

A Mouse *Keratin 1* Mutation Causes Dark Skin and Epidermolytic Hyperkeratosis

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Chemical mutagenesis in the mouse has increased the utility of phenotype-driven genetics as a means for studying different organ systems, developmental pathways, and pathologic processes. From a large-scale screen for dominant phenotypes in mice, a novel class of pigmentation mutants was identified by dark skin (Dsk). We describe a Dsk mutant, *Dsk12*, which models the human disease, epidermolytic hyperkeratosis (EHK). At 2 days of age, mutant animals exhibit intraepidermal blisters and erosions at sites of trauma, and by 2 weeks of age develop significant hyperkeratosis. We identified a missense mutation in mutant animals that predicts an S194P amino acid substitution in the 1A domain of *Keratin 1*, a known target for human mutations that cause EHK. *Dsk12* recapitulates the gross pathologic, histologic, and genetic aspects of the human disorder, EHK.

Journal of Investigative Dermatology (2006) **126**, 1013–1016. doi:10.1038/sj.jid.5700241; published online 9 March 2006

INTRODUCTION

Positional cloning of mouse mutations ascertained because of a specific phenotype is a powerful approach for studying genes involved in a defined cellular or developmental pathway. To increase the number of entry points towards this goal, large-scale mutagenesis projects have been initiated in several centers (Hrabe de Angelis *et al.*, 2000; Nolan *et al.*, 2000; Shiroishi, 2001; Clark *et al.*, 2004). The screen in Neuherberg, Germany, is a dominant screen focused on dysmorphology, in which nearly 10% of the mutations affect hair or skin color. We are interested in a new class of pigmentation mutants identified by virtue of exhibiting dark skin (Dsk) in the ears, footpads, and/or tails, and have previously described the pathologic and genetic characterization of 10 dominant Dsk mutants (Fitch *et al.*, 2003). While a majority of these mutants map to locations in the genome not known to be associated with cutaneous phenotypes, we previously identified mutations in *Keratin 2e* (*Krt2e*) and *epidermal growth factor receptor* (*Egfr*) in two Dsk mutants which serve as useful models of human disease: the rare Krt disorder, ichthyosis bullosa of Siemens (*Krt2e*), and epidermal growth factor receptor-dependent carcinogenesis.

Here we describe a new mutant, *Dsk12*, that models the human disease, epidermolytic hyperkeratosis (EHK). While *Dsk12* was identified by increased footpad pigmentation in

heterozygous animals at 2 months of age, marked blistering and hyperkeratosis develop during postnatal life, a phenotype very similar to the human condition EHK. We describe the whole animal, histologic, and molecular genetic characterization of *Dsk12*, and identify a missense alteration in the 1A domain of *Keratin 1* (*Krt1*).

RESULTS

Dsk12 was identified by the presence of excess pigmentation in heterozygous animals at 2 months of age (Figure 1a and b). The increase in pigmentation is located on the volar pads, the intervolar pad scales, and the digital tips. Histologic analysis of mutant footpads shows hyperkeratosis, acanthosis, and increased epidermal pigment located at the tips of the rete ridges and within the eccrine ducts (Figure 1c and d). These gross and histologic phenotypes are similar to other Class II mouse pigmentation mutants (a classification scheme described by Fitch *et al.* (2003)), which are characterized by an accumulation of epidermal pigment.

Immediately after birth, *Dsk12*/+ animals develop blisters and erosions at sites of friction, including the footpad and tail (Figure 1e and f and data not shown). Infrequently, *Dsk12*/+ animals have small erosions and scaling on the body, but, like other *Krt1* and *Krt 10* mouse mutants (Porter *et al.*, 1996; Arin *et al.*, 2001; Arin and Roop, 2004), scaling and blistering on the body diminishes with the onset of hair growth. Histological examination of lesional skin shows an intraepidermal blister and disintegration of spinous keratinocytes. After several days, serous crust and regenerating keratinocytes cover the ruptured blister (Figure 1g and h). By 2 weeks of age, erosions are replaced by marked scaling (Figure 1i and j), and the footpad epidermis is characterized by hyperkeratosis, acanthosis, and keratinocyte cytolysis (Figure 1k and l). Hyperkeratotic areas on the footpads develop into thick, brown hyperkeratotic plaques in adult animals.

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Abbreviations: Dsk, dark skin; EHK, epidermolytic hyperkeratosis; Krt, Keratin
Received 26 September 2005; revised 16 December 2005; accepted 2 January 2006; published online 9 March 2006



Figure 1. *Dsk12* heterozygous phenotype. (a, b) *Dsk12*/+ footpads are darker than nonmutant littermates at 2 months of age. (c, d) Histologic analysis of *Dsk12*/+ footpads shows hyperkeratosis, acanthosis, and increased epidermal pigment (black arrow). (e, f) At 2 days of age, blistering and erosions are apparent, and (g, h) an intraepidermal separation and serous crust are observed. (i, j) 2-week old *Dsk12*/+ footpads, are characterized by scaling. (k, l) Histology shows hyperkeratosis, acanthosis, and keratinocyte cytolysis. (a, b, i, j) Bar = 2 mm, (c, d) bar = 125 μ m, (e, f) bar = 500 μ m, (g, h) bar = 75 μ m, (k, l) bar = 50 μ m.



Figure 2. *Dsk12* homozygous phenotype. (a-c) *Dsk12*/*Dsk12* newborn pups show signs of skin fragility. (b) Some pups are born with small erosions, (c) while others have generalized bullae and erosions. (b-c) Bar = 2.5 mm.

We also characterized the phenotype of *Dsk12* homozygotes, which exhibit a more severe blistering phenotype than heterozygotes at birth, with small skin erosions in some pups and widespread desquamation in others (Figure 2a-c). Animals with extensive blistering appear dehydrated and either die spontaneously or are cannibalized; no *Dsk12*/*Dsk12* animals were recovered after 21 days of age (Table 1). We used a combined intercross and backcross strategy to map *Dsk12* to the distal end of chromosome 15. The proximal boundary is defined by a crossover between

Table 1. Observed genotype frequencies from *Dsk12*/+ intercrosses

Age	+/+ ¹	<i>Dsk12</i> /+	<i>Dsk12</i> / <i>Dsk12</i>
Newborn (P0.5)	17	31	7 ²
Adult (P21) ³	29	40	0

¹Animals were genotyped using flanking polymorphic markers on F1 intercross progeny or an *Mae*III restriction fragment length polymorphism on inbred animals.
²3/7 *Dsk12*/*Dsk12* newborn animals expired at birth.
³Values differ from expected Mendelian ratio ($P < 0.001$ using χ^2 statistical test).

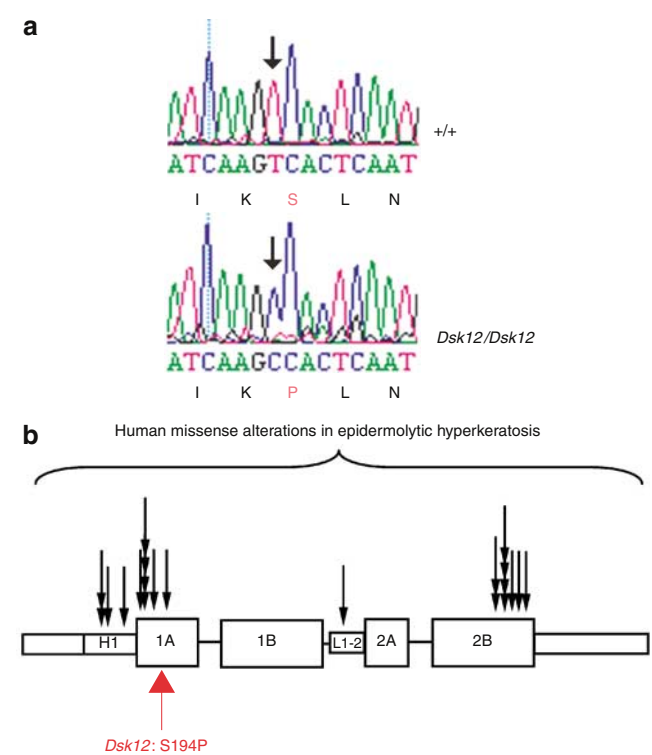


Figure 3. A mutation in basic *Krt1* in *Dsk12*. (a) Sequencing of genomic DNA from nonmutant and mutant animals reveals a T→C transition in exon 1 of *Krt1* (NM_008473). (b) The *Dsk12* base pair change predicts a S194P missense mutation in the highly conserved 1A domain of *Krt1* (red arrow). Human missense alterations in patients with EHK are indicated (black arrows) (<http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/128198.html>).

Dsk12 and D15Mit43 that was recovered in a *Dsk12*/+ animal; the distal boundary is defined by the end of chromosome 15. This defines a critical interval of ~5.6 Mb (from D15Mit43 at 98,066,137 to the end of chromosome 15 at 103,696,402) that contains ~100 genes. In this region, we considered *Krt1* as the most likely candidate because the *Dsk12*/+ phenotype mimics the gross and histologic features of *Krt1* (and *Krt10*) mutant mice: blistering at birth that diminishes with the onset of hair growth, leaving thick, brown, hyperkeratotic plaques on the paws (Bickenbach et al., 1996; Porter et al., 1996; Arin et al., 2001; Arin and Roop, 2004). We also considered *Krt2e* as a

candidate because, like *Krt1*, *Krt2e* is expressed in the suprabasal epidermis, and we have previously identified a missense mutation in *Krt2e* in another Dsk mutant (*Dsk2*) that causes epidermal thickening and suprabasal blistering (Fitch et al., 2003).

Using PCR amplification of genomic DNA and direct sequencing of *Krt1* and *Krt2e* exons from nonmutant and mutant animals, we identified a missense mutation that predicts a S194P substitution in the 1A domain of *Krt1* (Figure 3a). This region is known to be important for acidic Krt-basic Krt interactions during the formation of Krt intermediate filaments (Coulombe et al., 1990; Wilson et al., 1992), and is a domain where a number of missense mutations in human patients with EHK have been identified (Figure 3b). Surprisingly, this exact mutation has been reported in a human patient with EHK (McLean et al., 1994).

DISCUSSION

Dsk12 mutant mice exhibit many of the hallmarks of the human Krt disorder EHK (Bale et al., 1993). Like affected patients, *Dsk12* mutant animals present with blistering and erosions at birth; pathological examination of lesional skin shows suprabasal keratinocyte lysis and a thickened stratum corneum. Although *Dsk12* was identified as a pigmentary abnormality, humans affected with EHK also develop brown hyperkeratotic plaques in areas exposed to mechanical trauma later in life (DiGiovanna, 1999). Every reported case of EHK has been associated with a mutation in either *KRT1* or *KRT10*, and the mutations often affect the helix initiation or termination motifs (1A and 2B, respectively). Thus, *Dsk12* is a model for EHK at the whole animal, pathologic, and molecular genetic levels.

Secondary structure predictions carried out by McLean et al. (1994) indicate the S185P mutation in a human patient affected with EHK is likely to disrupt the α -helical structure of the 1A domain and interfere with *KRT1-KRT10* interactions during intermediate filament formation. Although we have not carried out biochemical studies on the Krt1 S194P mutation, it is orthologous to the S185P mutation in human *KRT1* and therefore likely to act in a similar manner. Finally, the mouse and the human conditions are manifest in heterozygotes. Although humans with homozygous EHK have not been described, the semidominant nature of *Dsk12* suggests that *KRT1* or *KRT10* missense alterations in the interaction domain would be more severe in homozygous than in heterozygous form.

Several approaches have been used to generate mouse models of EHK, including expression of a truncated *KRT14* transgene (Fuchs et al., 1992), expression of a truncated *KRT1* transgene (Bickenbach et al., 1996), and conditional expression of a mutant *Krt10* gene using gene targeting and site-specific recombination (Arin et al., 2001; Arin and Roop, 2004). These studies have led to substantial insight into the molecular pathogenesis of blistering diseases caused by Krt abnormalities, and provide the foundation for our speculations regarding the mechanism of action in *Dsk12* mutant mice.

The mechanism by which brown hyperkeratosis develops in *Krt1* or *Krt10* mouse mutants and in human patients with EHK is unknown. While the contribution of the thick epidermis to skin darkening has not been investigated, some investigators have proposed that scale pigmentation is due to a deficiency of melanosome degradation (Mesquita-Guimaraes, 1981). Our own studies of *Dsk2*, a *Krt2e* mutation that mimics ichthyosis bullosa of Siemens, suggest that skin darkening in *Dsk12* may be due to an increased number of melanocytes in affected skin (Fitch et al., 2003). Since epidermal thickening is apparent several weeks before dark skin in *Dsk2* and *Dsk12* animals, we speculate that proliferating basal keratinocytes release one or more paracrine factors that stimulate the proliferation of adjacent pigment cells.

Together with our earlier work (Fitch et al., 2003), the current studies of *Dsk12* suggest that dark skin in mice may provide a sensitive and specific signature for disorders of keratinization. Additional mutants similar to *Dsk2* and *Dsk12* will almost certainly lead to new Krt mutants, and, in addition, may provide a means to identify additional molecular components required for normal keratinization.

MATERIALS AND METHODS

Mouse genetics

Like other animals from the original mutagenesis project (<http://www.gsf.de/ieg>), *Dsk12/+* was generated using ethylnitrosourea, and maintained on a C3HeB/FeJ inbred background. For genetic mapping, genomic DNA from four mutant backcross progeny ((C3HeB/FeJ-*Dsk12/+* × C57BL/6J) F1 × C3HeB/FeJ) animals was pooled and compared to a pool of wild-type backcross progeny using a panel of 81 genome-wide simple sequence length polymorphisms spaced ~15 cM apart (marker sequences available on request). Linkage to distal chromosome 15 was verified using 18 backcross and 17 F2 intercross progeny that were scored for the Dsk phenotype at 2 months of age. (As described below, *Dsk12* mutant animals also exhibit blistering; however, the genetic mapping was based solely on the Dsk phenotype.) Relationship between genetic and physical distances was evaluated with the March 2005 release of the mouse genome sequence (<http://genome.ucsc.edu>). The experiments in this study were approved by the Stanford Administrative Panel on Laboratory Animal Care.

Histology

Skin from 2-day-, 2-week-, and 2-month-old mutant and nonmutant littermates was removed from the footpads and tails, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections of 10 μ m were taken, stained with hematoxylin and eosin, and examined by light microscopy. The footpad was sectioned in entirety for at least three mutant and nonmutant animals at each time point.

Molecular genetics

Genomic DNA from *+/+* and *Dsk12/Dsk12* animals was used for PCR amplification and direct sequencing of *Krt1* and *Krt2e* coding exons (NM_008473 and NM_010668). Animals were genotyped using an *Mae*III restriction fragment length polymorphism that is absent in mutant animals.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Hermie Manuel for preparing tail DNA, and the core facility of the GSF ethylnitrosourea mutagenesis screen for the care and organization of animal resources. Part of this work was supported by a grant from the German Human Genome Project (DHGP) and the National Genome Research Network (NGFN) to M.H.d.A. (01GR0430). K.A.M. is supported by a Mentored Clinical Scientist Development Investigator Award from the National Institutes of Health (AR050080-01). G.S.B. is supported by a Research Project Grant from the National Institutes of Health (GM068882).

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