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

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ABSTRACT
In humans, gain-of-function (GOF) mutations in RHBD2 cause the skin disease tylosis. We generated a mouse model of human tylosis and show that GOF mutations in RHBD2 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 RHBD2 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, RHBD2, Amphiregulin, Tylosis

INTRODUCTION
The role of RHBD2 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 RHBD2 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 RHBD2 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 Rhbd2 gene (Rhbd2cub/cub), GOF mutations in human RHBD2 (tylosis mutation p.I186T) promote increased secretion of AREG in vitro (Hosur et al., 2014). To determine in vivo whether the Rhbd2cub/cub mutation (Fig. 1A, loss of the cytosolic N-terminal domain of RHBD2) phenocopies human tylosis, and whether the tylosis p.I189L mutation (Fig. 1B, missense mutation in the cytosolic N-terminal domain of RHBD2) in RHBD2 increases AREG secretion, we generated mice carrying the human tylosis mutation p.I189L (p.I159L in mice) using CRISPR/Cas9-mediated targeting and homology-directed repair in C57BL/6J zygotes (Fig. 1C). We characterized the resulting Rhbd2P159L/P159L mice and observed that similarly to Rhbd2cub/cub mice (Hosur et al., 2014), mice carrying the human tylosis mutation present with complete hair loss (Fig. 1D). However, unlike Rhbd2cub/cub mice (Hosur et al., 2014; Siggs et al., 2014), which present complete hair loss throughout their lives, Rhbd2P159L/P159L mice developed a thin layer of curly hair coat that was noticeable in adult mice (Fig. 1E), indicating that the Rhbd2cub/cub phenotype is more severe than that of Rhbd2P159L. Nevertheless, Rhbd2P159L/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 Rhbd2P159L/P159L MEKs compared with Rhbd2+/+ MEKs (Fig. 2C).

RESULTS AND DISCUSSION
The human tylosis disease mutation enhances AREG secretion
Autosomal dominant mutations in the human RHBD2 gene cause tylosis (Blaydon et al., 2012; Saarinen et al., 2012). Substantial 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 similar to GOF mutations in the mouse Rhbd2 gene (Rhbd2cub/cub), GOF mutations in human RHBD2 (tylosis mutation p.I186T) promote increased secretion of AREG in vitro (Hosur et al., 2014). To determine in vivo whether the Rhbd2cub/cub mutation (Fig. 1A, loss of the cytosolic N-terminal domain of RHBD2) phenocopies human tylosis, and whether the tylosis p.I189L mutation (Fig. 1B, missense mutation in the cytosolic N-terminal domain of RHBD2) in RHBD2 increases AREG secretion, we generated mice carrying the human tylosis mutation p.I189L (p.I159L in mice) using CRISPR/Cas9-mediated targeting and homology-directed repair in C57BL/6J zygotes (Fig. 1C). We characterized the resulting Rhbd2P159L/P159L mice and observed that similarly to Rhbd2cub/cub mice (Hosur et al., 2014), mice carrying the human tylosis mutation present with complete hair loss (Fig. 1D). However, unlike Rhbd2cub/cub mice (Hosur et al., 2014; Siggs et al., 2014), which present complete hair loss throughout their lives, Rhbd2P159L/P159L mice developed a thin layer of curly hair coat that was noticeable in adult mice (Fig. 1E), indicating that the Rhbd2cub/cub phenotype is more severe than that of Rhbd2P159L. Nevertheless, Rhbd2P159L/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 Rhbd2P159L/P159L MEKs compared with Rhbd2+/+ MEKs (Fig. 2C).

Next, to examine whether mice carrying the human tylosis mutation phenocopy the Rhbd2cub/cub mutation (Hosur et al., 2014), we performed histological analysis of skin sections from Rhbd2P+/+ and Rhbd2P159L/P159L mice. Rhbd2P159L/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 Rhbd2P159L/P159L mice, we performed immunohistochemical analyses of skin sections from Rhbd2P+/+ and Rhbd2P159L/P159L mice. We observed a considerable increase in epidermal EGFR signaling pathway downstream effectors phospho-ERK1/2 and phospho-mTOR (Fig. 3A).
Similarly, immunoblot analyses of mouse embryonic fibroblasts (MEFs) isolated from Rhbd2P159L/− and Rhbd2P159L/P159L 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 Rhbd2P159L/P159L 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 Rhbd2P159L/P159L mice compared with those of Rhbd2+/− mice (Fig. 3C). Collectively, these data suggest that Rhbd2P159L/P159L 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 Rhbd2P159L/+ 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/P159L mice, we genetically deleted AREG in Rhbd2P159L/P159L mice by crossing Rhbd2P159L/P159L with AREG-null mice. Rhbd2P159L/P159L mice lacking AREG presented a full wavy coat rather than a partial hair-loss phenotype of Rhbd2P159L/P159L mice (Fig. 4A). We confirmed that these mice are null for AREG by measuring serum AREG levels in Rhbd2P159L/P159L, Rhbd2P159L/+ and Rhbd2P159L/P159L mice (Fig. 4B). Additionally, whereas homozygous mutant Rhbd2P159L/P159L 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 Rhbd2P159L/+ 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-Rhbd2P159L/P159L mice. Similar to the Rhbd2P159L/+ heterozygous mice (Hosur et al., 2014; Johnson et al., 2003; Siggs et al., 2014), the Rhbd2P159L/+ 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-Areg−/− Rhbd2P159L/+ 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-Areg−/− Rhbd2P159L/+ do not produce a functional AREG protein; hence, Areg−/− 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 (Lambeth, Sigma-Aldrich). Embryos were dissected in 15 ml sterile DMEM containing 10% fetal bovine serum and antibiotic/antimycotic (Thermo Fisher Scientific). Following overnight incubation at 4°C, epidermis was carefully separated from dermis and placed in a sterile petri dish containing 2 ml trypsin (TrypLE Select Enzyme, Thermo Fisher Scientific) for 30 min at room temperature. Subsequently, trypsin was neutralized with Defined Trypsin Inhibitor (Thermo Fisher Scientific), the cell suspension was centrifuged, and cells were seeded in collagen-coated six-well plates.
Fig. 2. Rapid wound healing and proliferative skin phenotype in Rhbdf2<sup>P159L/P159L</sup> mice. (A) Representative images of regenerating ear tissue in eight-week-old female Rhbdf2<sup>+/+</sup> and Rhbdf2<sup>P159L/P159L</sup> mice at 14 days postwounding. Magnification, 4×; scale bars: 1 mm. (B) Quantification of ear hole closures shown in A. Rhbdf2<sup>+/+</sup> (n=6) and Rhbdf2<sup>P159L/P159L</sup> (n=4). (C) ELISA quantitation of AREG levels in the supernatants of cultured Rhbdf2<sup>+/+</sup> and Rhbdf2<sup>P159L/P159L</sup> MEKs in response to stimulation with 100 nM PMA for the indicated times. (D) H&E-stained skin sections of female Rhbdf2<sup>+/+</sup>, Rhbdf2<sup>cub/cub</sup> and Rhbdf2<sup>P159L/P159L</sup> mice. Both the Rhbdf2<sup>cub/cub</sup> and Rhbdf2<sup>P159L/P159L</sup> mice present with significant hyperplasia (H), hyperkeratosis (arrow), enlarged sebaceous glands (S), and follicular dystrophy (F) compared with the Rhbdf2<sup>+/+</sup> 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 Rhbdf2^P159L/P159L^ mice. (A) Representative images of age-matched Rhbdf2^P159L/P159L_ and Rhbdf2^P159L/P159L_ Areg^-/- male mice. Rhbdf2^P159L/P159L_ mice show a loss of hair phenotype (1); Rhbdf2^P159L/P159L_ Areg^-/- mice show a full hair coat (2). (B) Percentage serum AREG levels in age-matched female Rhbdf2^+/+_, Rhbdf2^P159L/+_, Rhbdf2^P159L/P159L_ and Rhbdf2^P159L/P159L_ Areg^-/- mice. AREG was not detected (ND) in the serum of Rhbdf2^P159L/P159L_ Areg^-/- mice. Data represent mean±s.d. (n=4 mice per group). (C,D) H&E-stained skin sections of male Rhbdf2^P159L/P159L_ and Rhbdf2^P159L/P159L_ Areg^-/- mice. Follicular dystrophy, hyperplasia (H) and hyperkeratosis (arrow) observed in Rhbdf2^P159L/P159L_ mice are restored in Rhbdf2^P159L/P159L_ Areg^-/- mice. We found no evidence of follicular dystrophy (arrowhead), hyperplasia or hyperkeratosis of skin (asterisk) in Rhbdf2^P159L/P159L_ Areg^-/- mice. Scale bars: 200 μm (low magnification); 100 μm (high magnification).
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 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). Protein blots were 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 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). This work was supported by the National Institutes of Health (CA034196 and R21 OD023800-01 to M.V.W.).

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 stained with 3% hydrogen peroxide for 30 min followed by antigen retrieval with a Ventana Target Retrieval Solution for 30 min. 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 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). Protein blots were 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 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). This work was supported by the National Institutes of Health (CA034196 and R21 OD023800-01 to M.V.W.).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.H.; Methodology: 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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References


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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