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A genetic basis of variation in eccrine sweat gland and hair follicle density

Yana G. Kamberov, Elinor K. Karlsson, Gerda L. Kamberova, Daniel E. Lieberman, Pardis C. Sabeti, Bruce A. Morgan, and Clifford J. Tabin

Department of Genetics, Harvard Medical School, Boston, MA 02115; Department of Dermatology, Harvard Medical School and Cutaneous Biology Research Center, Mass General Hospital, Charlestown, MA 02129; The Broad Institute of MIT and Harvard, Cambridge, MA 02142; Department of Human Evolutionary Biology, Harvard University, Cambridge, MA 02138; Center for Systems Biology, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138; and Department of Computer Science, Hofstra University, Hempstead, NY 11549

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Among the unique features of humans, one of the most salient is the ability to effectively cool the body during extreme prolonged activity through the evaporative transpiration of water on the skin’s surface. The evolution of this novel physiological ability required a dramatic increase in the density and distribution of eccrine sweat glands relative to other mammals and a concomitant reduction of body hair cover. Elucidation of the genetic underpinnings for these adaptive changes is confounded by a lack of knowledge about how eccrine gland fate and density are specified during development. Moreover, although reciprocal changes in hair cover and eccrine gland density are required for efficient thermoregulation, it is unclear if these changes are linked by a common genetic regulation. To identify pathways controlling the relative patterning of eccrine glands and hair follicles, we exploited natural variation in the density of these organs between different strains of mice. Quantitative trait locus mapping identified a large region on mouse Chromosome 1 that controls both hair and eccrine gland densities. Differential and allelic expression analysis of the genes within this interval coupled with subsequent functional studies demonstrated that the level of En1 activity directs the relative numbers of eccrine glands and hair follicles. These findings implicate En1 as a newly identified and reciprocal determinant of hair follicle and eccrine gland density and identify a pathway that could have contributed to the evolution of the unique features of human skin.

Significance

Humans have the highest density of eccrine sweat glands of any mammal. Alongside hair follicles, eccrine glands are found throughout human skin and are indispensable for thermoregulation. Despite their importance, little is known about the biological factors that control eccrine gland formation and density. We investigated the genetic basis for the regulation of eccrine gland density in mice and find that eccrine gland and hair follicle densities are inversely regulated by the transcription factor En1. This work uncovers a previously unidentified biological factor that specifically promotes the development of eccrine glands when these organs are interspersed with hair follicles, and has the potential to inform efforts to elucidate the genetic basis for the evolution of increased eccrine gland density in humans.


References: C.L., NYU; and J.C.S., Cornell University.

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1 Present address: Program in Bioinformatics and Integrative Biology and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605.

2 To whom correspondence may be addressed. Email: tabin@genetics.med.harvard.edu or bruce.morgan@cbrc2.mgh.harvard.edu.

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challenging because invasive methods are required to accurately score these organs in hairy skin (10). However, experimental evidence indicates that the early development of ectodermally derived appendages is conserved in vertebrates (7, 11). We therefore turned to the laboratory mouse, a tractable genetic system. A rare exception among mammals, the mouse has both hair and eccrine glands interspersed in a distinct domain of the hindfoot where the density of the two appendage types can be easily scored. Genetic mapping of the differences in patterns of ectodermal appendages between mouse strains as well as functional modulation of expression levels, implicates the transcription factor En1 as a determinant of eccrine gland and hair follicle densities in mice. These results reveal a hereto unappreciated role for En1 in later patterning of the distal limb in mice and may provide clues as to the molecular pathways that were modified during human evolution to drive changes in eccrine gland density.

Results

Strain-Specific Differences in Ectodermal Appendage Densities of Mice. In mice, as in most other mammals, eccrine glands are not thermoregulatory, but instead serve a role in traction during locomotion and are restricted to the volar surfaces of the fore- and hind feet (Fig. 1A). The footpads of mice are densely populated with eccrine glands but devoid of hair follicles. However, the skin between the footpads exhibits both eccrine glands and hair follicles interspersed as they are found in human hairy skin. Within this region, the relative numbers of hair follicles and eccrine glands are fixed within an inbred strain, but highly variable between strains. Examination of the hind feet of two common strains of inbred mice, C57BL/6 and FVB/N, revealed that C57BL/6 have far more hair follicles (mean 65.4 ± 8.9) than FVB/N (mean 5.2 ± 3.3) in this region (unpaired t test P = 2.47e−24). In contrast, there are over fourfold more eccrine glands in this region in FVB/N than C57BL/6 (Mean: FVB/N 39.5 ± 8.9; C57BL/6 8.3 ± 2.3; unpaired t test P = 1.8e−24) (Fig. 1A–C). There is also a subtle but significant increase in the number of eccrine glands in the footpads of FVB/N mice relative to C57BL/6 (unpaired t test P < 0.01 for all footpads scored). Footpads lack hair follicles in both strains (Fig. 1A and C).

A Complex Genetic Basis for Strain Differences in Ectodermal Appendage Density. To examine the genetic architecture underlying differences in eccrine gland and hair follicle number in the two strains, we generated F2 animals from an FVB/N by C57BL/6 cross. Analysis of the trait distribution in the F2 population (n = 647) indicated a polygenic basis for both phenotypes (Fig. 1D and E). Comparison of eccrine gland and hair follicle number in F2 individuals shows that, at the extremes, there is an inverse correlation between the densities of the two appendage types (Fig. 1F). However, this inverse correlation is not a simple reciprocal relationship, as statistical analysis between hair follicle and eccrine gland number in F2 animals did not support a linear dependence between the two traits [correlation coefficient is −0.5622, with 95% confidence interval (−0.6127 to −0.5070)]. However, analysis of the P value to test for a correlation between hair follicle and eccrine gland number, P < 1e−16, indicated a very low probability that the observed covariation between the two traits occurred by chance.

Genetic Mapping Identifies Genomic Regions Controlling Eccrine Gland and Hair Follicle Traits. Whole genome quantitative trait locus (QTL) mapping in the F2 population using ninety individuals selected from the tails of both phenotype distributions (animals with the highest and lowest eccrine gland and hair follicle numbers) identified several significant QTLs for both eccrine gland and hair follicle number (P < 0.05) (Fig. 2A and B). We noted overlapping, major effect QTLs for the eccrine gland and hair follicle traits on Chromosome (Chr) 1 (1.5 LOD drop, mm10: 103.350236 Mb to 120.607746 Mb for eccrine glands; 91.653464 Mb to 131.092431 Mb for hair follicles; Fig. 2A and B). Given that the Chr1 QTLs for both traits were highly significant (LOD scores 27.9 and 18.6, respectively, for eccrine gland and hair follicle number) and explained a large percent of the phenotypic variance for each trait (76% and 61%, respectively for eccrine gland and hair follicle number) we focused on this overlapping interval.

Fig. 1. A polygenic basis for natural variation in mouse ectodermal appendage number. (A–C) Reciprocal patterns of eccrine gland (EG) and hair follicle (HF) densities in the interfootpad (IFP) space of C57BL/6 and FVB/N mice. (A) Epidermal preps of C57BL/6 and FVB/N ventral hind-feet; footpads (FP) are labeled; IFP is defined as the area circumscribed by all six FPs. Boxed area is detailed in (B); red arrow: EG; gray arrow: HF. (B) Detail of equivalent areas of C57BL/6 and FVB/N IFP spaces showing a reciprocal pattern of ectodermal appendage types between the two strains. (C) Quantification of ectodermal appendage number in FVB/N and C57BL/6 mouse hindfeet. Mean and SD for each trait are plotted. FP-3 P = 1.28e−24, FP-4 P = 1.22e−26, FP-5 P = 2.23e−13, FP-6 P = 1.51e−17, IFP-EG P = 1.82e−24, IFP-HF P = 2.47e−38. (D and E) Frequency distribution of EG (D) and HF (E) number in the F2 population is consistent with a polygenic specification for both traits. Data are presented in bins of ten, with x axis labels indicating the upper bound on EG (D) or HF (E) number per bin. (F) Relationship between IFP ectodermal appendage traits in the F2 population. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Differential Expression Screen Identifies En1 as a Candidate Causal Locus. The ~30 Mb region of Chr1 that spans the eccrine gland and hair follicle QTLs is typical of the mapping resolution achieved with an F2 intercross (12). As expected, this interval contains numerous annotated genes. To narrow down the list of plausible causal loci, we evaluated the relative expression of genes from the Chr1 QTLs in the developing plantar skin of C57BL/6 and FVB/N mice at P2.5, a stage when the early primordia of ectodermal appendages are morphologically evident in the interfootpad regions of both strains (Fig. 3 A and B). Of the genes evaluated, only En1 and Serpinb13 showed preferential expression in FVB/N, whereas Tmem177 showed preferential expression in C57BL/6. In preliminary studies, we were unable to discern expression of Tmem177, which encodes a transmembrane protein of unknown function, in the ventral developing hindlimb at a level detectable by in situ hybridization. Although Serpinb13 is expressed in mature eccrine glands (13), evaluation of its transcript localization by in situ hybridization did not reveal early placode-related expression.

In contrast, and consistent with reports that En1 is highly expressed in the basal layer of the epidermis of the footpads and footpad eccrine glands in later development (14), in situ hybridization revealed an abundance of En1 expression in these tissues at P2.5 in both the C57BL/6 and FVB/N strains (Fig. S1). Expression throughout the basal layer is strong in both strains within the footpad regions, which are devoid of hair follicle primordia. Expression in the same tissue layer is lower in the interfootpad regions, where the number of eccrine glands and hair follicles differ between the two strains. However, there is focal accumulation of En1 transcripts in the majority of ectodermal appendage primordia of FVB/N but not C57BL/6 mice (Fig. 3B). In more proximal and lateral regions where eccrine glands do not form, no En1 expression was observed, consistent with the later expression pattern of En1 (14). Thus, En1 expression is present during interfootpad development and high levels of En1 expression in the developing ectodermal appendages correlate with the development of eccrine glands.

Differential Cis-Regulation of En1 Underlies Expression Differences Between Mapping Strains. The observed difference in En1 expression between C57BL/6 and FVB/N strains could be a reflection of the preferential formation of eccrine glands, in which En1 is more highly expressed, or could itself play a causative role in driving the observed differences in phenotype. To distinguish between these alternatives, we evaluated the allelic expression of En1 in tissues of F1 animals of an FVB/N by C57BL/6 cross (Fig. 3C). In the F1 animal, the appendage composition of the skin is a constant and the En1 alleles derived from FVB/N and C57BL/6 origin are exposed to the same transregulatory factors. Consequently, a difference in the relative levels of expression of the FVB/N versus C57BL/6 copy of En1 transcript in this context would suggest a mechanism intrinsic to the En1 allele derived from each strain. We found that the FVB/N allele of En1 is expressed at a higher level than the C57BL/6 allele in the skin taken from the ventral surface of the feet of F1 animals at the time that placodes are forming (One way ANOVA, \( p = 7.96 \times 10^{-16} \), Fig. 3C). This difference is specific to the epidermis during the period when interfootpad appendages are forming and was not observed in forelimb tissues at earlier stages of limb development when En1 plays a role in dorsal ventral patterning (15) (Fig. 3C). As an additional control, allelic discrimination at Gapdh was evaluated in the P2.5 ventral hindlimb and did not
show an enrichment of the FVB/N allele, indicating that the observed skew toward the FVB/N allele is specific to the En1 transcript. Augmented expression of the FVB/N En1 allele is therefore the result of cis-regulatory changes, as both alleles are exposed to the same transacting factors in the F1 skin, a finding consistent with the model that cis-regulatory differences at the En1 locus are responsible for the ectodermal appendage phenotypes mapped to Chr1.

**Genetic Reduction of En1 Levels Leads to Reciprocal Alterations in Eccrine Gland and Hair Follicle Number.** A homeodomain transcription factor, En1 is a multifunctional protein with a classically described role in transcriptional repression that often relies on its interaction with Groucho/TLE corepressors (16). Efforts to test directly whether En1 acts to determine the type of appendage formed by the developing ectodermal placodes are hampered by the earlier developmental role of En1 in the specification of ventral identity (15). As eccrine glands are exclusively found on the ventral surface of the limb, there is a consequent absence of eccrine glands in the dorsaled distal limb of En1 homozygous knockout mice (15). In their place are large, dorsal-type hair follicles in what would have been the ventral skin, as well as hyperpigmentation and hypoplasia of footpads, circumferential nails and dorsally patterned tendons and muscles (15). Thus, any direct effect on eccrine gland development in the homozygous knock-out is obscured by the general dorsalization of the skin.

To analyze the phenotypic consequences of reduced En1 levels on ectodermal appendage patterning, we turned to animals heterozygous for an En1 null allele (En1<sup>Cki</sup>) (17), reasoning that if levels of En1 were important for specification of ectodermal appendage type, the decreased dose of En1 in the heterozygote might be informative. As previously described, En1 null heterozygotes show normal dorso-ventral limb patterning (Fig. 3D and E) (15, 17). Consistent with this phenotype, all footpads form normally (Fig. 3D). However, En1 heterozygotes have fewer eccrine glands and more hair follicles in the interfootpad region. This effect was observed in FVB/N by C57BL/6 F1 animals in which the C57BL/6 En1 allele was disrupted. In this context, interfootpad eccrine gland number was reduced from an average of 40 ± 5 to 17 ± 8 (unpaired t test: P = 3.22e⁻¹⁴) whereas hair follicle number increased from 33 ± 4 to 55 ± 8 (unpaired t test: P = 1.07e⁻⁹), respectively in wild-type animals versus those heterozygous for the En1<sup>Cki</sup> null allele (Fig. 3D and E). This effect was also observed in a C57BL/6 genetic background where eccrine gland number was reduced by half from 13 ± 3 to 5 ± 1 (unpaired t test: P = 1.67e⁻⁸), and hair follicle number increased from 74 ± 5 to 95 ± 9 (unpaired t test: P = 4.49e⁻⁷) (Fig. 3E). Thus, whether in the context of the high numbers of eccrine glands in an FVB/N by C57BL/6 hybrid, or the low numbers of eccrine glands characteristic of the C57BL/6

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**Fig. 3.** En1 is a determinant of eccrine gland and hair follicle number. (A) Quantitative RT-PCR on transcripts from the Chr1 QTL from C57BL/6 and FVB/N P2.5 ventral hindlimb skin. The log<sub>2</sub> of the fold change in the normalized expression of FVB/N:C57BL/6 transcript is plotted. Vertical bars indicate the 1.5-fold expression change boundary. Horizontal bar delineates the P < 0.05 significance boundary. Up-regulated transcripts (red); down-regulated transcripts (green); transcripts that did not show significant change (black). En1 is up-regulated 1.63-fold in FVB/N versus C57BL/6 tissue (P = 3.22e⁻²). (B) Focal up-regulation of En1 expression in FVB/N but not C57BL/6 interfootpad (IFP) ectodermal appendage primordia. Arrows indicate morphologically evident...
background, a reduction in En1 gene dosage shifts the balance from eccrine gland production to hair follicle production in the interfootpad region. These data provide functional evidence that En1 activity favors the formation of eccrine glands at the expense of hair follicles in the interfootpad space and that a difference in En1 is a causal component of the differences in eccrine gland and hair follicle densities we observed between C57BL/6 and FVB/N mice.

We also noted a subtle but significant decrease in eccrine gland number in the footpads of En1 null heterozygotes. This decrease was observed in all footpads scored in C57BL/6 mice, but only two of the four footpads scored in FVB/N by C57BL/6 F1 mice (unpaired t test P < 0.05) (Fig. 3 D and E). In the footpads, these reductions in eccrine gland number were not accompanied by the appearance of hair follicles. The relative insensitivity of the footpad eccrine glands to the loss of one functional En1 copy is most likely the result of the comparatively high level of expression of En1 in the footpad epidermis.

Discussion

Despite the evolutionary and functional importance of eccrine glands in humans, we know remarkably little about the molecular mechanisms that lead to the specific formation and patterning of these ectodermal appendages. In this study, we demonstrate that modulation of En1 levels is a driver of natural differences in eccrine gland and hair follicle density between mouse strains. This finding not only provides insight into a distinct molecular program that promotes increased eccrine gland density, but also reveals that this effect on eccrine development occurs at the expense of hair follicles in a tissue where the two appendage types are naturally interspersed.

Pursuing the Genetic Basis for Natural Variation as a Means to Elucidate Ectodermal Appendage Development. The discoveries that the interfootpad region contains the interspersed eccrine glands and hair follicles characteristic of human skin and that the patterns of these appendages are distinctive among inbred mouse strains provide a powerful approach to investigate the genetic regulation of cutaneous appendage type and density. We have observed that patterns of eccrine glands and hair follicles in the interfootpad skin vary widely between many mouse strains, yielding a rich resource to identify additional factors involved in specifying these traits. Community efforts to enhance the resolution of QTL mapping using newly developed mouse strains and mapping approaches will further facilitate this pursuit (18-20).

Genetic mapping using the two inbred strains analyzed in our study revealed that multiple loci contribute to the differences in ectodermal appendage phenotypes. The allele specific expression of En1 between C57BL/6 and FVB/N and the strength of the effect of modulating En1 levels on both eccrine gland and hair follicle densities suggest that En1 is one critical node in this genetic network and contributes to the effect of the Chr1 QTLs. Importantly, the Chr1QTLs explained less than 50% of the variance in either phenotype, emphasizing that although the En1-containing region of Chr1 is a major driver for our traits of interest, modifier loci located in other regions of the genome are contributing to the final phenotype observed. One such region was identified by our analysis: a significant QTL controlling hair follicle density on Chr7. Furthermore, a preliminary drop-out mapping analysis in which individuals with extreme phenotypes for each trait were removed from the mapping population further increased the significance of the Chr7 QTL and raised the significance of other minor peaks that did not surpass the α ≤ 0.05 significance threshold in our initial analysis. A complete understanding of the genetic basis for the specification of eccrine gland and hair follicle densities will thus require identification of these modifier loci and may be enhanced by increasing the number of interindividually sampled mice.

The natural variation in ectodermal appendage patterns among mouse strains provides a rich resource to further expand this catalog through the use of genetic mapping.

An Additional Developmental Role for En1. Taken together, our data indicate that differential cis-regulation of En1 is likely a contributor to the observed variation in ectodermal appendage distribution between C57BL/6 and FVB/N mapping strains. In conjunction with prior work, these results indicate that En1 has sequential functions in patterning the skin. First, En1 plays a well established role in determining ventral, as opposed to dorsal, identity in the distal limb (15). At subsequent stages, the expression of En1 is maintained in the basal layer of ventral epidermis in the medial and distal autopod until the ectodermal appendages form. At this point, En1 transcripts accumulate to a high level throughout the footpads. We propose that these higher levels of En1 serve to drive the formation of eccrine glands at the expense of hair follicles, thus patterning the footpads as hair follicle-free domains.

In contrast to the footpads, the interfootpad regions express lower levels of En1, in a range compatible with both hair follicle and eccrine gland specification and thus form a mixture of both appendages. Our data demonstrate that En1 levels modulate the relative proportions of these two types of ectodermal appendages within the interfootpad space in different genetic backgrounds. These findings suggest that differences in the activity of cis-regulatory regions of the of En1 gene play a significant role in dictating the preponderance of hair follicles and paucity of eccrine glands in the C57BL/6 background, drive the inverse trait distribution in the FVB/N background and, in the F1 animals of a C57BL/6 by FVB/N cross, result in the intermediate ratio of ectodermal appendage types.

In contrast to the interfootpad region, where alteration in En1 expression has a qualitative effect on the nature of skin appendages, the effect of altered En1 levels in the footpad is quantitative. Although fewer eccrine glands are formed when En1 expression is reduced in this region, they are not replaced by hair follicles. The footpad region is not inherently incapable of forming hair follicles, as transgenic mice expressing the BMP inhibitor Noggin in the basal layer of the developing epidermis have hair follicles in their somewhat flattened but nonetheless morphologically distinctive footpads (9). We infer that the specification of appendage type is sensitive to En1 levels within a range found in the interfootpad region but below that characteristic of the forming footpads. It will be of interest to determine whether the ability of Noggin to alter appendage type in the footpad is mediated by diminished En1 expression.

These results demonstrate that in the appropriate context, modest changes in En1 expression can have profound effects on the distribution and identity of cutaneous appendages. It is an intriguing possibility that changes in En1 itself or the pathways that regulate its expression may have similarly contributed both to altering the domain in which eccrine glands form and to the striking differences in eccrine sweat gland number and hair development that occurred as the human lineage evolved.

Materials and Methods

Mice. C57BL/6 mice were obtained from Taconic Biosciences, and FVB/N mice were obtained from Charles River Laboratories. F1 and F2 mice were generated at Harvard Medical School. En1<+/> mice were obtained on a C57BL/6 by FVB/N background from the laboratory of Susan Dyrecki (Harvard Medical School) and subsequently bred for one generation to FVB/N mice. The number of animals analyzed for trait distributions were as follows: C57BL6 n = 28, FVB/N n = 29, F1 n = 24, F2 n = 647. For analysis of the effects of En1 reduction on ectodermal appendage phenotypes the following numbers of mice were analyzed: +/- n = 14, En1<+/> n = 15. Mouse work was performed in accordance with protocols approved by the Harvard Medical Area Standing Committee on Animals.
Quantification of Eccrine Gland and Hair Follicle Number. Eccrine gland and hair follicle number was scored by averaging the number of each appendage type across the left and right hind feet in epidermal preparations from each assayed animal. Epidermal preparation method has been described in detail previously (21, 22). In brief, volar skin from both hind-feet was dissected and dissociated from the underlying tissue with Dispase II (Roche). Eccrine gland ducts and whole hair follicles remained associated with the epidermis. Epidermal preparations were stained with Nile Blue A (Sigma Aldrich) and Oil Red O (Sigma Aldrich) which stains the hair follicle-associated sebaceous glands, thus allowing unambiguous identification of each type of ectodermal appendage. Footpads 1 and 2 were not analyzed as their high eccrine gland density prevents accurate scoring. The number of eccrine glands or hair follicles was averaged across both hind-feet. Representative images were taken on a Leica MZFLIII stereomicroscope equipped with a Nikon DXM1200F camera. Phenotypes were assayed in 4- to 6-week-old mice.

QTL Mapping DNA Purification and Genotyping. Tail genomic DNA was purified using the Qiagen GentraPure kit according to the manufacturer’s protocol. Whole genome SNP genotyping of ninety two F2 mice was carried on the Illumina MD mouse panel which carried 833 informative SNPs to distinguish C57BL/6 and FVB/N strains. Genotyping was carried out by the Partners Healthcare Center for Personalized Medicine Genetic DNA Sequencing Facility using Illumina GoldenGate. Detection of the alleles was carried out using the Illumina iSCAN fluorescent reader and automated genotype calling was carried out using Illumina GenomStudio version 2010.2, which used GenCall software to automatically cluster, call genotypes and assign confidence scores panels.

Fine mapping of the Chr1 QTLs was carried out using Sequenom MassArray custom assays by the Genome Analysis Platform (Broad Institute of MIT and Harvard). Custom genotyping oligos were designed using software from Sequenom. Genotype calling was carried out using Sequenom Typer software, followed by manual review of any samples or SNPs that did not cluster or perform well. Ninety informative SNPs were genotyped spanning Chr1 76,375,266 bp to 148,980,277 bp (mm10 coordinates; Table S1 for a list of SNPs used). A total of 486 F2 individuals were genotyped; however, three individuals were dropped from the analysis due to missing genotype calls.

Quantitative RT-PCR. Assays were performed in biological triplicates consisting of six pooled ventral hindlimb skins from each genotype at P2.5, in technical duplicate. Skin included the footpads and interfootpad space. The expression of a subset of the annotated genes in the Chr1 QTLs was analyzed on the OpenArray platform (Life Technologies) using predesigned TaqMan expression assays (Table S2 for assays used). Results were analyzed using BioDass software v3.0 (Life Technologies). Expression was normalized to Actb and Gapdh housekeeping genes.

In Situ Hybridization. En1 in situ hybridization was performed according to standard protocols using an antisense probe spanning nucleotides 1 bp to 984 bp of the En1 mRNA.

Allelic Discrimination Assay. Allelic expression of the C57BL/6 and FVB/N alleles of En1 was analyzed from cDNA and gDNA of F1 mouse tissues. In brief, mRNA from E13.5 whole forelimb and P2.5 dissected footpad and interfootpad hindlimb skin was isolated by Trizol (Life Technologies) extraction followed by clean up and on column DNase treatment using the RNasey Mini Kit (Qiagen) according to the manufacturer’s instructions. En1 allelic expression was determined by the relative expression of FVB/N vs. C57BL/6 alleles as distinguished by genotype at rs3676156 and at rs30898354 for Gapdh. Allelic expression data were analyzed using the sequencing based Q5/Analyzer software (24) (dna.leeds.ac.uk/q5/) following amplification of cDNA and gDNA products using the following primers: En1 Forward 5’-GAGGAGCTG-CAGAGACTCAA-3’ and En1 Reverse 5’-CTCCGTCCTCTGTGCC-3’; Gapdh Forward 5’-TGGGTTGAGAACATGGTGCTG-3’ and Gapdh Reverse 5’-ATTGCAGTGGGCAGATTGTGG-3’. Sequencing was carried out using the aforementioned En1 Forward and Gapdh Forward primers. Normalization of electropherogram peak heights was carried out with respect to ten invariant nucleotides upstream and ten invariant nucleotides downstream of the variant SNP.

Statistical Analysis. MATLAB Statistical Toolbox function concorfe() was used to investigate whether the dependence between eccrine gland and hair follicle number could be approximated by a linear model, and to compute the correlation coefficient with upper and lower 95% confidence values and the related P value.

All other statistical analysis was carried out using the GraphPad Prism 6 software package. Correction for multiple testing was carried using Bonferroni’s multiple comparisons test when carrying out one-way ANOVA test for significance. Adjusted P values are reported.

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