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Genetic disorders of keratins and their associated proteins

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Abstract

It has recently been demonstrated that genetic defects in keratin genes cause a number of different skin disorders, including epidermolysis bullosa simplex (EBS), epidermolytic hyperkeratosis (EH), the EH form of epidermal nevi, epidermolytic and non-epidermolytic forms of palmoplantar keratoderma (EPPK and PPK) and pachyonychia congenita (PC). In this review, I describe the research that led to this discovery.

Keywords: Keratin; Genetic disorders; Epidermolysis bullosa simplex

1. Keratin

The major structural proteins of the epidermis and its appendages are keratins, members of the intermediate filament (IF) superfamily (for reviews, see [1,2]). In keratinocytes, keratins constitute $>10\%$ of the protein of dividing cells and 85% of the protein of terminally differentiated cells. Keratins are most abundant in the epidermis and its appendages, but they are found in all epithelial tissues [3]. There are ~ 30 different keratin genes subdivided into two distinct sequence types, which are differentially expressed as specific pairs [4–6]. Type I proteins include keratins 9–20 and the Ha keratins, and they tend to be acidic (pK_i 4–7) and smaller in size (40–63 kDa). Type II proteins include keratins 1–8 and the Hb keratins, and they tend to be basic (pK_i 7–9) and larger in size (44–67 kDa) (for review, see [3]).

Mitotically active keratinocytes of stratified squamous epithelia express keratins K5 and K14 [7]. In healthy skin, K5 and K14 are restricted to the basal layer [3,8]; in hyperproliferative disorders, K5 and K14 extend to suprabasal layers [9]. As epidermal keratinocytes commit to terminally differentiate and begin to move outward towards

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the skin surface, they switch off K5 and K14 and induce K1 and K10 [8]. As cells move further outward, they induce an additional type II keratin, K2e [10]. In palmar and plantar skin, K9 is also expressed suprabasally [8,11].

The pattern of keratins in the hair follicle is more complex than it is in the epidermis. K5 and K14 are expressed throughout the outermost layer of the outer root sheath (ORS), and in addition, in the inner layers of the lower portion of the ORS [12]. As the cells in the upper portion of the ORS differentiate, they move inward, inducing K6 and K16, a suprabasal pair of keratins that in epidermis are atypically induced in response to hyperproliferative disorders [6,9,13,14]. In the inner root sheath (IRS), K1 and K10 are expressed [15]. At the center and the base of the hair follicle are the relatively undifferentiated matrix cells, which maintain close contact with the dermal papillae. As these cells differentiate and move upward to form the cortex, they induce a set of hair-specific keratins, the Ha and Hb keratins [3,16]. These keratins form the elaborate and indestructible cytoskeleton of the terminally differentiated cells of the hair shaft.

While all keratin pairs form 10 nm filaments, the physical, chemical and biological properties are likely to be quite different for filaments composed of different keratin pairs. Given that the hypervariable regions of the keratins protrude along the surface of the keratin filaments [17], keratins are likely to be specifically tailored to suit the various structural needs of different epithelial cells. Indeed, epithelial cells do not simply contain bags of keratin filaments, but rather the keratin filaments are organized in a defined architecture within each cell. In epidermal keratinocytes, keratin IFs attach to hemidesmosomes and desmosomes through specific sequences that are not conserved among all keratins [18]. In terminally differentiating keratinocytes, IFs associate with filaggrin, which appears to catalyze bundling of keratin filaments into large macrofibrils [19]. IFs composed of keratins 1 and 10 also associate with the cornified envelope [20]. Different specialized contacts are also likely between Ha-Hb keratin IFs and the many different keratin filament-associated proteins of the hair follicle [21].

2. Keratin filament assembly

Keratin filaments assemble in the absence of any auxiliary proteins or factors [22]. Keratins form heterodimers, which assemble into obligatory heteropolymers [23–26]. The assembly of the heterodimer is based upon a central, largely α-helical 'rod' sequence which contains ~ 40 heptad repeats of hydrophobic residues, such that many a and d residues of the a b c d e f g repeats are often leucine, isoleucine or alanine (for review, see [1]). This creates a hydrophobic seal along the surface of the helix, enabling two such molecules to intertwine in a coiled-coil fashion. The coiled-coil heterodimer of type I and type II subunits is in-register and parallely aligned [5,27]. While some studies have suggested that keratin homodimers also exist [25,26], these subunits on their own do not appear to be competent for assembly into 10 nm filaments [25].

The rod domain of keratins is subdivided into four α-helices, termed 1A, 1B, 2A and 2B, that are interspersed with short non-helical linker segments, termed L1, L1-2 and L2 [5,27]. The functional significance of these short interruptions in the α-helix remains unknown. Within a keratin type, the rod domains share 50–90% sequence identity, while keratins of different types share only ~ 25–35% sequence identity in these regions ([5]; for review, see [1]). The residues at the beginning of helix 1A and the end of helix 2B are highly conserved among IF proteins and across the evolutionary kingdom. These rod ends are essential for IF assembly as judged by mutagenesis studies conducted in vivo and in vitro [28–34].

The rod is flanked by non-helical amino 'head' and carboxy 'tail' domains that are highly variable in sequence [5,17,35]. In contrast to the α-helical rod domain, the nonhelical head and tail segments of keratins play less of a role in filament assembly and more of a specialized role. Of the heads and tails of epidermal keratins, only the type II keratin head of K5 and K1 has deleterious effects on 10 nm filament assembly when deleted or mutated [33,36]. Thus, the heads and tails are likely to participate in IF-associated interactions, while the rod domains are central for 10 nm filament structure.

Table 1
Characteristics of epidermolysis bullosa simplex

Feature	Dowling-Meara	Koebner	Weber-Cockayne	With mottled pigmentation	With muscular dystrophy
Autosomal dominant	+	+	+	+	+
Skin blistering	Entire body	Body	Hands/feet	Body	Hands/feet
Basal cell cytolysis	+	+	+	+	+
Discernable abnormalities in basal keratin network	+	+	±	+	±
Keratin clumping in basal layer	+	–	–	–	–
Oral involvement	+	–	–	–	–

Typical of all IF proteins, keratin dimers align in a head-to-tail fashion to form linear arrays [36,37]. As judged by chemical crosslinking studies, the amino ends of helix 1A of one dimer overlap by about 10 residues with the carboxy ends of helix 2B of an adjacent dimer [36,38], thereby accounting for the importance of these ends to filament elongation, realized in earlier mutagenesis studies. Linear arrays of dimers are likely to be arranged in an antiparallel fashion, with a half-stagger relative to an adjacent dimer chain [20,27,36]. Under the electron microscope, keratin IFs appear as ropes of three or four strands, called protofibrils, each of which contain approximately eight keratin polypeptides, or four dimer chains [39,40]. While little is known about the sequences involved in lateral associations of linear dimer arrays, mutations in the non-helical linker segments have been shown to affect lateral interactions [34].

3. The discovery of the genetic basis of epidermolysis bullosa simplex: the first known keratin disorder

In the mid-1980s, it was known from molecular mutagenesis studies that most keratin mutations behave in a dominant negative fashion, i.e. they perturb filament assembly even in the presence of their wild type partner keratin [28–31,41]. To test the effects of disrupting keratin filament networks in skin in vivo, we used transgenic mouse technology to express mutant human keratin 14 genes in the skin [42–44]. In transgenic mouse technology, the mutant human keratin gene integrates randomly into one of the chromosomes of the mouse genome, leaving the two endogenous K14 alleles unharmed. The human K14 gene was faithfully expressed in mouse epidermis as it is in human epidermis. These mice expressing a severely disrupting mutant human keratin 14 gene displayed the clinical and pathological features of the Dowling-Meara (D-M) subtype of epidermolysis bullosa simplex (EBS) [42]. Of the three major subtypes of EBS (see Table 1), D-M EBS is the most severe, typified by mechanical stress-induced skin blistering due to cytolysis within the basal layer of the epidermis. Ultrastructurally, clumps or aggregates of keratin are present in the basal layer, while suprabasal layers are seemingly normal ([5] and references therein). Not only were these features present in the K14 mutant-expressing mice, but also the basal clumps of keratin labeled with antibodies against the transgene and endogenous K14 and K5 proteins [42]. If we now used a more mildly disrupting K14 mutant in transgenic technology, the mice exhibited features characteristic of Weber-Cockayne (W-C) EBS, where patients have milder blistering, most notable in the palmo and plantar skin [43]. As in W-C EBS, our mice displayed clinical features postnatally, and at the ultrastructural level, it was difficult to discern abnormalities in keratin architecture, despite the fact that a keratin mutant was expressed in the basal cells.

The similarities between the epidermis of our transgenic mice and of human EBS patients were striking, leading us to predict that EBS would be a disorder of keratins, namely of K14 and K5

[42]. We also predicted that the three major subtypes of EBS, D-M EBS, Koebner EBS and W-C EBS, would be genetically related based upon the location of the keratin mutations and the filament-disrupting capabilities of the particular mutations [42,43]. These predictions were consistent with early electron microscopy reports [46], suggesting that perturbations in keratin filament networks were early events in the blistering process, and with observations noting the similarities between cultured D-M EBS keratinocytes and keratinocytes transfected with mutant keratin genes [47].

We then focused our attention towards human EBS patients. Later in 1991, we demonstrated that humans with D-M EBS have point mutations in their K14 or K5 genes, and that the particular mutations were causative for the filament disruptions seen in the keratinocytes cultured from these patients [32]. Several months later, a report from Epstein's group [48] showed that defects in families with EBS map to chromosomes 17q12-21 and 12q11-12, where a graduate student in my lab had previously shown the K14 and K5 genes to reside [49,50]. Table 2 summarizes the locations of these mutations and their clinical severity. Intriguingly, the first two unrelated D-M EBS patients that we studied had mutations at the exact same arginine residue at position 125 (R125) in the K14 polypeptide. We now know that this residue is a hotspot for mutagenesis by methylation/deamination at a CpG dinucleotide [51]. Mutation to either a histidine (CpG methylation in the non-coding strand sequence) or a cysteine (CpG methylation in the coding strand sequence) accounts for the majority of the D-M EBS cases [32,52–54]. We also know that this arginine residue is highly conserved and is arginine even in snail IF protein, the most distantly related of all the IF proteins [55]. The residue also resides within the first heptad of helix 1A, i.e. in the putative region of head-to-tail dimer overlap predicted by Steinert et al. [20]. Thus, a mutagenic hotspot coupled with a residue critical for filament elongation, make this arginine an important player in Dowling-Meara epidermolysis bullosa simplex.

Table 2
Mutations found in patients with disorders of keratin

Gene	Disease	Mutation	Domain
K14	D-M EBS	R125H (9)	1A
K14	D-M EBS	R125C (6)	1A
K14	D-M EBS	R125S	1A
K14	D-M EBS	M119I	1A
K14	D-M EBS	Q120R	1A
K14	D-M EBS	Y129D	1A
K14	D-M EBS	A274D (3)	L12
K14	D-M EBS	I377N	2B
K14	D-M EBS	R388C	2B
K14	K EBS	L122F	1A
K14	K EBS	L384P	2B
K14	K EBS	M272R	L12
K14	K EBS	A247D	1B
K14	K EBS	ΔE375	2B
K14	K EBS	L122F	1A
K14	W-C EBS	V270M	L12
K14	EBS-rec	E144A	1A
K14	EBS-rec	TGAstop	1A,1B
K5	D-M EBS	E475G	2B
K5	D-M EBS	Δ30 aa	H1/1A
K5	K EBS	L463P	2B
K5	EBS-hom	K173N	1A
K5	W-C EBS	I161S (8)	H1
K5	W-C EBS	M327T	L12
K5	W-C EBS	D328V	L12
K5	W-C EBS	N329K	L12
K5	W-C EBS	R331C	L12
K5	M-P EBS	P24L (2)	
K10	EH	R156H (5)	1A
K10	EH	R156C (2)	1A
K10	EH	R156P	1A
K10	EH	R156S	1A
K10	EH	M150R	1A
K10	EH	N154H	1A
K10	EH	L161S	1A
K10	EH	Y160D	1A
K10	EH	Y160N	1A
K10	EH	L442Q	2B
K10	EH-mild	K439E	2B
K1	EH	S185P	1A
K1	EH	N187P	1A
K1	EH	N187S	1A
K1	EH	S192P	1A
K1	EH	Y481C	2B
K1	EH	E489Q	2B
K1	EH-mild	L160P	H1
K1	EH-mild	V154G	H1
K1	PPK-mild	K73I	V1
K2e	EH-mild	E493K (3)	2B
K2e	EH-mild	Q187P	1A
K2e	EH-mild	L490P	2B

Table 2 (continued)

Gene	Disease	Mutation	Domain
K9	EPPK	N160Y	1A
K9	EPPK	N160K	1A
K9	EPPK	R162Q	1A
K9	EPPK	R162W	1A
K17	PC	N92D	1A
K16	PC	L130P	1A
K6a	PC	ΔN170	1A
K13	WSN	L119P	1A
K4	WSN	ΔN170*	1A

*Equivalent mutation to the K6a PC mutation; exact residue number for K4 not known.
Δ, removal of residue; aa, amino acid.
Number in parentheses denotes total number of independent incidences identified.

While the first few EBS cases solved all involved mutations in the K14 gene, the obligatory heteropolymeric nature of keratin filaments predicted that K5 mutations should also exist. Indeed, in 1992, Lane and coworkers [56] discovered a mutation in the highly conserved carboxy end of helix 2B of the K5 polypeptide. Interestingly, Weber-Cockayne mutations are often in the K5 polypeptide [57–59].

Many mutations have now been found in the K5 or K14 polypeptides of patients with the three major subtypes of epidermolysis bullosa simplex. D-M EBS cases tend to have mutations within the highly conserved ends of the rod domain of K5 or K14 [32,52–54,56,60], K EBS cases are frequently proline residues and are located more centrally within the α-helical rod segments [48,61–63], and W-C mutations tend to be found either in the K5 head domain, just outside helix 1A [57,64], or in the L1-2 linker segment of K5 [58,59].

This clustering of mutations relative to disease severity correlates remarkably well with findings from random mutagenesis studies that had been conducted in the 5–7 years prior to the discovery of EBS [28–31,33,34]. Filament assembly and/or gene transfection assays with genetically engineered EBS mutations now reveal that the Dowling-Meara EBS mutations produce short filament rodlets, reflecting a defect in filament elongation [32,58,65], while the Weber-Cockayne EBS mutations in L1-2 assemble into filaments with a tendency to aggregate [58]. These data provide compelling evidence that the L1-2 sequence is involved in positioning the stagger of the dimer chains relative to one another, while the rod ends are involved in head-to-tail association of dimer subunits.

4. Epidermolysis bullosa simplex with mottled pigmentation

Recently, we have discovered that a very rare form of EBS, referred to as EBS with mottled pigmentation (MP), is also a keratin disorder [66]. Affected members of two seemingly unrelated families with EBS MP had a C to T point mutation in the second base position of codon 24 of one of two K5 alleles, leading to a Pro:Leu mutation. This mutation was not present in unaffected members, nor in 100 alleles from normal individuals. Linkage analyses mapped the defect to this type II keratin gene (peak lod score at $\phi = 0$ of 3.9), which is located on chromosome 12q11-q13. This provides strong evidence that this mutation is responsible for the EBS MP phenotype.

Only conserved between K5 and K6, and not among any of the other type II keratins, 24P is in the non-helical head domain of K5, and only mildly perturbs the length of 10 nm keratin filaments assembled in vitro. However, this part of the K5 head domain is likely to protrude on the filament surface, perhaps leading to additional aberrations in IF architecture and/or in melanosome distribution that are seen ultrastructurally in patients with the mutation.

5. Epidermolytic hyperkeratosis

At the time we predicted that EBS would be a disorder of K5 and K14, we also predicted that epidermolytic hyperkeratosis would be a disorder of K1 and K10 [42]. This disorder is nearly a mirror image of EBS, and is typified by clumping or abnormalities in the suprabasal keratin IF network, suprabasal cell cytolysis, often associated with mechanical stress, and seemingly normal basal cells [45,67]. Since it was well-

established that epidermal cells switch off K5 and K14 and switch on K1 and K10 as they terminally differentiate [8], it was straightforward to arrive at this analogy.

We next demonstrated that the clinical features of EH could be recapitulated in transgenic mice expressing a mutant K10 gene [44]. Genetic mapping data [68] provided further support for subsequent sequence analyses, which unequivocally added EH to the list of keratin disorders [69–71]. Many of the most severe cases of EH had mutations in the equivalent residues of K1 and K10 that were mutated in K5 and K14 in EBS cases [69–76]. For milder forms of EH, sometimes referred to as ichthyosis bullosa of Siemens, mutations in the K2e gene are frequently seen [77,78]. Given the late onset of K2e expression in differentiating keratinocytes [10], this leads to a mild phenotype even if the K2e mutation is severely disrupting to keratin filament assembly.

6. Epidermal nevi of the EH type

Several inherited disorders display clinically mosaic cutaneous patterns, often following a distribution along the lines of Blaschko. It has been suggested that these nonrandom patterns of lesional and nonlesional epidermis may be caused by postzygotic somatic mutations in epidermally expressed genes. To directly test this hypothesis, we determined the genetic basis of one such disorder, epidermal nevi of the epidermolytic hyperkeratosis type. Patients with this disease display a mosaic pattern of cutaneous changes similar to those of epidermolytic hyperkeratosis. Offspring of epidermal nevus patients may exhibit generalized epidermolytic hyperkeratosis.

After identifying the K10 mutations in the EH offspring, we analyzed keratinocytes that we cultured from affected and unaffected body sites. We showed that only affected regions carry the corresponding K10 mutation; the other areas are genetically wild-type [79]. In contrast, dermal fibroblasts harbored the K10 mutation but with no correlation between mutation and body site. This was consistent with the knowledge that the keratinocytes and fibroblasts from the skin migrate independently during embryogenesis.

It is likely relevant that no mosaic counterpart has yet been observed for EBS. In a mosaic disorder of mitotically active keratinocytes, wild-type cells in the basal layer can move laterally to fill a vacancy left by a degenerating mutant cell. Consequently, in a situation where 50% or greater of the basal cells are wild-type, the epidermis will quickly be taken over by the wild-type cells, leaving a diagnosis of clinically normal. In contrast, in a genetically mosaic disorder of differentiating keratinocytes, cells are already locked into a columnar upward movement by the time they first exhibit clinical signs of the disorder. In this case, no compensation can occur since wild-type spinous cells cannot move laterally. Thus, a diagnosis of epidermal nevi of the EH type is made.

7. Palmoplantar keratoderma

Patients with EPPK have palmoplantar skin blistering due to cytolysis in suprabasal layers. Given that K9 (63 kDa) is expressed specifically in plantar and palmar skin [8,11], it is not surprising that most cases of EPPK arise from mutations in the rod ends of the K9 polypeptide [80–83].

A non-epidermolytic case of palmoplantar keratoderma (PPK) was recently shown to arise from a mutation in the head domain of K1 [84], at a lysine residue involved in crosslinking K1- and K10-containing filaments to the cornified envelope [85]. This residue is conserved in K5, where it is likely to be involved in association of desmosomes with basal epidermal keratin filaments [18]. These observations are in agreement with the view that the head and tail segments of keratin polypeptides protrude along the surface of the IF, and participate in interactions between IFs and associated proteins.

The potential involvement of the K5 head domain in desmosomal interactions suggests that some cases of PPK may arise from mutations in desmosomal components. Interestingly, a family with PPK has recently been mapped to the chromosome 18 locus where the desmogleins and desmocollins reside, and a transgenic mouse engineered to express a mutant desmoglein 3 was found to exhibit a skin phenotype which in some respects, resembles human PPK [86].

8. Genetic disorders of keratin that affect tissues beyond the epidermis

Pachyonychia congenita (PC) is an autosomal dominant disorder involving hyperkeratosis in the epidermis, particularly near hair shafts and in palmoplantar skin, i.e. in regions where K6, K16 and K17 are known to be expressed. Oral and nail involvement are also prevalent, consistent with the known pattern of expression of these keratins [3]. In the past year, PC was shown to be a bona fide genetic disorder of K6, K16 and K17 [76,77,87,88].

Recently, White Sponge Nevus (WSN) was added to the growing list of keratin disorders [89,90]. This disease is autosomal dominant and is characterized by white, thick plaques in the oral mucosa, occasionally accompanied by esophageal, genitalial and/or rectal involvement. Given that epithelial cell cytolysis and keratin clumping are also hallmarks of this disorder, and that K4 and K13 are specifically expressed in the tissues affected in WSN, it was merely a matter of time before researchers found mutations in the K4 and K13 genes of patients with WSN. Finally, it is worth considering that there are more than 30 different keratin genes in the human genome. Many additional disorders, including genetic disorders of hair brittleness, fit the paradigm of a keratin disorder. It is just a matter of time before other keratin diseases are discovered as the list of keratin disorders continues to grow.

9. Using reverse genetics to elucidate the underlying bases of additional genetic disorders

A classical geneticist starts with a particular disease, and works her way down to the protein that is defective. With EBS, we began with keratin and worked our way up to epidermolysis bullosa simplex. We have begun to extend our studies beyond keratins in our use of reverse genetics to solve the underlying bases of other disorders. In some instances, these investigations have led us to important new insights into disorders of the skin.

Recently, we have been particularly interested in how keratin networks form inside cells. In this regard, we have been interested in the hemidesmosome, which connects the base of a basal epidermal cell to laminin 5 anchoring filaments and the basement membrane, and on the inside of the cell, it connects the membrane to keratin filaments [91]. The core of the hemidesmosome is composed of $\alpha 6\beta 4$ integrin [92]. Associated with this integrin are BP180, a transmembrane protein with a collagen-like domain [93], and BP230 and plectin, cytoplasmic proteins that are associated with the inner plate region of the hemidesmosome [91,94].

To understand the functional significance of the various proteins associated with the hemidesmosome, we have used gene knockout technology, a technique whereby both alleles of a particular gene are removed from the mouse genome. When we ablated the $\beta 4$ integrin gene, mice exhibited all of the clinical and morphological features of severe junctional epidermolysis bullosa (JEB) [95]. JEB is often referred to as EB letalis due to the high rate of infant mortality associated with this disorder. It is typified by gross separations between the epidermis and the basement membrane, with aberrant or absent hemidesmosomes. Most cases of JEB are rooted in defects in one of the laminin chains [96–101]. However, recently, heterozygous mutations were found in each of the $\beta 4$ alleles of a patient with JEB associated with pyloric atresia [102]. Our studies provide the first demonstration that a genetic lesion in a protein involved in hemidesmosomal attachment to the basement membrane is functionally responsible for the phenotype seen in JEB.

We also ablated the BPAG1 gene, encoding BP230 [103]. In striking contrast to the $\beta 4$ knockout mice, the BPAG1 null mice had hemidesmosomes which appeared otherwise normal, but they lacked the inner plate and had no keratin filaments attached. While not affecting cell growth or substratum adhesion, this compromised mechanical integrity, leading to a phenotype similar to mild epidermolysis bullosa simplex rather than junctional epidermolysis bullosa. Migration during wound-healing was also affected in these mice. Unexpectedly, the mice also developed severe peripheral nerve degeneration typical of *dystonia musculorum* (*dt/dt*) mice. While the human counterpart is most likely a severe ataxia, and as yet,

we have not found humans that have EBS with neurological disorders, it is intriguing that EBS with muscular dystrophy has recently been described, and that plectin, highly related to the brain form of BPAG1 that causes *dt/dt* [104], is expressed in muscle [105]. When taken together with our BPAG1 knockout studies, our findings point to the notion that EBS with muscular dystrophy may be a plectin disorder. As future studies are conducted, the extent to which this prediction will hold should become clear. However, it is already evident that the use of reverse genetics can be a powerful tool in solving the genetic bases of human skin disorders.

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