Figure legends

Fig 1. Clinical presentation of the patient. Tense blisters involve the fingers (A), forearms (B) and lips (C).
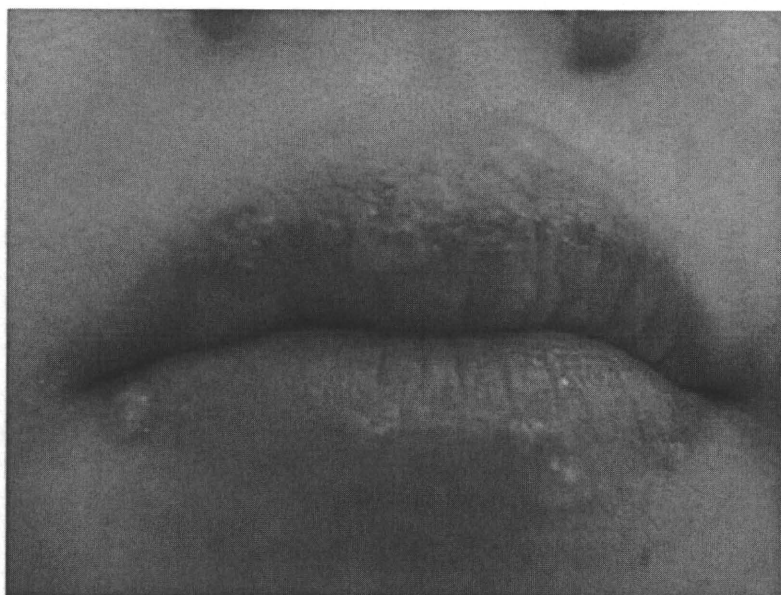
Fig 2. Histologic examination of skin specimens from the patient's left forearm. (A) Hematoxylin-eosin stain, subepidermal blister (x40) with infiltration of eosinophils (arrows) in the blister cavity (x200). Direct immunofluorescence of perilesional skin samples shows linear deposition of C3 (B) and IgG (C) at the dermo-epidermal junction (x40). Immunological characterization of autoantibodies. (D) Indirect immunofluorescence on 1M NaCl split skin. Circulating IgG antibodies bind to both the epidermal and the dermal sides (titer 1:20). (E) Immunoblot analysis using human epidermal extracts. Lane 1: a reference bullous pemphigoid serum reacting with 180-kDa (COL17) and 230-kDa (BP230) antigens; lane 2: a reference pemphigus vulgaris serum with positive bands at 130 kDa (Dsg3) and 160 kDa (Dsg1); lane 3: the patient's serum. IgG in the patient's serum reacts with BP230. (F) Immunoblot analysis using human dermal extracts. Lane 1: a reference EBA serum reacting with a 290-kDa molecule (type VII collagen); lane 2: a reference anti-laminin γ1

pemphigoid serum with a positive band at 200 kDa (p200, laminin γ1); lane 3: the patient's serum; lane 4: a reference normal serum. IgG in the patient's serum reacts with the 200-kDa antigen. (G) Immunoblot analysis using purified laminin-332 (a courtesy gift from Dr S. Amano, Shiseido Life Science Research Center, Yokohama, Japan). Lane 1: a reference of Ponceau S stain of laminin-332 consisting of α3 (165 kDa, 145 kDa), β3 (140 kDa), and γ2 (105 kDa) subunits; lane 2: the patient's serum; lane 3: a reference normal serum; lane 4: a reference of D4B5 (Millipore, Bedford, MA), a mouse monoclonal antibody against the γ2 subunit of laminin-332. IgG from the patient's serum and D4B5 reacts with the γ2 subunit of laminin-332 (105 kDa).

F

290kDa→ 200kDa→
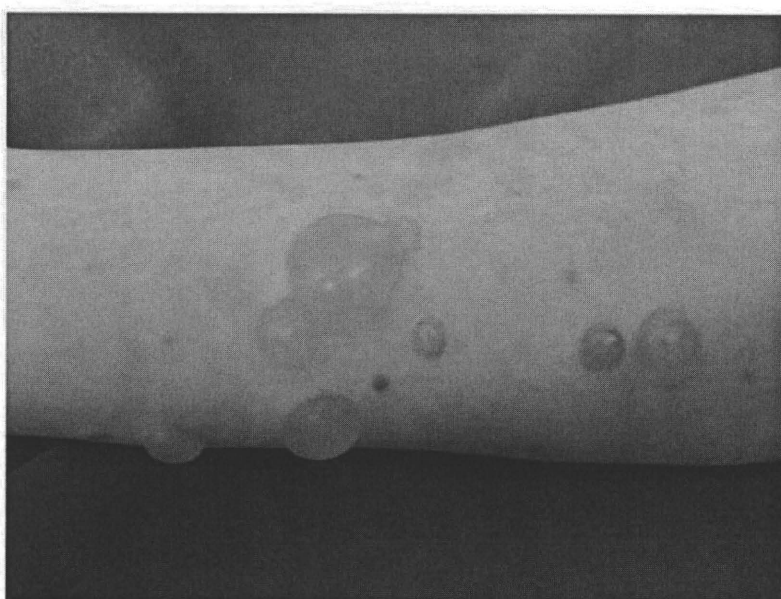
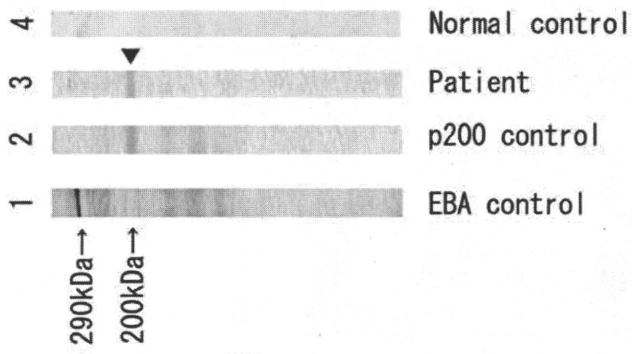1 EBA control
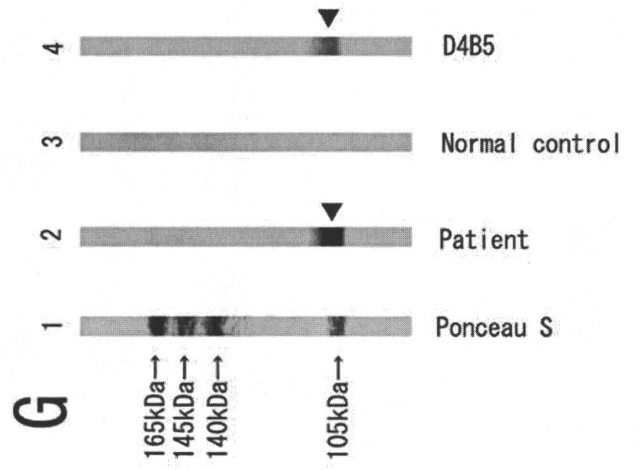2 p200 control
3 Patient
4 Normal control

G

165kDa→ 145kDa→ 140kDa→ 105kDa→

1 Ponceau S
2 Patient
3 Normal control
4 D4B5

E

230kDa→ 180kDa→ 160kDa→ 130kDa→

1 PV control
2 BP control
3 Patient
4 Normal control

A

B

C

D
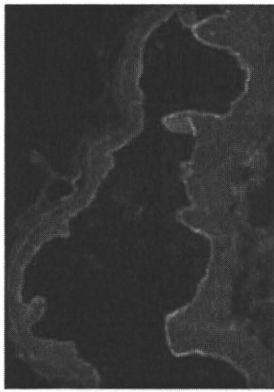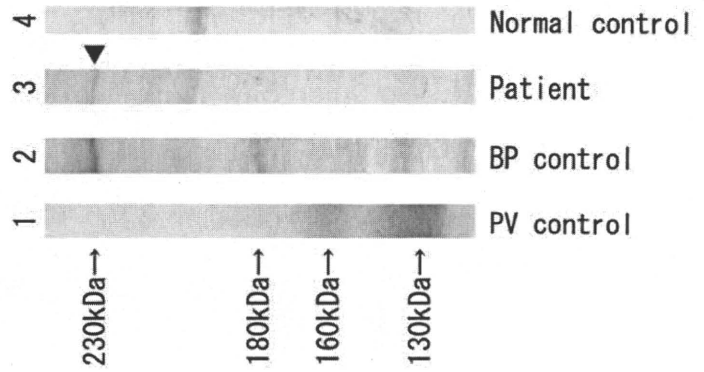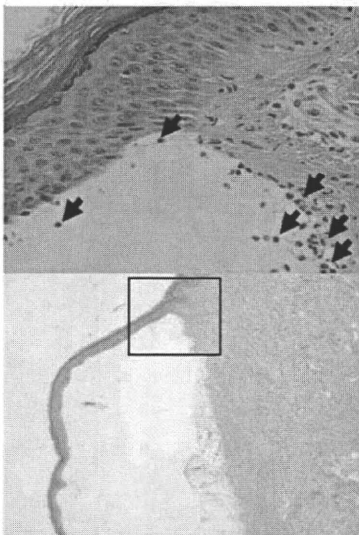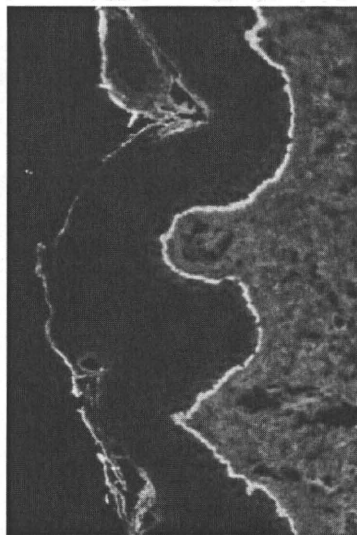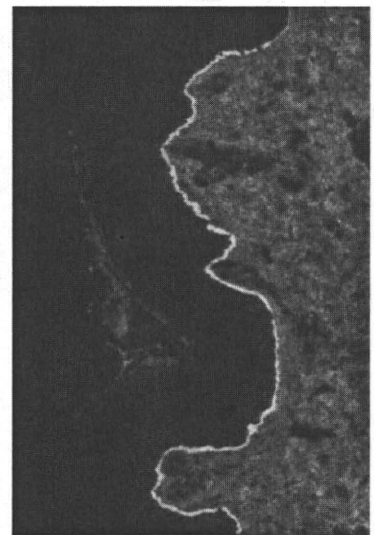
Update on filaggrin mutations and atopic dermatitis

Chao-Kai Hsu[1,2], Masashi Akiyama[†1] and Hiroshi Shimizu[1]

[1]Hokkaido University Graduate School of Medicine, Sapporo, Japan
[2]Institute of Clinical Medicine, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan
[†]Author for correspondence:
Tel.: +81 117 161 161
Fax: +81 117 067 820
akiyama@med.hokudai.ac.jp

Skin serves as a protective barrier against invasion by pathogens and harmful antigenic particles. Filaggrin is a key structural protein that facilitates terminal differentiation of the keratinocytes and formation of the skin barrier. Since the establishment of a sequencing method for the entire filaggrin gene (FLG) in 2006, approximately 40 loss-of-function FLG mutations have been identified in patients with ichthyosis vulgaris and/or atopic dermatitis (AD). Notably, there is a clear difference in filaggrin genetics between the European and Asian races. Overall, approximately 25–50% of AD patients have been found to harbor filaggrin mutations as a predisposing factor. In addition, filaggrin mutations are significantly associated with asthma. The restoration of skin barrier function seems a feasible and promising strategy for prophylactic treatment of AD patients with FLG mutations. This article reviews the discovery of filaggrin mutations; their association with AD, asthma and other atopic diseases; and FLG-related potential treatment strategies.

KEYWORDS: atopic dermatitis • eczema • filaggrin • FLG • ichthyosis vulgaris

Filaggrin, which is processed from profilaggrin, is a key structural protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. Mutations in *FLG*, the gene encoding filaggrin, have been identified as the cause of ichthyosis vulgaris (IV), a relatively common genetic keratinization disorder that is clinically characterized by scaling, especially on the extensor limbs, and palmoplantar hyperlinearity [1–3]. In 2006, the molecular basis and full sequencing of *FLG* were established [4]. Approximately 40 *FLG* mutations have been reported, and the prevalent ones are distinct in different populations [5]. Recent studies have shown that *FLG* mutations are also a key predisposing factor for atopic dermatitis (AD) [6], and for other atopic disorders, including asthma and allergic rhinitis [7]. This article reviews the discovery of filaggrin mutations and its association with AD.
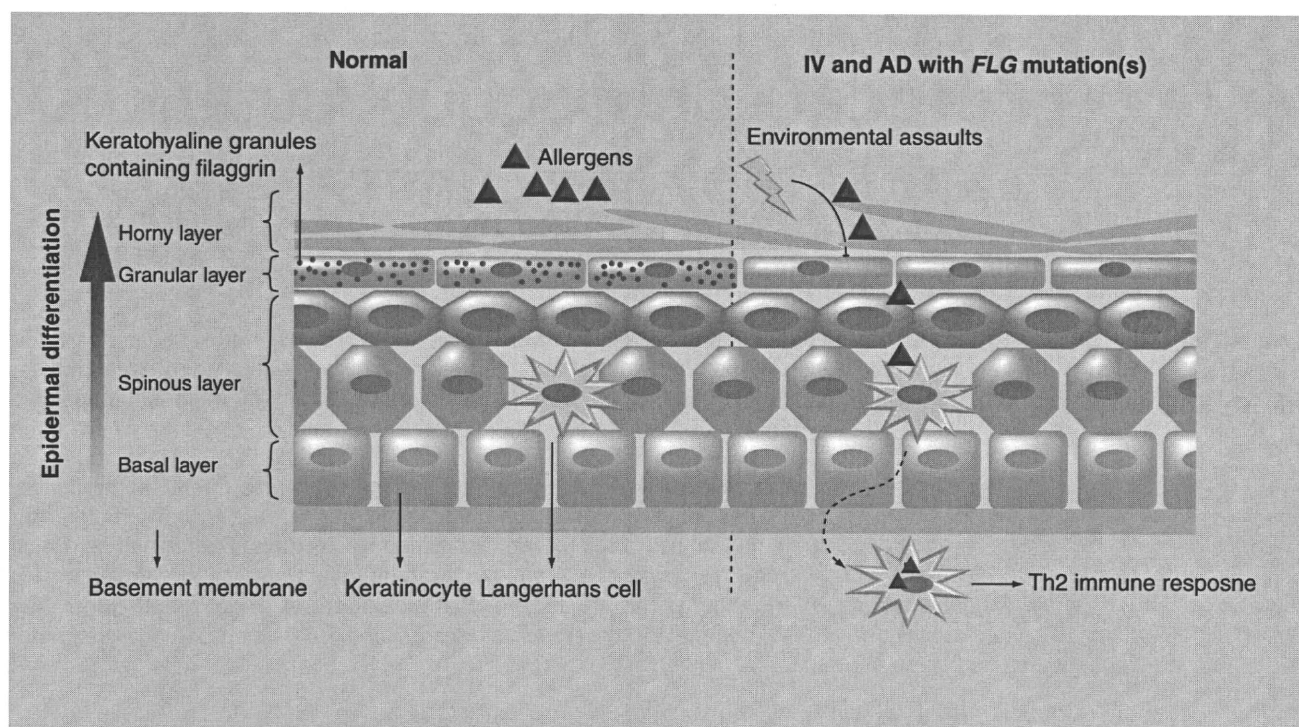
Skin barrier function

The primary function of the skin is to act as a protective barrier against invasion by harmful organisms, such as bacteria, viruses, fungi and other antigenic particles. Keratinocytes are the principal cells within the epidermis. The terminal differentiation of keratinocytes (FIGURE 1) results in the formation of an impenetrable barrier (the horny layer) that is the uppermost layer of the epidermis. The successive stages of keratinocytic differentiation in the epidermal layers are in the basal cell, spinous cell and granular cell layers (FIGURE 2). While spinous cells differentiate into granular cells, they begin to accumulate keratinocyte-specific proteins involved in terminal differentiation of the horny layer. There are three major components in the skin barrier of the horny layer: intercellular lipid layers; the cornified cell envelope; and the keratin network and keratohyaline granules [8]. Genetic defects in any of these components may result in various dermatoses, such as ichthyoses, which are usually characterized by dry, thickened, scaly or flaky skin ('ichthyosis' comes from the Ancient Greek word 'ichthys', meaning 'fish').

The keratin filament network is an important basic structure for maintaining the integrity and dimensions of the cornified cell, and the degraded product of keratohyalin granules – that is, filaggrin – aggregates keratin filaments in apoptosed keratinocytes into bundles and promotes the flattening of dead-cell remnants [9–12]. This layer of collapsed cells, which is reinforced by other structural proteins, forms an effective barrier against external allergens in normal skin.

Filaggrin

The term 'filaggrin' (a shortening of the phrase 'filament aggregation protein') first appeared in 1981 to describe a class of structural proteins that

10.1586/EDM.10.29 **1**

Figure 1. Anatomy and function of human skin barrier. The major cell population in the epidermis is keratinocytes, which undergo progressive differentiation from the basal layer to the granular layer, spinous layer and horny layer. In the granular layer, keratohyaline granules composed of profilaggrin predominate. Upon terminal differentiation of keratinocytes, the degraded product, filaggrin, aggregates keratin filaments and flattens the keratinocytes to form an effective barrier against external allergens in normal skin. In ichthyosis vulgaris and atopic dermatitis with *FLG* mutation, there is a reduction or complete absence of filaggrin. The defective skin barrier allows the external antigens to penetrate into the epidermis and interact with antigen-presenting cells, Langerhans cells and dermal dendritic cells, which might further initiate Th2 immune response and lead to atopic disorders. Modified from [2].

are isolated from the horny layer [9]. Filaggrin is initially synthesized as profilaggrin, an approximately 500-kDa, highly phosphorylated, histidine-rich polypeptide that consists of an amino-terminal S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats, as well as of a carboxy-terminal domain (Figure 2) [13,14]. During the post-translational processing of profilaggrin, the 10–12 individual 37-kDa filaggrin polypeptides cleave proteolytically and then dephosphorylate. As mentioned above, the liberated filaggrin subsequently and highly efficiently aggregates the keratin filaments, which causes the keratinocytes in the stratum corneum to collapse [9,12]. Filaggrin subsequently degrades into amino acids, which act in retaining epidermal moisture [12,15]. The aspartate-specific protease caspase 14 plays an important role in the cleavage of profilaggrin [16]. Caspase-14-knockout mice show an abnormal accumulation of filaggrin fragments with a low molecular mass (12–15 kDa) within the stratum corneum [17]. Filaggrin is a key protein during terminal differentiation, and it is essential for the formation of an intact, protective and properly moisturized skin barrier [8,12].

Filaggrin loss-of-function mutations in ichthyosis vulgaris

Ichthyosis vulgaris (OMIM 146700) is a common inherited skin disorder that is estimated to affect one in 250 individuals. IV is characterized by generalized dry and scaly skin prominent on the extensor surfaces of limbs, and is associated with palmoplantar hyperlinearity (Figure 3) [1,4]. Histologically, IV is characterized by a decrease in the size and number of keratohyaline granules in the granular layer, or in their complete absence there (Figure 3) [1,18]. An association between IV and profilaggrin had long been suspected, but the gene that encodes profilaggrin, *FLG*, proved to be technically challenging to sequence. *FLG* resides on human chromosome 1q21 within the so-called epidermal-differentiation complex (EDC). The EDC contains an area of 1.62 megabases harboring more than 70 genes that are expressed during terminal differentiation of keratinocytes [19,20]. These EDC proteins, such as loricrin, involucrin, small protein-rich proteins and late envelop proteins, share similar important sequences, and phylogenic study suggests that these proteins derive from a common ancestor [21]. Of these EDC proteins, filaggrin is the key member.
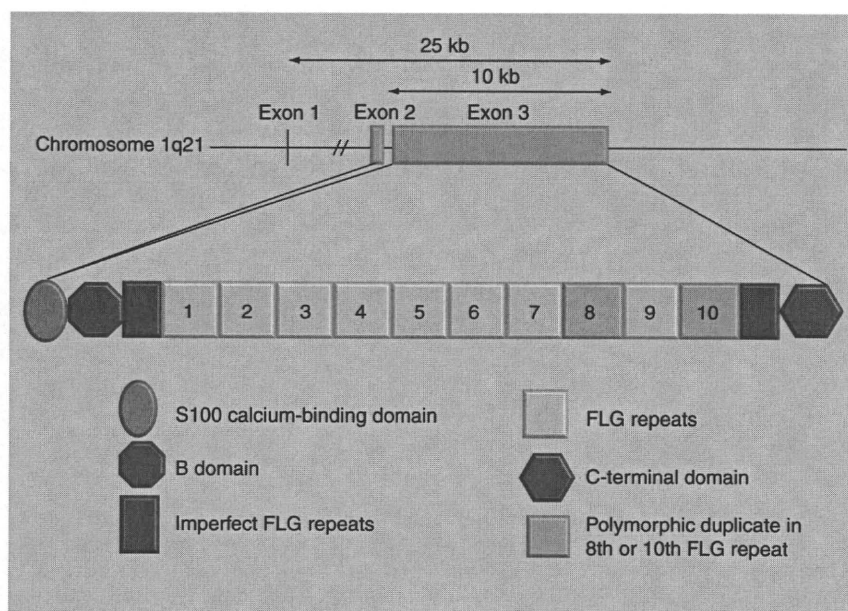
The initiation codon of the *FLG* gene is in exon 2, and most of the profilaggrin protein is encoded by exon 3 (Figure 2). Exon 3 is extremely large (>12 kb) and it encodes most of the profilaggrin polypeptides, with almost completely homologous 10, 11 or 12 repeats. There exist polymorphic variations in the number of filaggrin repeats. Some individuals have a duplication of the

eighth and/or tenth domain. The huge size, polymorphic variations in the number of filaggrin repeats and highly repetitive nature prevent the entire gene from being sequenced. However, the improvements in PCR strategy that involve long-range sequencing and multiple-alignment techniques that permit comprehensive sequencing of the entire *FLG* gene have recently been developed [4,13]. Smith *et al.* first identified the homozygous or compound heterozygous *FLG* mutations R501X and 2282del4 as the cause of moderate or severe IV in 15 kindreds [4]. Those investigators also demonstrated that IV is a semi-dominant condition with incomplete penetrance (~90% in homozygotes). Homozygotes or compound heterozygotes had a severe form of IV, whereas heterozygotes displayed mild or no IV phenotype.

A fewer number of *FLG* repeat domains might be associated with the dry-skin phenotype [22]. Individuals with an absence of the 12-repeat profilaggrin allele (i.e., with allelotypes 10, 10; 10, 11; or 11, 11) were at least four-times more likely to report skin dryness than those who carried one or two 12-repeat alleles (i.e., 10, 12; 11, 12; or 12, 12 allelotypes) [22]. The genotype

Figure 2. Profilaggrin and filaggrin gene structure. The *FLG* gene, which is within the epidermal differentiation complex on chromosome 1q21, spans approximately 25 kb of genomic DNA and comprises three exons and two introns. Profilaggrin contains a S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking ten essentially identical filaggrin repeat domains, as well as a C-terminal domain. There exist polymorphic variations in the number of filaggrin repeats. Some individuals have duplication of the eighth and/or tenth filaggrin repeat. Modified from [28,79].

and phenotype correlation in *FLG* mutations is still lacking. *FLG* mutations at any site were reported to result in similarly severe deficiency of profilaggrin/filaggrin processing [13]. Currently, it is hypothesized that the profilaggrin C-terminal region is essential for proper profilaggrin processing. The hypothesis is supported by the finding of nonsense mutation p.Lys4022X in the C-terminal incomplete filaggrin repeat. In the epidermis of patients carrying this mutation, levels of profilaggrin/filaggrin peptides are remarkably reduced, even though *FLG* mRNA expression is not reduced significantly and expresses mRNA-inclusive messages derived from both the wild-type alleles and the mutant alleles [23]. All the truncation mutations are now generally regarded as leading to serious loss of filaggrin peptides, resulting in absence of genotype/phenotype correlations with respect to *FLG* mutations in IV or AD.

Prevalent filaggrin mutations: distinct in each race

Since the establishment of sequencing methods for the entire *FLG* coding region in 2006 [4,13,24], approximately 40 loss-of-function *FLG* mutations have been identified in IV and/or AD [5,21] (Figure 4). The *FLG* mutations were initially identified in European families [4,24,25]. Using this methodology, we identified two novel *FLG* mutations (3321delA and S2554X) in four Japanese families with IV [26]. Subsequently, six additional *FLG* mutations in Japanese have been identified [26–31]. The study was repeated for other Asian populations, including Chinese [32], Taiwanese [28] and Korean

populations [33]. Only two mutations (R501X and E2422X) were reported in both European and Asian populations [31,32]. Further haplotype analysis of the European-specific mutation R501X in the Japanese family showed that the mutation was not inherited from a European ancestor, but recurred *de novo* in Japan [31]. In Asian populations, 3321delA was found in all four East Asian populations [26,28,31–33], and Q2417X was reported in both Chinese and Taiwanese populations [28,32]. These results revealed the differences in filaggrin population genetics between Europe and Asia (Figure 4).

Filaggrin mutations: a major predisposing factor for AD

Atopic dermatitis is a common chronic pruritic inflammatory skin disease with high prevalence in developed countries, and it is responsible for a notable share of morbidity and health service costs [34,35]. A systematic review estimated the annual costs of treating AD in the USA at US$364 million–3.8 billion [36]. The costs will likely increase in proportion to the increasing prevalence of the disease [36].

The clinical manifestations of AD vary with age [35,37]. In infancy, the lesions are generally more acute and usually present on the face and scalp. Serous exudates or crusted erosions frequently appear secondary to scratching. During childhood, AD lesions involve flexures, nape and the dorsal aspects of the limbs. In adolescence and adulthood, lichenified plaques usually affect flexures, head and neck.

Atopic dermatitis has been regarded as a genetically complex disorder with a strong environmental component [2]. There are two
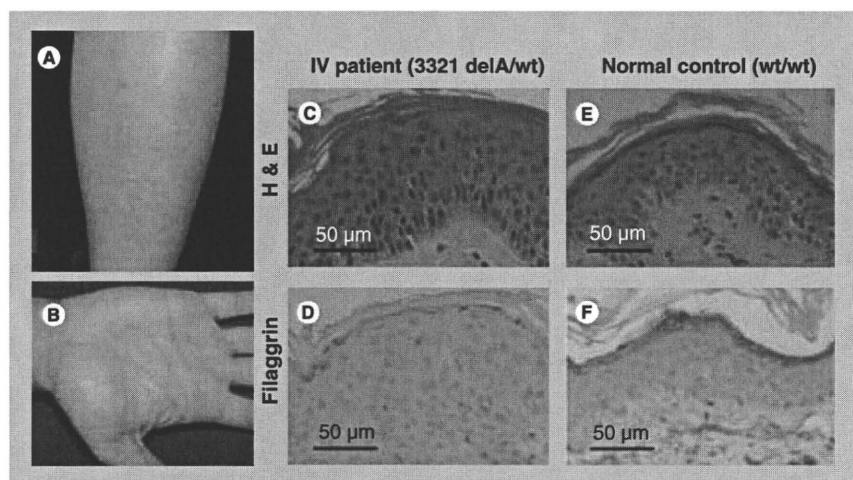
Figure 3. Clinicopathological features of ichthyosis vulgaris. (A) Ichthyosis vulgaris (IV) with dry and scaly skin on the pretibial region, and IV associated with **(B)** apparent palmoplantar hyperlinearity. **(C & E)** Hematoxylin and eosin staining. An IV patient heterozygous for 3321delA **(C)** shows a lack of granular layers in the epidermis, where only a small amount of basophilic substance, resembling keratohyalin, is occasionally present. In contrast, normal control skin **(E)** has abundant keratohyalin granules in the granular layers. **(D & F)** In immunohistochemical staining, a 3321delA heterozygote **(E)** shows a marked reduction in staining for filaggrin, whereas normal control skin **(F)** stains strongly.

proposed hypotheses explaining the mechanism [35]. One suggests that the primary defect is immuno-aberration, evidenced by serum IgE elevation and eosinophilia; thus, skin barrier dysfunction is a consequence of local inflammation. The other proposes that AD originates from an intrinsic defect of epithelial cells that leads to barrier dysfunction; thus, the immunologic aspects are epiphenomena. A main hallmark of AD is xerosis. Transepidermal water loss, the measurement of skin barrier function, was reported to increase in AD patients due to skin barrier defect [38,39]. Significant correlations were observed between penetration rates of a hydrophilic dye and elevated IgE levels in patients with severe AD [40]. Taken together, these findings strongly support the hypothesis that patients with AD have a skin barrier defect.

Before 2006, despite considerable efforts to elucidate genes associated with AD susceptibility, no gene with strong, reproducible effect was identified [41]. There were three clues suggesting that *FLG* mutation plays an important role in the pathogenesis of AD. First, to dermatologists, it has been well understood that AD often occurs in IV patients, although the precise mechanisms of this co-occurrence remain unknown [42–44]. Second, the linkage of AD to a chromosome locus on 1q21 where *FLG* resides was also demonstrated [45]. Third, the skin in patients with AD also demonstrates decreased filaggrin expression at both the mRNA and the protein levels [46,47]. In addition, it has been long proposed that the permeability barrier abnormality in AD is not just an epiphenomenon, but rather is an important driver of disease activity [48], and that the severity of the permeability barrier abnormality precisely parallels the AD severity [39,49]. Therefore, the two loss-of-function mutations in *FLG* found initially in IV were soon applied in the genetic investigation of families with AD [24].

Palmer *et al.* first reported that decreased or absent *FLG* expression due to loss-of-function mutations leads to impaired barrier function that manifests as AD [24]. They found that AD manifested in heterozygous carriers of two null *FLG* mutations (R501X and 2282del4) with a relative risk (odds ratio [OR]) for AD of 3.1, suggesting a causal relationship. Thereafter, numerous studies established *FLG* as a major genetic predisposing factor for AD [13,50–56]. Baurecht *et al.* performed a meta-analysis of nine studies of *FLG* mutations and AD, focusing on the mutations prevalent in Europeans (R501X or 2282del4) [41]. They found an overall OR of 4.09 (95% CI: 2.64–6.33) from the case–control studies and a summary OR of 2.06 (95% CI: 1.76–2.42) from the family studies [41]. The strong association between *FLG* mutations and AD was a milestone in the genetic study of the complex allergic disorders. The *FLG* gene is the most likely candidate as a predisposing gene for AD so far. Based on the information of population-specific *FLG* mutations, many cohort studies on *FLG* mutations in AD were performed, and approximately 25–50% of AD patients were revealed to harbor *FLG* mutations as a predisposing factor [5].

One factor affecting the frequency of *FLG* mutation is the number of identified mutations among specific population. For example, we first identified two null mutations (p.Ser2554X and c.3321delA) among 11 patients from seven Japanese IV families, but only 5.6% of 143 AD patients carried either or both of these *FLG* mutations [26]. We identified two additional novel *FLG* mutations (S2889X and S3296X) in seven Japanese families with IV [27], and more than 20.6% of patients in 102 AD cases carried either or both of these *FLG* mutations [27]. Eight *FLG* variants have been identified in the Japanese population, including six that are prevalent, and we found that approximately 27% of the patients in our Japanese AD case series carry at least one of these eight *FLG* mutations and that these variants are also carried by 3.7% of Japanese general control individuals [23]. Thus, information on population genetics of *FLG* mutation is essential for global *FLG* mutation screening in AD patients.

As mentioned above, every population is likely to have a unique set of *FLG* mutations. *FLG* mutation screening in one population using the *FLG* mutations reported in other populations may result in false-negatives. For example, Ching *et al.* found that the *FLG* mutations that are prevalent in Caucasian and non-Chinese Asian populations are rarely found in childhood AD among the Chinese [57]. It is therefore important to identify novel *FLG* mutations in different populations by sequencing this important AD candidate gene in order to establish global population genetic maps that will facilitate research into that gene's pathogenetic roles for AD.

Flaky tail (ft/ft) is a spontaneous autosomal-recessive mutation
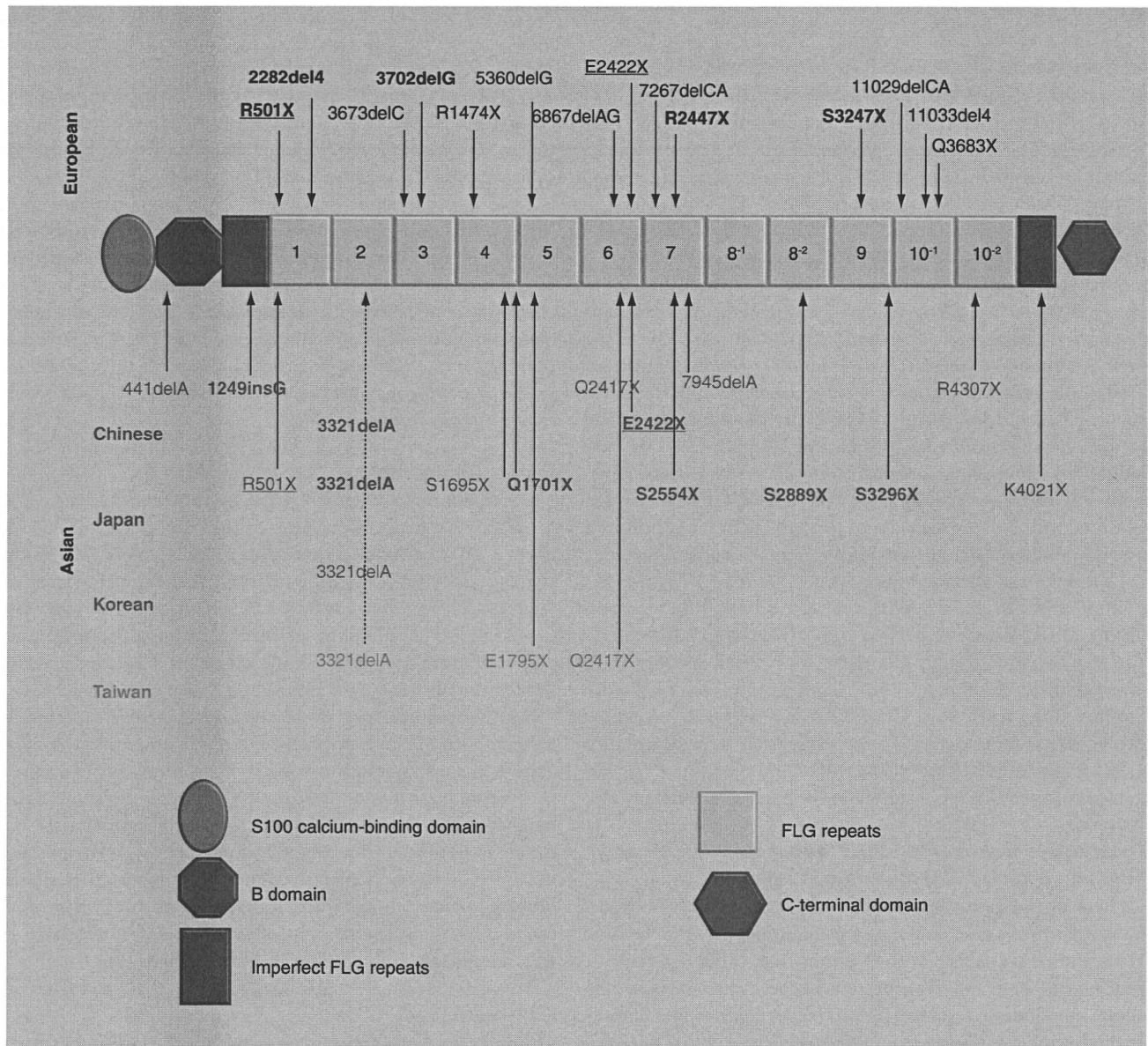
Figure 4. Locations of reported FLG mutations in the profilaggrin peptide. Several of these mutations are rare, but a number of recurrent mutations have been identified (bold text). FLG mutations appear to differ between the European and Asian populations. Only two mutations (R501X and E2422X) were reported in both European and Asian populations (underlined text). We further analyzed FLG mutations in Asian populations (Chinese, marked blue; Japanese, marked red; Korean, marked purple; and Taiwanese, marked green). 3321delA was found in all four East Asian populations, and Q2417X was reported in the Chinese and Taiwanese populations. The duplication of the eighth and tenth filaggrin repeats is represented as 8–1, 8–2, 10–1 and 10–2. Modified with permission from [5,28].

in mice that results in dry, flaky skin and annular tail in the neonatal period. Presland *et al.* demonstrated that ft/ft mice express a lower-molecular-weight form of profilaggrin (220 kDa) instead of the normal high-molecular profilaggrin (~500 kDa). In addition, the abnormal profilaggrin is not proteolytically processed into profilaggrin intermediates or into filaggrin. The absence of filaggrin and, in particular, the hygroscopic filaggrin-derived amino acids that function in epidermal hydration, underlies the

dry, scaly skin characteristic of ft/ft mice. This animal model provides a tool for understanding the role of filaggrin in normal epidermal function [58]. Recently, Fallon *et al.* demonstrated that topical application of allergen to flaky-tail (ft/ft) mice results in cutaneous inflammatory infiltrates and enhanced cutaneous allergen priming, resulting in development of allergen-specific antibody and cytokine responses mimicking human AD. These data provide experimental evidence for the barrier hypothesis of

AD pathogenesis [59].

FLG mutations also associated with asthma

Atopic dermatitis is typically the first clinical manifestation of allergic diseases, followed by the development of asthma and allergic rhinitis. Atopic diseases progress in the so-called 'atopic march', which suggests that these various atopic diseases share a common etiology. Previous studies demonstrated that 70% of patients with severe AD developed asthma, compared with 30% of patients with mild AD and approximately 8% of the general population [60].

Filaggrin is expressed in the vestibulum of the nose, but not in the nasal or tracheal mucosae [61,62]. How does the *FLG* mutation confer the pathogenesis of asthma? An AD animal study found that dysfunction of the skin barrier not only enhances sensitization to allergens, but also leads to systemic allergic responses such as increased IgE levels and airway hyperreactivity [63]. Recent studies hypothesized that skin barrier defects caused by *FLG* mutations allow allergens to penetrate the epidermis and interact with antigen-presenting cells, the Langerhans cells and dermal dendritic cells, which might further initiate Th2 immune response and lead to the development of atopic disorders including AD, asthma and allergic rhinitis [64,65].

Studies in European populations have reported that variants in the *FLG* gene are associated with eczema and concomitant asthma [50–54] or eczema alone [25]. One recent meta-analysis showed that *FLG* mutations are significantly associated with asthma (OR: 1.48; 95% CI: 1.32–1.66). However, although strong effects for the compound phenotype asthma plus eczema (OR: 3.29; 95% CI: 2.84–3.82) were observed, there appears to be no association with asthma in the absence of eczema [66].

Prospective treatments for AD based on skin barrier function & recent *FLG* mutation studies

In light of the discussion above, the restoration of skin barrier function seems a feasible and promising strategy for prophylactic treatment of AD patients with *FLG* mutation. Clinically, there are efficient methods for restoring skin barrier function, including topical application of general moisturizer or specific lipid replacement therapy [67]. When used under nursing supervision, moisturizers have been shown to alleviate the need for topical steroids [68]. In addition, the topical application of ceramide dominant lipid replacement therapy has been proven effective in improving skin barrier defects and reducing AD severity significantly in childhood AD patients [39]. Most *FLG* mutations are caused by premature termination codons, which account for numerous genetic disorders, such as thalassemia and cystic fibrosis. Recently, several pharmaceuticals targeting nonsense mutations in genetic diseases have been developed [69]. For example, PTC124, a small molecule designed to induce ribosomes to selectively read through premature stop codons during mRNA translation, has been proven effective in restoring the function of the *CFTR* gene, whose mutation accounts for some cases of cystic fibrosis [70]. Skin diseases, such as IV and AD, might be even more feasible targets through topical application of similar pharmacological agents.

A large number of patients with severe AD do not have the *FLG* mutation, and there are healthy subjects with *FLG* mutations who do not express AD or IV lesions. This fact suggests additional factors modulating the expression of the *FLG* gene. The skin lesion of AD is characterized by the overexpression of Th2 cytokines, including IL-4 and IL-13 [71,72]. Howell *et al.* showed that *in vitro* keratinocytes exhibited significantly reduced filaggrin gene expression in the presence of IL-4 and IL-13 [73]. Therefore, it is possible that correction of the Th2 immune response could increase filaggrin gene expression and thereby restore the skin barrier function. For example, Kootiratrakarn *et al.* found that oligodeoxynucleotides containing CpG motifs prevented the development of Th2-mediated responses in a new, unique mouse cutaneous eosinophilic inflammation model [74]. The screening of other compounds or approaches to restore filaggrin expression in the epidermis may lead to the new development of efficient treatments for IV and AD.

Expert commentary

The concept that epidermal barrier dysfunction caused by *FLG* mutations is a major contributor to the pathogenesis of AD has opened up a new era. As mentioned above, most *FLG* mutations are specific to each population, such as Europeans [13], Japanese [27,29–30], Singaporean–Chinese [32] and Taiwanese [28]. It is therefore important to establish global population genetics maps of *FLG* mutations for the development of better diagnostic tests or the further design of novel treatments for IV and AD.

No genotype/phenotype correlation has been observed in patients with *FLG* mutations. Mutations at any site within *FLG* appear to cause significant reductions in profilaggrin/filaggrin peptide amounts in the epidermis. Our recent study showed that mutations in C-terminal imperfect filaggrin repeats also contribute to significant phenotypes, which supports the hypothesis that the C-terminal region is essential for proper processing of profilaggrin into filaggrin peptides [29]. Further study on the exact functions of each genetic component within *FLG* is necessary for a better understanding of skin barrier function.

Five-year view

Although methods are underway to restore skin barrier function, the concept of *FLG* mutation has not yet translated into therapeutic advances. Two therapeutic strategies focusing on *FLG* mutation were proposed, and related research is well underway in McLean's laboratory [75]. One strategy is to upregulate *FLG* gene expression by small molecules acting on pathways controlling *FLG* gene expression, and the other strategy is to read through premature termination codon mutations by interfering 'nonsense-mediated decay', which is a cellular mechanism of mRNA surveillance that functions to detect nonsense mutations [76]. We expect therapeutic modalities focusing on *FLG* mutation, especially topical agents, to evolve in coming years.

Atopic dermatitis is a genetically complex disorder complicated by a strong environmental component [2], so developing diagnostic criteria and classification is challenging. Although various validated sets of diagnostic criteria have been developed over the past few decades, there is disagreement about these [77].

Brenninkmeijer *et al.* performed a methodological review of 27 validation studies of various diagnostic criteria for AD [78]. Two frequently quoted criteria focusing on clinical presentation showed variable sensitivity and specificity. Hanifin and Rajka diagnostic criteria sensitivity and specificity ranged from 87.9 to 96.0% and from 77.6 to 93.8%, respectively. The UK diagnostic criteria showed sensitivity and specificity ranging from 10 to 100% and 89.3 to 99.1%, respectively [78]. The *FLG* mutation study is expected to have a major impact on the diagnostic criteria. In addition, we expect that in the future, classification of AD may be based on the presence or absence of *FLG* mutations. Such disease classification and treatment focusing on *FLG* mutation will complement each other.

Key issues

- Filaggrin, processed from profilaggrin, is a key structural protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. Filaggrin aggregates keratin filaments in apoptosed keratinocytes into bundles and promotes the flattening of dead-cell remnants.
- *FLG* spans approximately 25 kb of genomic DNA and resides on human chromosome 1q21, within the so-called epidermal-differentiation complex.
- *FLG* comprises three exons and two introns. Exon 3 is extremely large (>12 kb) and encodes most of the profilaggrin polypeptides with almost completely homologous 10, 11 or 12 repeats.
- Ichthyosis vulgaris is a semi-dominant condition with incomplete penetrance (~90% in homozygotes). Homozygotes and compound heterozygotes have a severe form of ichthyosis vulgaris, whereas heterozygotes display mild or no phenotypic abnormality.
- There exist differences in filaggrin population genetics between Europe and Asia. Only two identical mutations (R501X and E2422X) were reported in both European and Asian populations.
- Approximately 25–50% of atopic dermatitis (AD) patients were revealed to harbor filaggrin mutations as a predisposing factor.
- A meta-analysis study showed that *FLG* mutations are significantly associated with asthma accompanied by AD (odds ratio: 1.48; 95% CI: 1.32–1.66).
- The restoration of skin barrier function seems a feasible and promising strategy for prophylactic treatment of AD patients with *FLG* mutations.

References

1. Sybert VP, Dale BA, Holbrook KA. Ichthyosis vulgaris: identification of a defect in synthesis of filaggrin correlated with an absence of keratohyaline granules. *J. Invest. Dermatol.* 84(3), 191–194 (1985).

2. McGrath JA, Uitto J. The filaggrin story: novel insights into skin-barrier function and disease. *Trends Mol. Med.* 14(1), 20–27 (2008).

3. McGrath JA. Filaggrin and the great epidermal barrier grief. *Australas. J. Dermatol.* 49(2), 67–73; quiz 73–64 (2008).

4. Smith FJ, Irvine AD, Terron-Kwiatkowski A *et al.* Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat. Genet.* 38(3), 337–342 (2006).

5. Akiyama M. *FLG* mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. *Br. J. Dermatol.* 162(3), 472–477 (2010).

6. Irvine AD, McLean WH. Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. *J. Invest. Dermatol.* 126(6), 1200–1202 (2006).

7. Van Den Oord RA, Sheikh A. Filaggrin gene defects and risk of developing allergic sensitisation and allergic disorders: systematic review and meta-analysis. *BMJ* 339, b2433 (2009).

8. Akiyama M, Shimizu H. An update on molecular aspects of the non-syndromic ichthyoses. *Exp. Dermatol.* 17(5), 373–382 (2008).

9. Steinert PM, Cantieri JS, Teller DC, Lonsdale-Eccles JD, Dale BA. Characterization of a class of cationic proteins that specifically interact with intermediate filaments. *Proc. Natl Acad. Sci. USA* 78(7), 4097–4101 (1981).

10. Dale BA, Resing KA, Lonsdale-Eccles JD. Filaggrin: a keratin filament associated protein. *Ann. NY Acad. Sci.* 455, 330–342 (1985).

11. Listwan P, Rothnagel JA. Keratin bundling proteins. *Methods Cell. Biol.* 78, 817–827 (2004).

12. Sandilands A, Sutherland C, Irvine AD, McLean WH. Filaggrin in the frontline: Role in skin barrier function and disease. *J. Cell Sci.* 122(Pt 9), 1285–1294 (2009).

13. Sandilands A, Terron-Kwiatkowski A, Hull PR *et al.* Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat. Genet.* 39(5), 650–654 (2007).

14. Gan SQ, Mcbride OW, Idler WW, Markova N, Steinert PM. Organization, structure, and polymorphisms of the human profilaggrin gene. *Biochemistry* 29(40), 9432–9440 (1990).

15. Rawlings AV, Harding CR. Moisturization and skin barrier function. *Dermatol. Ther.* 17(Suppl. 1), 43–48 (2004).

16. Denecker G, Ovaere P, Vandenabeele P, Declercq W. Caspase-14 reveals its secrets. *J. Cell Biol.* 180(3), 451–458 (2008).

17. Denecker G, Hoste E, Gilbert B *et al.* Caspase-14 protects against epidermal UVB photodamage and water loss. *Nat.*

Cell Biol. 9(6), 666–674 (2007).

18 Fleckman P, Brumbaugh S. Absence of the granular layer and keratohyalin define a morphologically distinct subset of individuals with ichthyosis vulgaris. *Exp. Dermatol.* 11(4), 327–336 (2002).

19 South AP, Cabral A, Ives JH *et al.* Human epidermal differentiation complex in a single 2.5 mbp long continuum of overlapping DNA cloned in bacteria integrating physical and transcript maps. *J. Invest. Dermatol.* 112(6), 910–918 (1999).

20 Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat. Rev. Immunol.* 4(12), 978–988 (2004).

21 O'Regan GM, Sandilands A, McLean WH, Irvine AD. Filaggrin in atopic dermatitis. *J. Allergy Clin. Immunol.* 124(3 Suppl. 2), R2–R6 (2009).

22 Ginger RS, Blachford S, Rowland J, Rowson M, Harding CR. Filaggrin repeat number polymorphism is associated with a dry skin phenotype. *Arch. Dermatol. Res.* 297(6), 235–241 (2005).

23 Nemoto-Hasebe I, Akiyama M, Nomura T, Sandilands A, McLean WH, Shimizu H. Flg mutation p.Lys4021x in the c-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema. *Br. J. Dermatol.* 161(6), 1387–1390 (2009).

24 Palmer CN, Irvine AD, Terron-Kwiatkowski A *et al.* Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* 38(4), 441–446 (2006).

25 Sandilands A, O'Regan GM, Liao H *et al.* Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. *J. Invest. Dermatol.* 126(8), 1770–1775 (2006).

26 Nomura T, Sandilands A, Akiyama M *et al.* Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J. Allergy Clin. Immunol.* 119(2), 434–440 (2007).

27 Nomura T, Akiyama M, Sandilands A *et al.* Specific filaggrin mutations cause ichthyosis vulgaris and are significantly associated with atopic dermatitis in Japan. *J. Invest. Dermatol.* 128(6), 1436–1441 (2008).

28 Hsu CK, Akiyama M, Nemoto-Hasebe I *et al.* Analysis of Taiwanese ichthyosis vulgaris families further demonstrates differences in *FLG* mutations between European and Asian populations. *Br. J. Dermatol.* 161(2), 448–451 (2009).

29 Nemoto-Hasebe I, Akiyama M, Nomura T, Sandilands A, McLean WH, Shimizu H. *FLG* mutation p.Lys4021x in the c-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema. *Br. J. Dermatol.* 161(6), 1387–1390 (2009).

30 Nomura T, Akiyama M, Sandilands A *et al.* Prevalent and rare mutations in the gene encoding filaggrin in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J. Invest. Dermatol.* 129(5), 1302–1305 (2009).

31 Hamada T, Sandilands A, Fukuda S *et al.* De novo occurrence of the filaggrin mutation p.R501x with prevalent mutation c.3321dela in a Japanese family with ichthyosis vulgaris complicated by atopic dermatitis. *J. Invest. Dermatol.* 128(5), 1323–1325 (2008).

32 Chen H, Ho JC, Sandilands A *et al.* Unique and recurrent mutations in the filaggrin gene in Singaporean Chinese patients with ichthyosis vulgaris. *J. Invest. Dermatol.* 128(7), 1669–1675 (2008).

33 Kang TW, Lee JS, Oh SW, Kim SC. Filaggrin mutation c.3321dela in a Korean patient with ichthyosis vulgaris and atopic dermatitis. *Dermatology* 218(2), 186–187 (2009).

34 Gupta R, Sheikh A, Strachan DP, Anderson HR. Burden of allergic disease in the UK: secondary analyses of national databases. *Clin. Exp. Allergy* 34(4), 520–526 (2004).

35 Bieber T. Atopic dermatitis. *N. Engl. J. Med.* 358(14), 1483–1494 (2008).

36 Mancini AJ, Kaulback K, Chamlin Sl. The socioeconomic impact of atopic dermatitis in the United States: a systematic review. *Pediatr. Dermatol.* 25(1), 1–6 (2008).

37 Leung Dy, Bieber T. Atopic dermatitis. *Lancet* 361(9352), 151–160 (2003).

38 Aalto-Korte K. Improvement of skin barrier function during treatment of atopic dermatitis. *J. Am. Acad. Dermatol.* 33(6), 969–972 (1995).

39 Chamlin SL, Kao J, Frieden IJ *et al.* Ceramide-dominant barrier repair lipids alleviate childhood atopic dermatitis: changes in barrier function provide a sensitive indicator of disease activity. *J. Am. Acad. Dermatol.* 47(2), 198–208 (2002).

40 Hata M, Tokura Y, Takigawa M *et al.* Assessment of epidermal barrier function by photoacoustic spectrometry in relation to its importance in the pathogenesis of atopic dermatitis. *Lab. Invest.* 82(11), 1451–1461 (2002).

41 Baurecht H, Irvine AD, Novak N *et al.* Toward a major risk factor for atopic eczema: meta-analysis of filaggrin polymorphism data. *J. Allergy Clin. Immunol.* 120(6), 1406–1412 (2007).

42 Wells RS, Kerr CB. Genetic classification of ichthyosis. *Arch. Dermatol.* 92(1), 1–6 (1965).

43 Kuokkanen K. Ichthyosis vulgaris. A clinical and histopathological study of patients and their close relatives in the autosomal dominant and sex-linked forms of the disease. *Acta Derm. Venereol. Suppl. (Stockh.)* 62, 1–72 (1969).

44 Tay YK, Khoo BP, Goh CL. The epidemiology of atopic dermatitis at a tertiary referral skin center in Singapore. *Asian Pac. J. Allergy Immuno.l* 17(3), 137–141 (1999).

45 Compton JG, Digiovanna JJ, Johnston KA, Fleckman P, Bale SJ. Mapping of the associated phenotype of an absent granular layer in ichthyosis vulgaris to the epidermal differentiation complex on chromosome 1. *Exp. Dermatol.* 11(6), 518–526 (2002).

46 Sugiura H, Ebise H, Tazawa T *et al.* Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. *Br. J. Dermatol.* 152(1), 146–149 (2005).

47 Seguchi T, Cui CY, Kusuda S, Takahashi M, Aisu K, Tezuka T. Decreased expression of filaggrin in atopic skin. *Arch. Dermatol. Res.* 288(8), 442–446 (1996).

48 Elias PM, Feingold KR. Does the tail wag the dog? Role of the barrier in the pathogenesis of inflammatory dermatoses and therapeutic implications. *Arch. Dermatol.* 137(8), 1079–1081 (2001).

49 Sugarman JL, Fluhr JW, Fowler AJ, Bruckner T, Diepgen TL, Williams ML. The objective severity assessment of atopic dermatitis score: an objective measure using permeability barrier function and stratum corneum hydration with computer-assisted estimates for extent of disease. *Arch. Dermatol.* 139(11), 1417–1422 (2003).

50 Morar N, Cookson WO, Harper JI, Moffatt MF. Filaggrin mutations in children with severe atopic dermatitis. *J. Invest. Dermatol.* 127(7), 1667–1672 (2007).

51 Marenholz I, Nickel R, Ruschendorf F

et al. Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J. Allergy Clin. Immunol.* 118(4), 866–871 (2006).

52 Ruether A, Stoll M, Schwarz T, Schreiber S, Folster-Holst R. Filaggrin loss-of-function variant contributes to atopic dermatitis risk in the population of Northern Germany. *Br. J. Dermatol.* 155(5), 1093–1094 (2006).

53 Weidinger S, Illig T, Baurecht H *et al.* Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J. Allergy Clin. Immunol.* 118(1), 214–219 (2006).

54 Barker JN, Palmer CN, Zhao Y *et al.* Null mutations in the filaggrin gene (*FLG*) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. *J. Invest. Dermatol.* 127(3), 564–567 (2007).

55 Stemmler S, Parwez Q, Petrasch-Parwez E, Epplen JT, Hoffjan S. Two common loss-of-function mutations within the filaggrin gene predispose for early onset of atopic dermatitis. *J. Invest. Dermatol.* 127(3), 722–724 (2007).

56 Weidinger S, Rodriguez E, Stahl C *et al.* Filaggrin mutations strongly predispose to early-onset and extrinsic atopic dermatitis. *J. Invest. Dermato.l* 127(3), 724–726 (2007).

57 Ching GK, Hon KL, Ng PC, Leung TF. Filaggrin null mutations in childhood atopic dermatitis among the Chinese. *Int. J. Immunogenet.* 36(4), 251–254 (2009).

58 Presland RB, Boggess D, Lewis SP, Hull C, Fleckman P, Sundberg JP. Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. *J. Invest. Dermatol.* 115(6), 1072–1081 (2000).

59 Fallon PG, Sasaki T, Sandilands A *et al.* A homozygous frameshift mutation in the mouse flg gene facilitates enhanced percutaneous allergen priming. *Nat. Genet.* 41(5), 602–608 (2009).

60 Spergel JM, Paller AS. Atopic dermatitis and the atopic march. *J. Allergy Clin. Immunol.* 112(6 Suppl.), S118–S127 (2003).

61 De Benedetto A, Qualia CM, Baroody FM, Beck LA. Filaggrin expression in oral, nasal, and esophageal mucosa. *J. Invest. Dermatol.* 128(6), 1594–1597 (2008).

62 Ying S, Meng Q, Corrigan CJ, Lee TH. Lack of filaggrin expression in the human bronchial mucosa. *J. Allergy Clin. Immunol.* 118(6), 1386–1388 (2006).

63 Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J. Clin. Invest.* 101(8), 1614–1622 (1998).

64 Hudson TJ. Skin barrier function and allergic risk. *Nat. Genet.* 38(4), 399–400 (2006).

65 Callard RE, Harper JI. The skin barrier, atopic dermatitis and allergy: a role for langerhans cells? *Trends Immunol.* 28(7), 294–298 (2007).

66 Rodriguez E, Baurecht H, Herberich E *et al.* Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J. Allergy Clin. Immunol.* 123(6), 1361–1370 (2009).

67 Elias PM, Hatano Y, Williams ML. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J. Allergy Clin. Immunol.* 121(6), 1337–1343 (2008).

68 Cork MJ, Britton J, Butler L, Young S, Murphy R, Keohane SG. Comparison of parent knowledge, therapy utilization and severity of atopic eczema before and after explanation and demonstration of topical therapies by a specialist dermatology nurse. *Br. J. Dermatol.* 149(3), 582–589 (2003).

69 Rowe SM, Clancy JP. Pharmaceuticals targeting nonsense mutations in genetic diseases: progress in development. *BioDrugs* 23(3), 165–174 (2009).

70 Kerem E, Hirawat S, Armoni S *et al.* Effectiveness of ptc124 treatment of cystic fibrosis caused by nonsense mutations: a prospective Phase II trial. *Lancet* 372(9640), 719–727 (2008).

71 Fiset PO, Leung DY, Hamid Q. Immunopathology of atopic dermatitis. *J. Allergy Clin. Immunol.* 118(1), 287–290 (2006).

72 Ong PY, Ohtake T, Brandt C *et al.* Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* 347(15), 1151–1160 (2002).

73 Howell MD, Kim BE, Gao P *et al.* Cytokine modulation of atopic dermatitis filaggrin skin expression. *J. Allergy Clin. Immunol.* 120(1), 150–155 (2007).

74 Kootiratrakarn T, Fujimura T, Sano K *et al.* Development of a novel Ag-specific immunotherapy using CpG oligodeoxynucleotides in a new, unique mouse cutaneous eosinophilic inflammation model. *Eur. J. Immunol.* 35(11), 3277–3286 (2005).

75 Brown SJ, Mclean WH. Eczema genetics: current state of knowledge and future goals. *J. Invest. Dermatol.* 129(3), 543–552 (2009).

76 Ainsworth C. Nonsense mutations: running the red light. *Nature* 438(7069), 726–728 (2005).

77 Williams HC, Grindlay DJ. What's new in atopic eczema? An analysis of systematic reviews published in 2007 and 2008. Part 1. Definitions, causes and consequences of eczema. *Clin. Exp. Dermatol.* 35(1), 12–15 (2009).

78 Brenninkmeijer EE, Schram ME, Leeflang MM, Bos JD, Spuls PI. Diagnostic criteria for atopic dermatitis: a systematic review. *Br. J. Dermatol.* 158(4), 754–765 (2008).

79 Brown SJ, Irvine AD. Atopic eczema and the filaggrin story. *Semin. Cutan. Med. Surg.* 27(2), 128–137 (2008).

Article

Cell Press

Hair Follicle Stem Cells Provide a Functional Niche for Melanocyte Stem Cells

Shintaro Tanimura,[1,2,9] Yuko Tadokoro,[1,9] Ken Inomata,[1,3] Nguyen Thanh Binh,[1] Wataru Nishie,[2] Satoshi Yamazaki,[4] Hiromitsu Nakauchi,[5] Yoshio Tanaka,[1] James R. McMillan,[2] Daisuke Sawamura,[6] Kim Yancey,[7] Hiroshi Shimizu,[2] and Emi K. Nishimura[1,2,8,*]

[1]Department of Stem Cell Medicine, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan
[2]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan
[3]Fundamental Research Laboratories, KOSÉ Corporation, 1-18-4 Azusawa, Itabashi-ku, Tokyo 174-0051, Japan
[4]Japan Science and Technology Agency (JST), ERATO, Sanbacho, Chiyoda-ku, Tokyo 102-0075, Japan
[5]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[6]Department of Dermatology, Hirosaki University, School of Medicine, Hirosaki, Aomori 036-8562, Japan
[7]Department of Dermatology, University of Texas Southwestern Medical Center in Dallas, Dallas, TX 75390-9069, USA
[8]Department of Stem Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kanda Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan
[9]These authors contributed equally to this work
*Correspondence: nishscm@tmd.ac.jp
DOI 10.1016/j.stem.2010.11.029

SUMMARY

In most stem cell systems, the organization of the stem cell niche and the anchoring matrix required for stem cell maintenance are largely unknown. We report here that collagen XVII (COL17A1/BP180/BPAG2), a hemidesmosomal transmembrane collagen, is highly expressed in hair follicle stem cells (HFSCs) and is required for the maintenance not only of HFSCs but also of melanocyte stem cells (MSCs), which do not express Col17a1 but directly adhere to HFSCs. Mice lacking Col17a1 show premature hair graying and hair loss. Analysis of Col17a1-null mice revealed that COL17A1 is critical for the self-renewal of HFSCs through maintaining their quiescence and immaturity, potentially explaining the mechanism underlying hair loss in human COL17A1 deficiency. Moreover, forced expression of COL17A1 in basal keratinocytes, including HFSCs, in Col17a1-null mice rescues MSCs from premature differentiation and restores TGF-β signaling, demonstrating that HFSCs function as a critical regulatory component of the MSC niche.

INTRODUCTION

The stem cell microenvironment, or niche, is critical for stem cell maintenance (Li and Xie, 2005; Moore and Lemischka, 2006). Accumulating evidence has confirmed that cell-cell and cell-extracellular matrix adhesion within the niche is essential for the establishment and maintenance of niche architecture in different stem cell systems (Raymond et al., 2009). Adhesion to the underlying extracellular matrix has been suggested as an important factor in epidermal stem cell maintenance (Green,

1977; Watt, 2002), but a specific stem-cell anchoring matrix for stem cell maintenance has not yet been identified. Hair follicle stem cells (HFSCs) are found in the hair follicle bulge, a distinct area of the outer root sheath that overlies the basement membrane at the lower permanent portion in mammalian hair follicles (Blanpain and Fuchs, 2006; Cotsarelis, 2006). The HFSC population is composed of multipotent keratinocyte stem cells and is responsible for the cyclic regeneration of hair follicles as well as a transient supply of progeny to the interfollicular epidermis (IFE) and to sebaceous glands after wounding (Blanpain and Fuchs, 2006; Cotsarelis, 2006; Oshima et al., 2001). The HFSC population in the bulge area normally supplies a short-term reservoir to the secondary hair germ (subbulge area), which is located just below the bulge area but above the dermal papilla and corresponds to the lowermost portion of resting hair follicles (Figure S1A available online; Greco et al., 2009). Melanocyte stem cells (MSCs), which are originally derived from the neural crest, also reside in the follicular bulge-subbulge area (Figure S1A). MSCs supply pigment-producing melanocytes to the hair matrix during each hair cycle to maintain hair pigmentation (Nishimura et al., 2002). Therefore, the bulge-subbulge area houses at least two distinct stem cell populations with different origins. However, it is still unclear to what extent these two different stem cells interact to promote each other's maintenance.

Hemidesmosomes are multiprotein adhesion complexes that promote stable epidermal-dermal attachments. The transmembrane protein collagen XVII (COL17A1/BP180/BPAG2) is a structural component of the outer hemidesmosomal plaque, which projects beneath hemidesmosomes in epidermal basal keratinocytes into the underlying basement membrane to mediate anchorage (Masunaga et al., 1997; Nishizawa et al., 1993; Powell et al., 2005). In patients with COL17A1 deficiency, a subtype of congenital junctional epidermolysis bullosa blistering disease, hemidesmosomes are poorly formed (McGrath et al., 1995; Nishie et al., 2007) and there is a characteristic premature hair loss (alopecia) with hair follicle atrophy (Darling et al.,
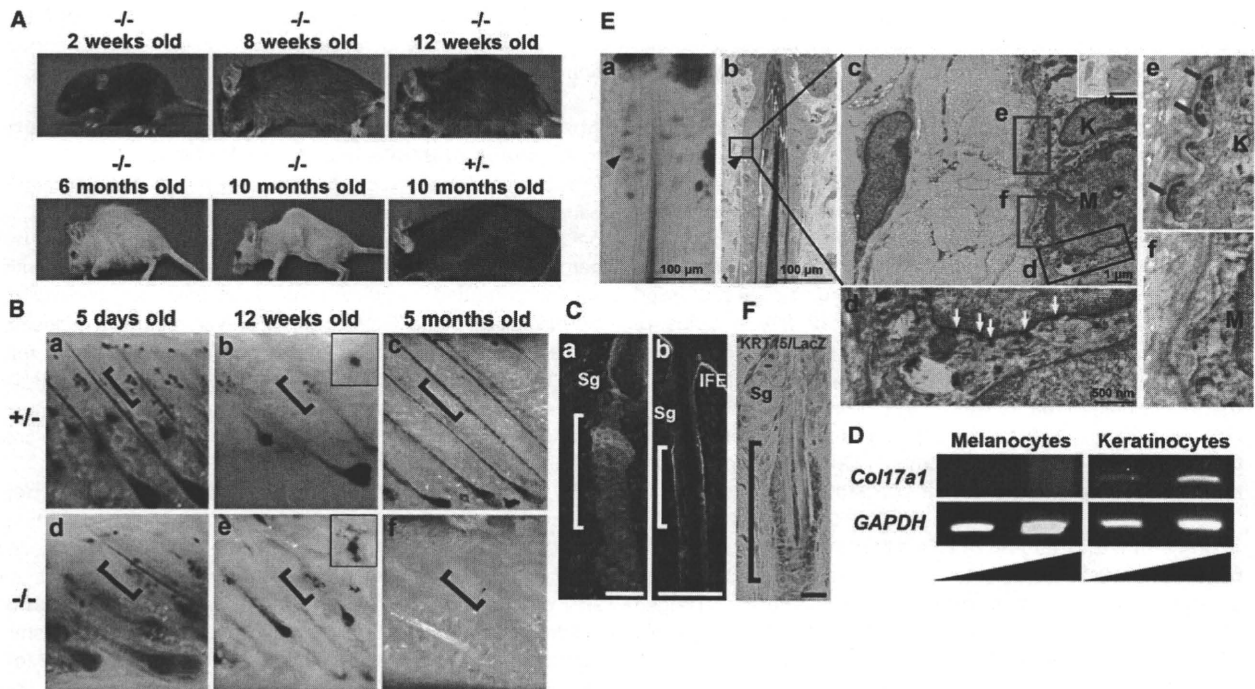
Figure 1. Hair Graying and Hair Loss Are Preceded by Depletion of MSCs in *Col17a1* Deficiency

(A) Macroscopic phenotype of *Col17a1*$^{-/-}$ mice at different times as noted and of *Col17a1*$^{+/-}$ littermates at 10 months of age.

(B) Deletion of *Col17a1* affects the maintenance of MSCs in the bulge-subbulge area. The distribution and morphology of *Dct-lacZ*-expressing melanoblasts is normal in the bulge-subbulge area of 5-day-old *Col17a1*$^{-/-}$ mice (a and d). At 12 weeks of age, abnormal melanocytes with dendritic morphology were found in the bulge area of *Col17a1*$^{-/-}$ anagen follicles (e). Inset in (e) shows magnified view of ectopically pigmented melanocytes in the hair follicle bulge-subbulge of *Col17a1*$^{-/-}$ mice. By 5 months of age, *Dct-lacZ*-expressing cells were lost both in the bulge-subbulge area and in the hair bulb (f). Bulge-subbulge areas are demarcated by brackets.

(C) COL17A1 expression in the bulge area (demarcated by brackets). (a) COL17A1 (a: red) is expressed in KRT15 (a: green)-expressing bulge keratinocytes and in basal cells of the IFE (b: green) in wild-type skin. (b) *Dct-lacZ*-expressing melanoblasts (b: red) are located close to COL17A1$^+$ basal cells (b: green) in wild-type follicles. Scale bars represent 40 µm.

(D) RT-PCR analysis; the level of *Col17a1* mRNA is below the detection limit in flow cytometry-sorted GFP-tagged melanoblasts.

(E) Light and electron micrographs of *Dct-lacZ*-expressing melanoblasts in the bulge area (MSCs). The arrowheads in (a) and (b) point to *Dct-lacZ*-expressing melanocytes in the bulge areas in semithin sections of the skin. The ultrastructural high-power view is of the boxed areas shown in (b) and (c). X-gal reaction products accumulated in association with the nuclear membrane (d: white arrows). *Dct-lacZ*-expressing melanocytes lack hemidesmosome formation in the basement membrane zone (f), whereas adjacent keratinocytes form mature hemidesmosomes in the bulge area (e: red arrows).

(F) *Dct-lacZ*-expressing melanoblasts (blue) are in direct contact with keratin 15 (KRT15)-expressing keratinocytes in the bulge area (yellow brown). Scale bar represents 20 µm.

M, melanocytes in bulge; K, keratinocytes in bulge; Sg, sebaceous gland; IFE, interfollicular epidermis. See also Figure S1.

1997; Hintner and Wolff, 1982) that suggests that COL17A1 plays a role in hair follicle homeostasis. We previously reported premature hair loss in *Col17a1*-deficient mice (Nishie et al., 2007), although the precise underlying mechanism is unknown. In this study, we used *Col17a1* knockout mice and *COL17A1*-expressing transgenic mice to show that *Col17a1* plays essential roles in the maintenance of HFSCs, which provide a functional niche for MSCs.

RESULTS

Defective MSC Maintenance and Resultant Hair Graying in *Col17a1*-Null Mice

To understand the role of collagen XVII in hair follicle homeostasis, we performed a careful chronological analysis of *Col17a1*-deficient mice. As shown in Figure 1A, *Col17a1* null mice showed premature hair loss generally preceded by extensive hair graying. Conversely, heterozygous mice displayed a normal phenotype. The hair coats in *Col17a1*-null mice were indistinguishable from control littermates for 8 weeks after birth. However, progressive hair graying started from the snout at around 12 weeks of age and then became pronounced on their backs at around 4–6 months of age and was associated with a sparser hair distribution that was subsequently followed by progressive and more extensive hair loss (Figure 1A). It is notable that these hair changes were not accompanied or preceded by any apparent changes in the skin. Skin friction, such as attempting to artificially peel neonatal skin, can induce skin erosions in *Col17a1*-null mice (Nishie et al., 2007) but did not significantly accelerate hair graying or hair loss. Thus, it is unlikely that the hair changes are a secondary outcome of skin detachment but is more likely that the hair graying and hair loss are programmed through the

Col17a1 deficiency in the hair follicles. Our previous studies demonstrated that defective maintenance of MSCs in the hair bulge causes hair graying (Nishimura et al., 2005). Thus, we first examined the distribution and morphology of MSCs in *Col17a1*-null mice by using a melanocyte-targeted *Dct-lacZ* transgene (Mackenzie et al., 1997). As shown in Figures 1Ba and 1Bd, *Dct-lacZ*-expressing cells showed a normal morphology and distribution in the bulge area during hair follicle morphogenesis until initiation of the hair regeneration cycle both in *Col17a1*$^{+/-}$ and in *Col17a1*$^{-/-}$ mice. At around 12 weeks after birth, pigmented melanocytes with a dendritic morphology that expressed melanocyte markers appeared in the hair follicle bulge of *Col17a1*$^{-/-}$ mice (Figure 1Be; Figure S1Be). At 5 months of age, *Dct-lacZ*-expressing cells were almost completely lost in the follicle bulge area as well as in the hair bulbs of *Col17a1*-null mice (Figure 1Bf; Figure S1Bf). These data demonstrate that MSC maintenance is defective in *Col17a1*-deficient mice and that this mechanism results in progressive hair graying.

Preferential Expression of COL17A1 in HFSCs but Not in MSCs

Collagen XVII is a hemidesmosomal transmembrane collagen expressed by basal keratinocytes of the IFE (McGrath et al., 1995). However, neither the expression of mouse *Col17a1* nor hemidesmosome assembly in melanocyte lineage cells and/or in bulge keratinocytes has been reported, so we first examined the expression of mouse COL17A1 protein in hair follicles by using immunohistochemistry. As shown in Figure 1Ca and Figures S1C and S1D, mouse COL17A1 was preferentially localized along the dermal-epidermal junction of bulge keratinocytes that express markers for HFSCs but not in follicular keratinocytes outside of the bulge area. However, the localization of COL17A1 in basal cell surface of MSCs could not be determined via normal immunohistochemical methods, because the attachment site of MSCs to the basement membrane is limited (Figure 1Cb). We therefore examined *Col17a1* expression by using RT-PCR in flow cytometry-sorted GFP$^+$ cells from melanocyte lineage-tagged GFP transgenic mouse skin (Osawa et al., 2005). In sharp contrast to the significant expression of *Col17a1* in control keratinocytes, expression in GFP$^+$ melanocytes was not detectable (Figure 1D). To support this finding, we used transmission electron microscopy to check whether *Dct-lacZ*-expressing melanoblasts within the bulge area in wild-type animals have hemidesmosomes. As shown in Figure 1E, hemidesmosomes, which form regularly spaced electron-dense structures along the epidermal basement membrane zone (McMillan et al., 2003), were completely absent in *Dct-lacZ*-expressing melanoblasts in the bulge (Figures 1Ed and 1Ef), whereas typical hemidesmosomes were seen overlying the basal plasma membrane in surrounding bulge keratinocytes (Figure 1Ee). As these bulge, keratinocytes adjacent to *Dct-lacZ*-expressing melanoblasts express HFSC markers (Figure 1F), these data indicate that HFSCs but not MSCs are anchored to the underlying basement membrane via hemidesmosomes. We also confirmed the localization of COL17A1 to hemidesmosomes in basal keratinocytes but not in melanocytes by immunogold electron microscopic analysis of human epidermis (Figure S1E). Therefore, we conclude that MSCs do not express COL17A1 and do not assemble any discernible hemidesmosomal structures at their surface. These findings suggested that the depletion of MSCs in *Col17a1*-null mice is caused by defects in the HFSC population that forms the main supportive cells surrounding MSCs.

Abrogated Quiescence and Immaturity of HFSCs Result in Depletion of HFSCs in *Col17a1*-Null Mice

Previous studies on wild-type mouse skin reported that mature hemidesmosomes exist at the follicular-dermal junction just below the level of sebaceous glands (Hojiro, 1972) and in hair germs of telogen hair follicles (Greco et al., 2009). Consistently, we found mature hemidesmosomes at these junctions within the hair follicle bulge (Figure 1Ee). However, mature hemidesmosomes have not been found in the transient portion of hair follicles (Hojiro, 1972), where COL17A1 expression is undetectable. These data suggested that hemidesmosome formation is important for anchoring of HFSCs located in the bulge-subbulge area of hair follicles to the basal lamina.

To test whether the abnormalities observed in *Col17a1* deficiency are specifically caused by any functional defects of HFSCs or by their detachment from the basal lamina, we first carefully examined the junctions of hair follicles in the dorsal skin of *Col17a1*-null mice and their controls by transmission electron microscopy (TEM). A significant number of hemidesmosomes are poorly formed in the bulge keratinocytes of *Col17a1*-deficient mice (Figure S2B), as seen in epidermal keratinocytes of those mice (Nishie et al., 2007). However, we did not find any significant microscopic separation at the follicular-dermal junction in sections of trunk skin from *Col17a1*-null mice (Figure 2; Figures S2A and S2B). Furthermore, we did not find significant inflammatory cell infiltrates or any signs of cell death, such as the appearance of eosinophilic cell bodies or TUNEL-positive or cleaved caspase 3-positive cells, at the follicular-dermal junction area of *Col17a1*-null mouse skin (Figure S2C and data not shown). Basement membrane thickening/reduplication, a sign of repeated regeneration of the epidermal and dermal junction, was also not found. These findings suggested that the hair graying and hair loss phenotypes in *Col17a1*-null mice cannot be explained simply by HFSC detachment from the basal lamina but instead may result from dysregulation or altered cell properties of HFSCs caused by *Col17a1* deficiency.

To examine whether HFSCs show any dysregulation caused by *Col17a1* deficiency, we carefully examined the hair follicle cycle progression, which alternates phases of growth (anagen), regression (catagen), and rest (telogen) in synchronization with the activation status of HFSCs, in *Col17a1*-null mice. While the first short telogen phase was transiently seen around 22 days after birth both in *Col17a1*-null mice and in control littermates, the second telogen phase was significantly shortened in *Col17a1*-null mice (Figure 2, summarized on the right side). At 6 weeks of age, just before normal hair follicles on the dorsal skin enter the second telogen phase, most hair follicles in *Col17a1*$^{-/-}$ mice were not distinguishable from those in *Col17a1*$^{+/-}$ mice either in morphology or in hair cycle progression. The second telogen phase is normally seen at around 7 weeks after birth and lasts about 4–5 weeks over the entire skin surface of wild-type mice (Paus and Cotsarelis, 1999; Paus et al., 1999). This phase was shortened to less than 2 weeks in all *Col17a1*$^{-/-}$ mice examined at 8–12 weeks of age, whereas such an aberrant pattern was seen in only 14.3% of *Col17a1*$^{+/-}$ mice. The subsequent
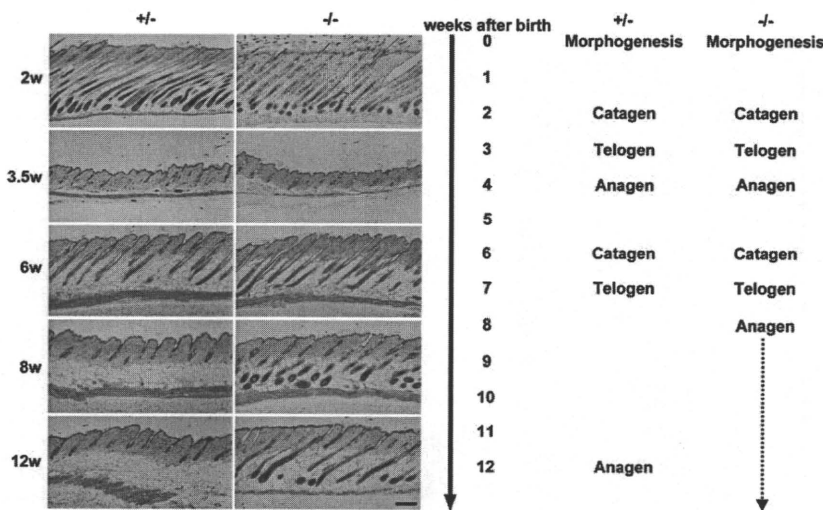
Figure 2. Loss or Shortening of the Resting State of Hair Follicles in *Col17a1*-Deficient Skin

Representative H&E images of dorsal skin sections (left) and time-scale for the hair cycle (right) from *Col17a1*$^{-/-}$ mice and from *Col17a1*$^{+/-}$ littermates during the first 12 weeks after birth. In *Col17a1*$^{-/-}$ follicles, the second telogen was significantly shortened, and the second anagen lasted longer. Scale bar represents 200 μm. See also Figure S2.

anagen phase was rather prolonged in *Col17a1*$^{-/-}$ mice compared to their control littermates. These findings suggest that HFSCs are unable to remain quiescent for a sufficient time from the second telogen phase and thereafter in the absence of *Col17a1*.

To search for early events or changes in HFSCs in *Col17a1*-null mice, we performed immunohistochemical analysis with four different markers for HFSCs, keratin 15 (KRT15), CD34, α6-integrin, and S100A6, at different stages (Figure 3A; Figure S3; Morris et al., 2004; Tumbar et al., 2004). At 5 weeks of age, there was no difference in the expression of HFSC markers or the number of HFSC marker-positive cells between control and *Col17a1*-null mice. However, at around 8 weeks of age, HFSC marker-expressing cells were absent in the bulge area in selected null mouse hair follicles (Figures 3A and 3B; Figure S3A), and the number of these marker-deficient follicles increased over time. By 6 months of age, the HFSC population had been lost in most hair follicles of *Col17a1*-null mice (Figure S3B). Flow cytometric analysis also confirmed that the α6-integrin^{high} CD34$^+$ population (Blanpain and Fuchs, 2006), which represents basal HFSCs in the bulge area, was diminished (Figure 3C). Hair follicle atrophy with the loss of hair follicle structures were also observed once the HFSC population was diminished (Figure 3D). These data indicate that *Col17a1*-null HFSCs fail to maintain their stem cell characteristics, including their quiescence and immaturity, after the second telogen phase, resulting in hair follicle atrophy. Conversely, epidermal hyperplasia was also transiently found in some focal areas of the *Col17a1*-null skin at around 6 months of age (Figure 3D, arrowheads) but was normalized and subsequently became atrophic at later stages, which suggests that the epidermal stem cell population might also be gradually losing its self-renewing potential with age in *Col17a1* deficiency compared to controls.

To examine whether HFSC maintenance fails because the cells lose their immaturity or quiescence in the absence of *Col17a1*, we analyzed the expression of markers for keratinocyte differentiation and proliferation in *Col17a1*-null hair follicles. Interestingly, keratin 1 (KRT1), a differentiation marker for the IFE, was ectopically expressed in the bulge area of *Col17a1*-

null mice at 8 weeks of age (Figure 4A). KRT1-positive cells in the bulge areas of affected hair follicles were found in 60% of *Col17a1*-null mice but not in control mice at that age. Ectopic expression of other epidermal differentiation markers, such as involucrin and KRT10, was also present in the bulge areas of *Col17a1*-null mice at 8 weeks of age (Figure S4A). Furthermore, Ki67-positive cells were located in the bulge area of *Col17a1*-null mice, and those Ki67-positive cells showed an absent or reduced level of KRT15 expression (Figure 4A).

The maintenance of quiescence and immaturity of somatic stem cells in tissues is a prerequisite for sustained stem cell self-renewal, and which can be assessed for HFSCs by means of a colony-formation assay in vitro (Barrandon and Green, 1987; Oshima et al., 2001). We therefore took advantage of the type of assay by using neonatal epidermal keratinocytes, which contain the presumptive HFSC population (Nowak et al., 2008), to assess the self-renewal potential of that population in *Col17a1*-null mice. As shown in Figures 4Ba and 4Bb, *Col17a1* homozygous null keratinocytes showed defects in colony-forming ability on 3T3-J2 feeder cells compared to keratinocytes from control mice. Colonies larger than 0.5 mm in diameter were significantly decreased in number with *Col17a1*-null keratinocytes (Figure 4Bc). Although *Col17a1*-null keratinocytes showed defective binding ability to collagen I-coated dishes (Figure S4B), they showed no detectable defects in their ability to directly adhere to 3T3-J2 feeder cells (Figure 4Bd). These data strongly suggest that *Col17a1*-null keratinocytes have a much lower renewal capability than control keratinocytes. Taken together with the in vivo findings, we conclude that COL17A1 is critical for the self-renewal of HFSCs by maintaining their immaturity and quiescence.

Loss of TGF-β Expression by HFSCs and the Associated Differentiation of Adjacent MSCs

To examine whether the early changes in HFSC in *Col17a1* mutant mice affects the maintenance of MSCs in the hair follicle bulge, we carefully examined MSCs in hair follicle bulge areas in *Col17a1*-null mice beginning to show HFSC defects. At 8 weeks of age, when HFSCs in *Col17a1*-null mice are prematurely activated, KIT$^+$ melanoblasts within the bulge area prematurely coexpressed TYRP1, a melanocyte differentiation marker, in *Col17a1*-null mice but not in control mice (Figure S5A). At around 12 weeks of age, pigmented melanocytes with a mature
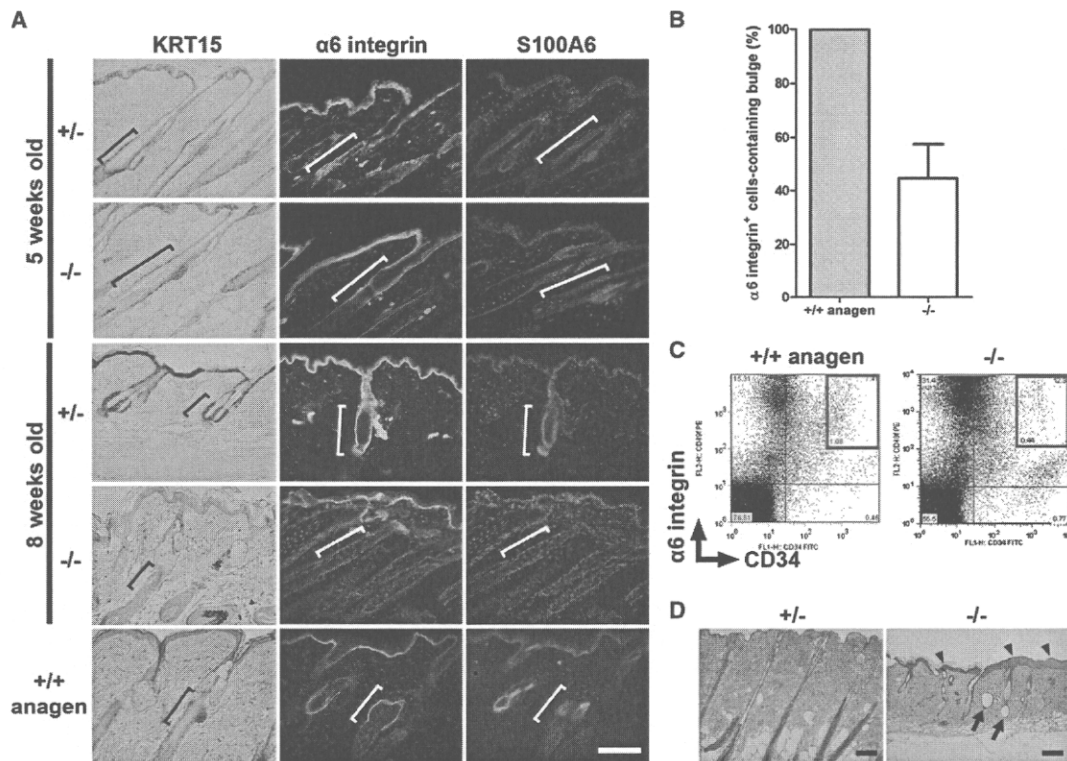
Figure 3. HFSC Depletion in COL17A1-Deficient Mice

(A) Immunostaining of the dorsal skin from $Col17a1^{-/-}$ and from $Col17a1^{+/-}$ mice with HFSC markers. The bulge areas are demarcated by brackets. HFSC marker (KRT15, α6-integrin, and S100A6)-expressing cells were still maintained at 5 weeks of age in $Col17a1^{-/-}$ mice, whereas follicles without HFSC marker-positive cells appeared at 8 weeks of age.

(B) Ratio of hair follicles with α6-integrin$^+$ cells in the bulge areas of skin from control mice and from 8- to 10-week-old $Col17a1^{-/-}$ mice. In $Col17a1^{-/-}$ mice, many hair follicles without α6-integrin$^+$ cells in the bulge areas were found (n = 3).

(C) Flow cytometric analysis of α6-integrin and CD34 double-labeled keratinocytes. α6-integrin$^+$ CD34$^+$ cells are almost completely lost in the skin of 9-month-old $Col17a1^{-/-}$ mice.

(D) H&E-stained histological sections of $Col17a1^{-/-}$ and of $Col17a1^{+/-}$ mouse skin. At 6 months of age, there was a diminution of hair follicle bulbs, degeneration of the hair follicles (arrows), and epidermal hyperplasia (arrowheads) in $Col17a1^{-/-}$ skin. As a control for the anagen phase in (A), (B), and (C), dorsal skin at 5 days after hair-plucking of telogen follicles was used.

Scale bars represent 100 μm. See also Figure S3.

dendritic morphology and expressing TYRP1 in addition to Dct-$lacZ$ and KIT were aberrantly found within the bulge area in mid-anagen hair follicles (Dct-$lacZ$-expressing cells in Figure 1B, Figure S1B, arrow in Figure 5A; KIT$^+$/TYRP1$^+$ cells in Figures 5B and 5C and Figure S5B). Conversely, only nonpigmented melanoblasts expressing Dct-$lacZ$ and KIT but not TYRP1 and with small cell bodies (MSCs) were found in control littermates (Figure 1B; Figures S1B and S5B). Similar morphological changes were previously described as ectopic MSC differentiation within the niche (Inomata et al., 2009; Nishimura et al., 2005). These ectopically differentiated melanocytes were found in the bulge area at 12–13 weeks of age, prior to the hair graying seen in $Col17a1$-null mice (Figure 5D). Furthermore, it is notable that the ectopically differentiated melanocytes in $Col17a1$-deficient mice were typically found in association with early changes in bulge keratinocytes including the enlarged morphology of surrounding bulge keratinocytes (Figure 5A, arrowheads) and an increased number of Ki67-expressing bulge keratinocytes in midanagen follicles (Figures 4A and 5E and data

not shown). The appearance of ectopically differentiated melanocytes within the bulge area was followed by progressive hair graying in $Col17a1$-null mice (Figures 1A and 1B; Figure S1B).

TGF-β signaling is activated in the hair follicle bulge and is involved in but is not essential for the maintenance of HFSCs (Guasch et al., 2007; Qiao et al., 2006; Yang et al., 2005, 2009). Our recent study showed that the signal is required for the maintenance of MSCs through promoting MSC immaturity and quiescence (Nishimura et al., 2010), but it was not clear whether the signal is derived from HFSCs or MSCs. As similar changes in MSCs, such as the appearance of ectopically differentiated melanocytes in the niche and the subsequent depletion of MSCs seen in $Col17a1$-null mice, were found in $TGFbRII$ conditional knockout mice (Nishimura et al., 2010), we hypothesized that the defective renewal of MSCs in $Col17a1$-null mice might be mediated by defective TGF-β signaling from the surrounding HFSCs. To test this model, we examined the involvement of TGF-β signaling in the defects of MSCs in $Col17a1$-null mice and their controls. We found that KRT15-expressing
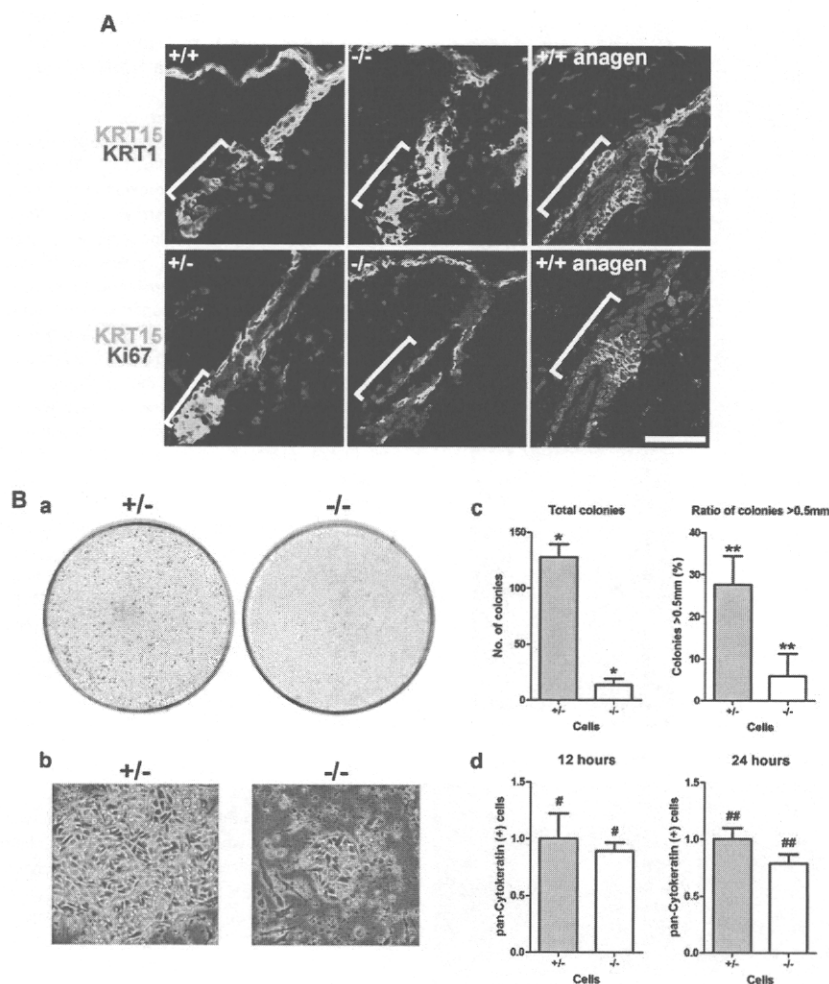
Figure 4. Deficient Stemness of HFSCs in Col17a1-Null Skin

(A) Immunostaining of the dorsal skin from 8-week-old $Col17a1^{-/-}$ and from control mice with the IFE differentiation marker keratin 1 (KRT1) and Ki67. The bulge areas are demarcated by brackets. Top: In $Col17a1^{-/-}$ mice, cells coexpressing KRT15 (green) and KRT1 (red) appeared within the bulge area. Bottom: Cells in the bulge area of $Col17a1^{-/-}$ mice proliferated abnormally. As a control for anagen phase, dorsal skin at 5 days after hair-plucking of telogen follicles was used. Scale bar represents 50 μm.

(B) Loss of keratinocyte clonal growth potential resulting from Col17a1 deficiency. (a) Clonal growth assays of keratinocytes from $Col17a1^{-/-}$ and from $Col17a1^{+/-}$ mice; representative dishes are shown. (b) $Col17a1^{-/-}$ keratinocytes formed only small colonies. (c) Colonies from $Col17a1^{-/-}$ skin were significantly fewer and smaller than those from $Col17a1^{+/-}$ control mice. *, **p < 0.05. (d) Keratinocytes from $Col17a1^{-/-}$ skin did not show decreased binding to 3T3-J2 feeder cells. #p = 0.6653, ##p = 0.162.
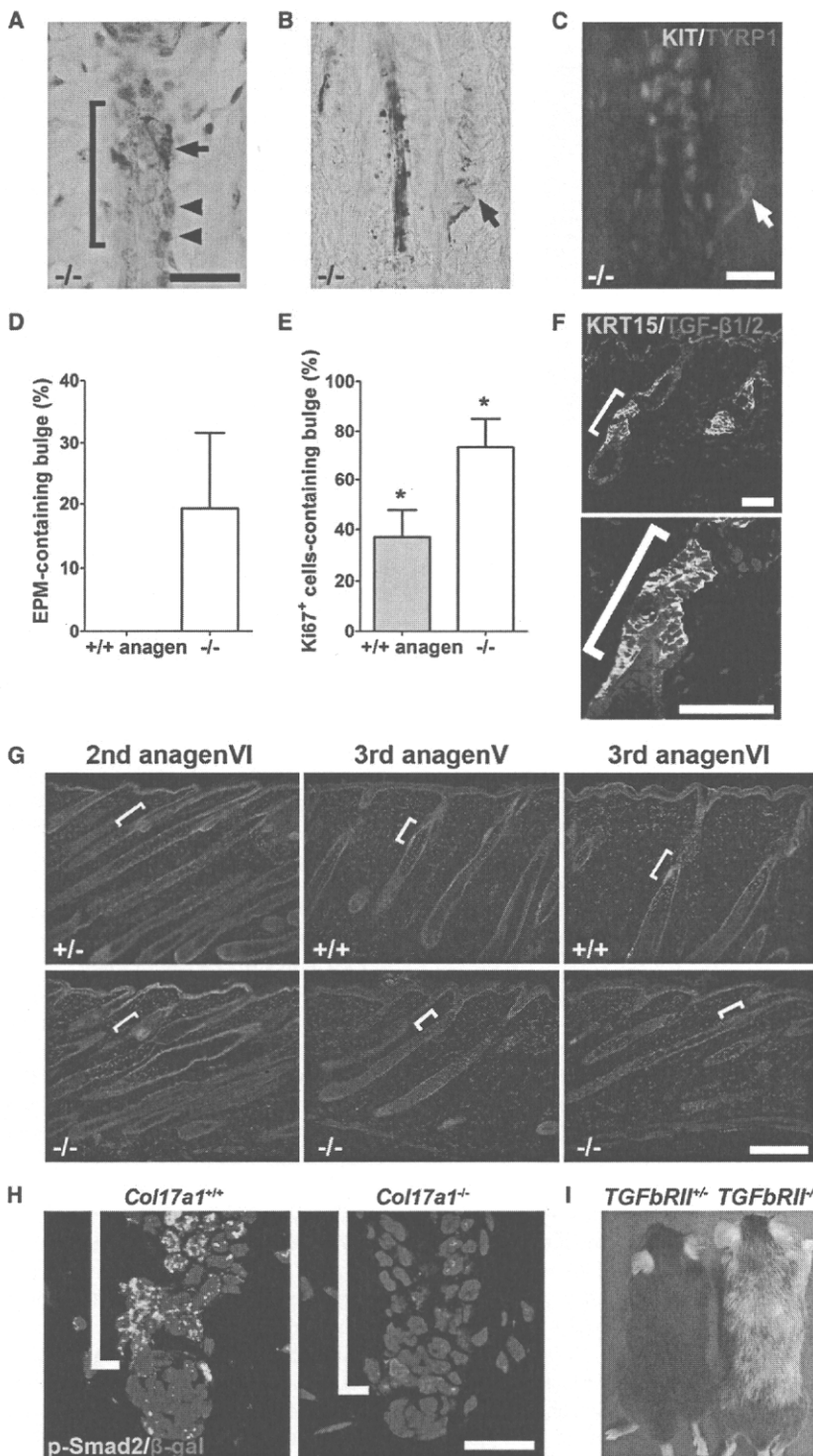
See also Figure S4.

MSCs resulting from the loss of TGF-β production from HFSCs affects MSC maintenance in Col17a1 mutant mice and that HFSC-derived TGF-β signaling mediates the niche function of HFSCs for MSC maintenance.

Human COL17A1-Mediated Rescue of HFSCs Normalizes Maintenance of MSCs in Col17a1-Null Mice

Finally, to address whether the defects in Col17a1-null HFSCs induce the ectopic differentiation and eventual depletion of MSCs in the bulge area, leading to hair graying, we studied the impact of the transgenic rescue of Col17a1-null mice and in particular the HFSC phenotype resulting from forced expression of human COL17A1 under control of the Keratin 14 (Krt14) promoter (Olasz et al., 2007). In these rescued mice, human COL17A1 expression was restricted to basal keratinocytes and not to the melanocyte lineage (Figure S6A). As shown in Figure 6A, the hair coat of these mice was quite similar to that of $Col17a1^{+/-}$ mice and did not show progressive hair depigmentation or hair loss at 6 months of age, or even at 1 year of age (data not shown), whereas control Col17a1-null mice demonstrated the hair graying and other typical changes described above. Interestingly, both the distribution and morphology of Dct-lacZ-expressing melanoblasts in the bulge area were normal in the $Col17a1^{-/-}$; Krt14-hCOL17A1 rescued mice (Figure 6B). Furthermore, the aberrant expression of Ki67 and KRT1, downregulation of TGF-β1/2 expression, and inactivation of TGF-β signaling in bulge keratinocytes were all also normalized (Figures 6C and 6D; Figure S6B). These findings demonstrate the dual critical roles of COL17A1 in HFSCs for their maintenance and for providing a niche for MSC maintenance through HFSC-derived TGF-β signaling (Figure 7).

keratinocytes coexpress TGF-β1/2 in wild-type hair follicles (Figure 5F), demonstrating that HFSCs produce TGF-β1/2 in the bulge area. At 6 weeks of age, the expression of TGF-β1/2 was similar in bulge keratinocytes in the control and in Col17a1-null mice (Figure 5G). At 8 weeks of age or later, however, the hair follicle bulge exhibited significantly downregulated expression of TGF-β1/2 in Col17a1-null mice, although the hair follicle bulge in control mice showed a normal expression pattern (Figure 5G). Furthermore, phospho-Smad2 signals were not found either in bulge keratinocytes or in melanocytes of Col17a1-null mice but were present in control mice (Figure 5H). These findings demonstrate that niche features, including the loss of TGF-β1/2 production, are defective in Col17a1-null HFSCs. We reported previously that TGFbRII conditional knockout in mice via a bitransgenic system causes mild hair graying with incomplete penetrance (73.3% within 10 months after birth) possibly because of incomplete CRE-mediated recombination (Nishimura et al., 2010). In this study, we found that TGFbRII straight knockout mice (with a Rag2-null background for the inhibition of multiorgan autoimmunity) show a severe hair graying phenotype with 100% penetrance within 5–6 weeks of age (Figure 5I). Thus, these data suggest that defective TGF-β signaling in

Cell Stem Cell

Role of Hair Follicle Stem Cells as Niche Cells

Figure 5. Ectopic Differentiation of MSCs in the Bulge Area with Diminished TGF-β Signaling Resulting from *Col17a1* Deficiency

(A–E) Ectopic differentiation of MSCs and surrounding keratinocytes in the bulge areas of *Col17a1*$^{-/-}$ follicles at 12 weeks of age. The bulge areas are demarcated by brackets. Ectopically pigmented melanocytes (A; arrow) are in direct contact with enlarged keratinocytes with large nuclei (A; arrowheads) in an anagen VI follicle; these ectopically pigmented melanocytes (arrow) are KIT$^+$/TYRP1$^+$ cells with a dendritic morphology (B and C). Ectopically pigmented melanocytes were detected only in the bulge-subbulge area of *Col17a1*$^{-/-}$ follicles (D), and the proliferation of *Col17a1*$^{-/-}$ bulge keratinocytes at 12–13 weeks of age was abnormally accelerated compared with that of control anagen V follicles (E). *p < 0.05. Scale bars represent 30 μm in (A) and 20 μm in (B) and (C).

(F) Localization of TGF-β1/2 expression (red) in *Col17a1*$^{+/+}$ hair follicles. Plucked dorsal skins (4 days after hair plucking in telogen skin from 7-week-old *Col17a1*$^{+/+}$ mice were used. KRT15-expressing keratinocytes (shown in green) express TGF-β1/2 (red). Scale bars represent 50 μm.

(G) *Col17a1*$^{-/-}$ mouse hair follicles from 5-week-old mice showed normal TGF-β1/2 expression patterns (left). However, at 8 weeks of age or later in *Col17a1*$^{-/-}$ mice, the TGF-β1/2 expression was downregulated (right and middle). Scale bar represents 200 μm.

(H) Phosphorylated Smad2 (shown in green) was not detected at 8 weeks in the *Col17a1*$^{-/-}$ hair follicle bulge. Dct-lacZ-expressing melanocytes in the bulge area are shown in red. Bulge areas are demarcated by brackets. Scale bar represents 20 μm.

(I) *TGFbRII* straight knockout mice (*TGFbRII*$^{-/-}$) (right) show severe hair graying phenotype at 6 weeks of age.

See also Figure S5.

maintenance of stem cell properties (Li and Xie, 2005; Moore and Lemischka, 2006). Although previous in vitro studies suggested some correlation in keratinocytes between integrin-mediated extracellular matrix adhesion and proliferation potential, in vivo ablation studies of major integrins in basal keratinocytes have not provided data on stem cell-specific depletion phenotypes (Dowling et al., 1996; Georges-Labouesse et al., 1996; Raghavan et al., 2000; van der Neut et al., 1996; Watt, 2002). In the present study, we demonstrated that COL17A1, a hemidesmosomal transmembrane collagen, is highly expressed in HFSCs within hair follicles and is required for the self-renewal of HFSCs. We found that *Col17a1* ablation in mice results in premature hair loss almost homogeneously over the entire body surface without showing any specific association

DISCUSSION

Interactions between somatic stem cells and their surrounding niche microenvironment are critical for the establishment and