B). Also, there was a two- to threefold increase in the levels of the proliferation marker Ki-67 in the skin of Rhbd2P159LP159L mice compared with those of Rhbd2+/+ mice (Fig. 3C). Collectively, these data suggest that Rhbd2−/− mice and Rhbd2P159L/− mice are GOF mutations in the Rhbd2 gene, and that these mutations enhance secretion of AREG to cause tylosis.

AREG deficiency restores the normal skin phenotype in Rhbd2P159LP159L mice

Mice carrying the human tylosis GOF mutation Rhbd2P159L elicit enhanced AREG secretion (Fig. 2). To test whether AREG drives the skin disease phenotype in Rhbd2P159L/− mice, we genetically deleted AREG in Rhbd2P159LP159L mice by crossing Rhbd2P159LP159L with AREG-null mice. Rhbd2P159LP159L mice lacking AREG presented a full wavy coat rather than a partial hair-loss phenotype of Rhbd2P159LP159L mice (Fig. 4A). We confirmed that these mice are null for AREG by measuring serum AREG levels in Rhbd2P159L/−, Rhbd2P159L/−, Rhbd2P159LP159L, and Rhbd2P159LP159L Areg−/− mice. As expected, we found no detectable levels of AREG in Rhbd2P159LP159L Areg−/− mice (Fig. 4B). Additionally, whereas homozygous mutant Rhbd2P159LP159L mice had various degrees of follicular dystrophy or hair loss, the homozygous mutant mice with no AREG had no follicular dystrophy or hair loss (Fig. 4C). Also, there was no evidence of hyperplasia or hyperkeratosis of skin in Rhbd2P159LP159L Areg−/− mice (Fig. 4D). Collectively, these data strongly suggest that AREG mediates the skin disease phenotype of mice carrying the human tylosis mutation Rhbd2P159L, and that AREG is thus a potential therapeutic target for treating the skin disease tylosis.

MATERIALS AND METHODS

Animals

Mice were bred and maintained under modified barrier conditions at The Jackson Laboratory (JAX). The Animal Care and Use Committee at JAX approved the experimental procedures. For the tylosis mutation, we targeted the Rhbd2 locus in C57BL/6J zygotes by pronuclear microinjection of Cas9 mRNA (60 ng/μl), single guide RNA (sgRNA) (30 ng/μl), and single-stranded oligonucleotide DNA (ssDNA) (1 ng/μl). A total of 10 pups were born, and five of the 10 pups exhibited partial to complete hair loss. Tail DNA was amplified using PCR and then sequenced, and subsequently founder mice carrying the desired modified alleles were backcrossed to C57BL/6J mice to validate germline transmission. The resulting offspring heterozygous for the Rhbd2P159L allele were intercrossed to generate homozygous C57BL/6J-Rhbd2P159LP159L mice. Similar to the Rhbd2−/− heterozygous mice (Hosur et al., 2014; Johnson et al., 2003; Sigg et al., 2014), the Rhbd2P159LP159L heterozygous mice present with a normal hair coat, and moreover there is no evidence of epidermal hyperplasia or hyperkeratosis. We recently showed that B6.Cg-AregMcub Rhbd2P159L/J mice carry a T-to-G point mutation that disrupts the coding frame and introduces a premature stop codon in the Areg gene (Hosur et al., 2014). B6. Cg-AregMcub Rhbd2P159L/J do not produce a functional AREG protein; hence, AregMcub mice are referred to as Areg−/− in this manuscript.

Ear hole closure and histology

Ear punch closure, histology and histological analyses of hematoxylin and eosin (H&E)-stained sections were performed as previously described (Hosur et al., 2014). Slides were scanned using a Nanozoomer digital slide scanner (2.0-HT, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with the help of Dr Rosalinda Doty, a board-certified pathologist at JAX.

Isolation of fibroblasts and keratinocytes

MEFs were isolated from 13.5 days postcoitum mouse embryos using Liberase DL (Sigma-Aldrich). Embryos were dissected in 15 ml sterile Ear hole closure, histology and histological analyses of hematoxylin and eosin (H&E)-stained sections were performed as previously described (Hosur et al., 2014). Slides were scanned using a Nanozoomer digital slide scanner (2.0-HT, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with the help of Dr Rosalinda Doty, a board-certified pathologist at JAX.

Isolation of fibroblasts and keratinocytes

MEFs were isolated from 13.5 days postcoitum mouse embryos using Liberase DL (Sigma-Aldrich). Embryos were dissected in 15 ml sterile serum-free DMEM and miniced with a razor blade in a 100 mm petri dish. The DMEM medium was replaced with fresh serum-free DMEM containing 10% fetal bovine serum and antibiotic/antimycotic (Thermo Fisher Scientific). The cell suspension was centrifuged, washed three times with sterile PBS, re-suspended in collagen-coated six-well plates.
Fig. 2. Rapid wound healing and proliferative skin phenotype in Rhbdf2P159L/P159L mice. (A) Representative images of regenerating ear tissue in eight-week-old female Rhbdf2+/+ and Rhbdf2P159L/P159L mice at 14 days postwounding. Magnification, 4×; scale bars: 1 mm. (B) Quantification of ear hole closures shown in A. Rhbdf2+/+(n=6) and Rhbdf2P159L/P159L (n=4). (C) ELISA quantitation of AREG levels in the supernatants of cultured Rhbdf2+/+ and Rhbdf2P159L/P159L MEKs in response to stimulation with 100 nM PMA for the indicated times. (D) H&E-stained skin sections of female Rhbdf2+/+, Rhbdf2cub/cub and Rhbdf2P159L/P159L mice. Both the Rhbdf2cub/cub and Rhbdf2P159L/P159L mice present with significant hyperplasia (H), hyperkeratosis (arrow), enlarged sebaceous glands (S), and follicular dystrophy (F) compared with the Rhbdf2+/+ mice. Scale bars: 100 μm (low magnification); 50 μm (high magnification).
Fig. 3. Enhanced EGFR signaling in Rhbdf2<sup>P159L/P159L</sup> mice. (A) Immunohistochemical staining of Rhbdf2<sup>+/+</sup> and Rhbdf2<sup>P159L/P159L</sup> skin sections with phospho-ERK1/2 and phospho-mTOR. Arrows indicate increased levels of phospho-ERK1/2 and phospho-mTOR staining. Scale bars: 100 μm. (B) Immunoblot analysis of cell lysates obtained from Rhbdf2<sup>+/+</sup> and Rhbdf2<sup>P159L/P159L</sup> MEFS. Actin served as a loading control. Band intensities were quantified using ImageJ (https://imagej.nih.gov/ij/). (C) Immunohistochemical staining of Rhbdf2<sup>+/+</sup> and Rhbdf2<sup>P159L/P159L</sup> skin sections with proliferation marker Ki-67. Quantification of proliferating cells was performed as described previously (Almeida et al., 2012). Scale bars: 100 μm.
Fig. 4. Genetic deletion of Areg restores the normal skin phenotype in Rhbd2\textsuperscript{P159L/P159L} mice. (A) Representative images of age-matched Rhbd2\textsuperscript{P159L/P159L} and Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} male mice. Rhbd2\textsuperscript{P159L/P159L} mice show a loss of hair phenotype (1); Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice show a full hair coat (2). (B) Percentage serum AREG levels in age-matched female Rhbd2\textsuperscript{+/+}, Rhbd2\textsuperscript{P159L/+}, Rhbd2\textsuperscript{P159L/P159L} and Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice. AREG was not detected (ND) in the serum of Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice. Data represent mean±s.d. (n=4 mice per group). (C,D) H&E-stained skin sections of male Rhbd2\textsuperscript{P159L/P159L} and Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice. Follicular dystrophy, hyperplasia (H) and hyperkeratosis (arrow) observed in Rhbd2\textsuperscript{P159L/P159L} mice are restored in Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice. We found no evidence of follicular dystrophy (arrowhead), hyperplasia or hyperkeratosis of skin (asterisk) in Rhbd2\textsuperscript{P159L/P159L} Areg\textsuperscript{−/−} mice. Scale bars: 200 μm (low magnification); 100 μm (high magnification).
AREG ELISA
MEKs seeded in collagen-coated six-well dishes were stimulated with 100 nM PMA (R&D Systems, Minneapolis, USA) for the indicated times. AREG protein levels in the conditioned medium were measured using a Mouse Amphiregulin DuoSet ELISA Developmental Kit (#DY989, R&D Systems). A spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, USA) was used to determine the optical density.

SDS/PAGE and immunoblotting
SDS/PAGE and immunoblotting assays were performed as described previously (Hosur et al., 2014). Briefly, MEFs grown in six-well dishes were lysed with RIPA buffer (Cell Signaling Technology) and protein concentrations were determined using a Qubit Fluorometer (Life Technologies). After loading 50 μg of protein onto 4-20% (wt/vol) precast gels (Lonza), proteins were transferred to a PVDF membrane, before blocking with 5% milk for 1 h at room temperature (RT). Membranes were then exposed to EGFR (#2232; 1:1000), phospho-ERK1/2 (#4370; 1:1000), phospho-AKT (#4060; 1:1000), phospho-S6 (#4858; 1:1000 dilution) or actin (#4970; 1:1000) antibodies (Cell Signaling Technology) overnight at 4°C. Subsequently, membranes were washed in TBST for 2 h, exposed to secondary antibodies (Santa Cruz Biotechnology; 1:10,000) for overnight at 4°C. Subsequently, membranes were washed in TBST for 2 h, exposed to secondary antibodies (Santa Cruz Biotechnology; 1:10,000) for 1 h at RT, and washed for 2 h in TBST. Membranes were then exposed to Luminol reagent (Santa Cruz Biotechnology) for 3 min and visualized using a gel documentation system (G:BOX F3, Syngene, Frederick, USA).

Immunohistochemistry
Immunohistochemical staining of skin sections with specific antibodies [phospho-p44/42 MAPK (ERK1/2) (#4370, Cell Signaling Technology; 1:400), phospho-mTOR (#2976, Cell Signaling Technology; 1:100)] and Ki-67 (Thermo Fisher Scientific; prediluted) was performed on a Ventana Discovery XT automated IHC research slide staining system (Roche, Tucson, USA). For antigen unmasking, deparaffinized slides were pretreated with Cell Conditioning Solution (Ki-67) or citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) (phospho-ERK1/2 and phospho-mTOR). Ki-67 proliferation index was determined as described previously (Almeida et al., 2012).

Statistical analysis
GraphPad Prism v6 was used to analyze data. Student’s t-test was used to assess the statistical difference between two groups. P<0.05 was considered statistically significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.H.; Methodology: V.H.; Investigation: V.H., B.E.L.; Writing - original draft: V.H.; Writing - review & editing: V.H., L.D.S., M.V.W.; Supervision: L.D.S., M.V.W.; Funding acquisition: L.D.S., M.V.W.

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