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Corneocyte Proteomics: Applications to Skin Biology and Dermatology

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Abstract

Advances in mass spectrometry-based proteomics now permit analysis of complex cellular structures. Application to epidermis and its appendages (nail plate, hair shaft) has revealed a wealth of information about their protein profiles. The results confirm known site-specific differences in levels of certain keratins and add great depth to our knowledge of site specificity of scores of other proteins, thereby connecting anatomy and pathology. An example is the evident overlap in protein profiles of hair shaft and nail plate, helping rationalize their sharing of certain dystrophic syndromes distinct from epidermis. In addition, inter-individual differences in protein level are manifest as would be expected. This approach permits characterization of altered profiles as a result of disease, where the magnitude of perturbation can be quantified and monitored during treatment. Proteomic analysis has also clarified the nature of the isopeptide cross-linked residual insoluble material after vigorous extraction with protein denaturants, nearly intractable to analysis without fragmentation. These structures, including the cross-linked envelope of epidermal corneocytes, are comprised of hundreds of protein constituents, evidence for strengthening the terminal structure complementary to disulfide bonding. Along with other developing technologies,

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Author Contributions

All authors have provided experimental samples, data or data analysis, have been engaged in reviewing and editing the manuscript, and approve the submitted version.

Conflict of interest

Dr. Sundberg has sponsored research projects with Biocon, Bioniz, Curadim, Takeda and Theravance, none of which are related to the work presented here. The other authors state they have no conflicts of interest.

Focus Theme issue “Skin barrier”

proteomic analysis is anticipated to find use in disease risk stratification, detection, diagnosis, and prognosis after the discovery phase and clinical validation.

Keywords

Cross-linked envelopes; Hair shaft; Keratins; Mass spectrometry; Transglutaminase

Introduction

Advances in genomics, proteomics, metabolomics and other technology-driven specialties provide a remarkable amount of detail on the fundamental mechanisms of life and are helping develop connections between anatomy and pathology. Mass spectrometry-enabled proteomics permits comparative profiling, characterization of post-translational modifications, elucidation of protein-protein interactions and networks and development of biomarkers of disease[1], especially in the last case integrated with other omics technologies.[2] For example, the depth of knowledge of the keratin gene superfamily, with distinct groups of keratin proteins found in a variety of epithelia, makes keratins a fundamental marker for all epithelia. We can now interrogate the protein content relatively noninvasively of the epidermis, hair shaft, and nail plate to understand not only their keratin compositions but also the wealth of other proteins located in them and how their structures differ in health and disease.[3] The results summarized below, from straightforward application of protein profiling to epidermal callus, hair shaft and nail plate,[4] provide a foundation for improved analysis of disease of the integument and monitoring of treatment.

Keratinocytes exhibit an intricate maturation program to yield mature corneocytes. These cells and the structures they form have presented a substantial challenge to characterization at the protein level due to the high density of disulfide bonding and the prevalence of transglutaminase-mediated isopeptide cross-linking. Traditional biochemical methods permitted isolation of keratins from corneocytes by solubilization with strong denaturants in the presence of reducing agents. The identities of proteins in the fraction (10–20%) that could not be solubilized remained mysterious until the advent of mass spectrometry-mediated analysis of complex protein mixtures. Reproducible fragmentation through tryptic proteolysis and the ability to match resulting fragments to a peptide database generated *in silico* has resulted in a dramatic advance in our information-gathering ability.

Nevertheless, several technical factors must be kept in mind to exploit this advance. Because keratinocytes, and especially corneocytes, are designed by nature to be tough, cohesive and resistant to their environment, the structures they form often need vigorous treatment with strong denaturants under reducing conditions, and the cysteine residues then are alkylated for stability. With care, the solubilizable protein can be separated from the heavily isopeptide cross-linked (insoluble) fraction if desired.[4] When the tryptic digests are matched in the peptide database to identify protein constituents, the presence of shared peptides needs attention to improve the quantitation. Thus peptides with the same sequence may be found in different proteins, not a rare phenomenon among closely related proteins such as the keratins, and can be distributed by a weighting process.[5] Spectral counting (weighted) is

satisfactory for comparing relative amounts of a given protein among samples, but label free methods are becoming common for comparing amounts of different proteins.[6] Although the work described below has been performed in shotgun or discovery mode, a focus on given proteins for a specific purpose could speed up the analysis and sensitivity. As the sensitivity of mass spectrometry increases, more proteins are anticipated to be detected, increasing the need for scrupulous technique (e.g., sample processing in laminar flow hoods and, where possible, passage of reagent solutions through C18 solid phase extraction columns). Higher sensitivity brings with it more opportunity for detecting contamination, a particular risk for corneocyte samples inasmuch as shed corneocytes from epidermis and hair are a large fraction of dust in the human environment.

Hair shaft

Shotgun proteomics identified a score of keratins in the solubilized fraction of hair shaft protein. It also revealed a previously unappreciated complexity of the isopeptide cross-linked fraction that could not be solubilized with strong denaturants under reducing conditions. Some 300 proteins were identified that mapped to the various compartments of the cell.[7] In addition to comprising the overwhelming majority of protein in the solubilized fraction, keratins also appeared to comprise a majority of the protein in the insoluble fraction as well. Removal of the solubilized keratins, however, assisted detection of the remaining non-keratin proteins in the insoluble fraction.

Application of this technology to inbred mouse strains did not permit establishment of “the” mouse hair shaft proteome. Rather, it revealed that strains differed substantially in their pelage hair proteomes and that single gene alterations could produce considerable alteration in the hair protein profile.[8] The corollary proposition that individuals in an outbred population would differ in hair protein profile was then substantiated for humans.[9] The variation among individuals within ancestral subpopulations appears greater than among subpopulations. A survey of hair samples from 10 human monozygotic twins showed that observed differences in hair protein profile among individuals has a genetic basis.[10] No clear evidence for male/female differences was obtained in this limited sample. However, the VSIG8 protein, recently found to be prominent in hair shaft and nail plate in humans[11] and mice[12] was seen only at very low levels in two of the 5 hair samples from female subjects (Supporting Figure S1). Using this information in a forensic context to distinguish among individuals seems feasible, although detection of genetically variant peptides in the hair shaft appears to offer a greater power of discrimination.[13]

The hair of AKR/J inbred mice provides an example of protein profiling as a useful complement to study of disease by genetic approaches. Superficially resembling human pili annulati, the hair appears softer than that from other inbred albino mouse strains. The hair shafts are similar externally but the inside appears to have bubbles, not unlike cooked human hair (“bubble hair”).[14] This abnormal mouse hair was termed “hair interior defect”.[15] When mapping the responsible mutation, the hair interior defect phenotype behaved like a simple autosomal recessive trait when affected mice were crossed with four unrelated strains, resulting in about 25% affected progeny. The fifth strain, which was the first one used, had only 14.62% affected progeny recovered. Mutations in sterol O-acyltransferase 1

(*Soat1*) result in hair interior defects in AKR/J mice, and linkage analysis indicated that at least 6 modifier genes affect this phenotype.[16] To help address the basis for the peculiar phenotype, proteomic analysis of the hair shafts showed that hair from AKR/J mice, in comparison with two other strains without the defect, was deficient in trichohyalin. This deficiency seemed responsible for the lack of proper orientation of cells in the medulla and thus its unusual appearance.[17]

Alopecia areata is a cell mediated autoimmune disease directed against actively growing anagen stage hair follicles in humans, mice, rats, cattle, horses, and probably even chickens (growing feathers). Disruption of the hair follicle root sheaths results in a deformed hair shaft, commonly referred to as “exclamation point hair”. The defective hair shaft breaks off at or near the skin surface resulting in the clinical presentation of hair loss (alopecia). In fact, if the disruptive effect of the immune system is removed the hair grows back. The C3H/HeJ is a well-accepted mouse model for human alopecia areata.[18] Other inbred strains of mice also develop alopecia areata but usually far less frequently and less severely.[19, 20] Hair samples from AKR/J and 4 other mouse strains were analyzed to find whether a relation to incidence or severity of alopecia areata was discernable. MRL/MPJ and SJL/J are susceptible, and C3H/HeJ are susceptible as they age. Not susceptible are AKR/J and STOCK *a/a Tmem79ma Flg*^{ft}*/J,* a mouse model for atopic dermatitis with mutations in transmembrane protein 79 (*Tmem79*) and filaggrin (*Flg*) genes.[21, 22] Previous work had pointed to cysteine-rich secretory protein 1 (*Crisp1*), coding for a hair structural protein as a candidate gene for alopecia areata.[23] These 5 strains did not differ in their CRISP1 protein levels judging by mass spectrometry, but functionality of the protein was not assessed. Levels of several other proteins differed, notably keratins 1 (KRT1) and 10 (KRT10) (Figure 1). These were reported in human hair cuticle in some[9, 24] but not other reports,[25] while KRT10 has been found in sheep wool cuticle.[26] Figure 1 indicates they are detectable in the hair shaft of some (MAFT, SJL/J) but not other mouse strains.

Nail plate

Proteomic analysis of nail plate showed that, like hair shaft, the overwhelming majority of the proteins solubilized by detergent under reducing conditions were keratins.[11] Also similar to hair shaft, keratins comprised a majority of the insoluble fraction as well, but numerous cytoplasmic, membrane and junctional proteins and histones were identified in addition. As indicated by previous work,[27, 28] the nail plate keratin composition was intermediate between epidermis and hair shaft containing some keratins abundant in epidermis but lacking in hair shaft and vice versa. The overlap of the nail plate and hair proteomes helps rationalize hair and nail dystrophies as seen in mice and humans with defects in the forkhead box N1 (*FOXN1*) gene.[29, 30] Illustrating the effect of programmed maturation on the corneocyte proteome, mice with a specific autophagy deficiency in the nail unit showed minimal effects on the content of keratin, keratin-associated or desmosomal proteins, but substantially higher levels of diverse enzymes and other proteins.[31]

Epidermis

Extension of shotgun proteomics to epidermis led to study of ichthyosis by sampling the stratum corneum with tape stripping. To provide a basis for comparison of afflicted skin at different sites, the dependence of the protein profile on bodily location was surveyed. Dependence on the site of sampling was observed generally, but the most dramatic contrast was between the palm and elsewhere (forearm, forehead, lower leg, upper back, abdomen) in content of keratin 9 and hornerin (HRNR).[32] Keratins, especially KRT9,[33] are well known to show anatomic site specificity.[34] Such differences, evident from transcriptomic studies, could affect susceptibility to infectious disease.[35]

Samples were collected and compared to controls at the same sites from subjects with ichthyosis vulgaris (FLG deficiency), recessive X-linked ichthyosis (steroid sulfatase, STS, deficiency) and lamellar ichthyosis (transglutaminase 1, k polypeptide, TGM1, deficiency). Sampled lesions were distinguishable in appearance and in their protein profiles from each other and from control samples. Effects of mutations in the *STS* and gap junction protein beta 2 (*GJB2*) genes on appearance of ichthyosis vulgaris lesions and their protein profiles were also evident. The degree of departure from normal in the profile was parallel to that in the appearance. Indeed, this parallel was evident as well in normal and ichthyosis human epidermal samples grafted to mouse skin.[36] In this work, individual differences were clear in expression levels of certain proteins. This finding is also evident in a more recent survey of four individuals, where some individuals were distinguishable by protein profiling (Figure 2) in two way comparisons as previously performed with hair samples.[7]

A survey was undertaken analyzing the profiles of callus from the ball of the foot in individuals suffering from pachyonychia congenita. Gain of function mutations in keratins 6A, 6B, 6C, 16 or 17 lead to palmoplantar keratoderma, abnormalities in the nail unit and various other symptoms.[37, 38] Comparisons with samples from unaffected control subjects revealed the protein profiles were most altered in samples from individuals with KRT6 or KRT16 mutations, while those from KRT6C or KRT17 mutations displayed few protein alterations.[39] The degree of departure from normal generally fit the observed severity of the syndrome for the keratin gene categories. Although not clearly contributing to understanding the mechanism of pain generation, a debilitating symptom, the results do provide a quantitative basis for noninvasive monitoring of treatment effectiveness.

A mouse model for severe keratoderma was developed by inhibiting AP1 transcription factor action in the suprabasal epidermis through expression of a dominant negative jun proto-oncogene (*Jun*) construct, producing hyperplasia, hyperkeratosis, parakeratosis and impaired barrier function.[40] Perturbation of the protein profile was found to be severe as well.[41] Analysis of the disulfide and isopeptide cross-linked fraction revealed suppression of epidermal keratins, filaggrin, filaggrin family member 2, late cornifed envelope proteins and keratin-associated proteins but stimulation of hyperproliferation-associated keratins, desmosomal linker, small proline rich and S100 proteins. The results suggest the genetic modification reduced expression of late differentiation genes that was compensated by increased expression of early differentiation genes.

Although giving rise to a mild phenotype, effects of targeting a single gene are illustrated well by studies of a loricrin null mouse.[42] Proteomic analysis showed that keratins comprised ≈55% of the total protein of the cross-linked envelope in the control and null, with keratins 1 and 10 as primary constituents (30–40%).[43] In envelopes from the newborn (4 day) wild type mice, LOR was estimated as 11.5% of the total protein (Table 1). Its absence in the null mice led to altered incorporation of some 40 proteins into cross-linked envelopes, notably reductions of FLG, CSTA and CASP14 and increases in KPRP, IVL, JUP, DSG1 and EPS8L1 (Figure 3). SERPINB3 was reduced, while SERPINB2 was increased. Thus, the mild phenotype could be attributed to compensatory stimulation of alternate envelope proteins, some of which are encoded by NFE2L2 target genes such as *Lce* family members.[44]

An extension of this work compared the profile of human epidermal callus to profiles from buccal cells obtained by cheek swab and from biopsy material from esophageal squamous epithelium. Figure 4 shows the striking variation observed among keratins, junctional and various other proteins. Statistical analysis showed that the majority of proteins were expressed at significantly different levels in comparisons of sample types (Supporting Figure S2). Site specificity of keratin expression is well known,[45] which these findings reflect. Some of the differences are potentially influenced by the different state of the sampled tissues. Unlike terminally differentiated epidermal callus samples, which are subjected to proteolytic remodeling, human esophageal specimens reflect the living interactive lining tissue. The sampled superficial buccal cells were of an intermediate state but closer to epidermal callus in terminal state. Efficiency of detection of proteins could be changed by the action of transglutaminase incorporating them into cross-linked envelopes, but envelopes comprise only ≈10% of the total cellular protein.

Corneocyte cross-linked proteome

Mature keratinocytes of the epidermis, hair shaft and nail plate are remarkably resistant to physical damage. They consist largely of keratin intermediate filaments with abundant disulfide bonding and are bounded by a cross-linked protein envelope. In the epidermis, this envelope serves as a scaffold to which a lipid barrier is attached that prevents transepidermal water loss [46] and where glycerol originating from sebaceous glands maintains hydration. [47] Loss of TGM1 activity localized at the plasma membrane results in defective envelopes and thus deficient barrier function and manifests as a prominent cause of autosomal recessive congenital ichthyosis.[48, 49] Defects in genes affecting lipid processing also give rise to scaly skin.[46]

The mechanism of envelope formation has been studied intensively. In addition to TGM1 activity, the substrate proteins being cross-linked have received considerable attention. An inability to reverse the isopeptide bonding sufficiently to isolate individual protein components made such investigation quite difficult. The advent of mass spectrometric analysis of peptide fragments from complex protein structures, however, has provided considerable insight. Identification of at least 300 proteins from the isopeptide cross-linked fraction of hair shaft,[7] and similar results with nail plate,[11] suggest analogous complexity for epidermal corneocytes.

Exhaustive extraction of hair shaft, nail plate and epidermal callus with SDS under reducing conditions removes the protein held together by disulfide and minimal isopeptide bonding.[4] Analysis of the remainder (10–20% of the total protein), where an estimated 15–20% of the lysines participate in isopeptide bonding in epidermal keratinocytes,[50] revealed that a large fraction, roughly two-thirds, was keratin. Keratin participation in envelopes from epidermal callus had been reported,[51] and the ablation of keratin genes in mice provided clear evidence for their essentiality.[52]

Some proteins can serve as substrates for transglutaminase-mediated cross-linking through available glutamine residues, but most are capable of participating though their lysine residues. Thus, those with abundant glutamines (e.g., involucrin and small proline rich proteins) have been proposed to facilitate the incorporation of other proteins. In addition, protein-protein interactions could influence substrate availability leading up to cross-linking, for example, by increasing their representation in the vicinity of TGM1 at the plasma membrane. Moreover, the large array of proteins that participate suggests that flexibility in envelope composition is possible, particularly in the ability to compensate for missing components. Such considerations provide a rationale for the minimal effects of loricrin gene ablation on mouse epidermis.[42] They also explain the analogous lack of effect of involucrin gene ablation[53] and the mild phenotype observed when it is accompanied by ablation of envoplakin (*Evpl*) and periplakin (*Ppl*) genes as well.[54]

Future prospects

At present, shotgun proteomics offers an overview of protein level changes for the most prevalent several hundred proteins. With respect to the epidermis and appendages, it permits viewing the extent of perturbation, providing a quantitative description of visible changes. Application to related epithelia, including that of esophagus, appears feasible. Now that the anatomic site specificity of protein levels is appreciated, and applications to disease thus far indicate its potential usefulness, extension to analyzing environmental influences appears possible, including atmospheric pollutants[55] and well known therapeutic agents such as coal tar.[56] This direction has been explored in cultured human epidermal keratinocytes treated with the persistent halogenated aromatic contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin. The results provide a rationale for a hyperkeratotic response (*in vivo*) due to reduction in certain differentiation markers while stimulating cross-linked envelope formation.[57] Such experiments may be valuable in finding the influence of modifier genes in culture samples from different individuals.

Use of mouse models can greatly assist elucidating the genetic basis for disease, including genodermatoses, and effects of modifier genes. Clinical subtypes of so-called “single gene based diseases” are now amenable to study. For example, a number of single gene mutations that occur or were created on a variety of inbred mouse backgrounds develop few to no lesions when crossed with the FVB/NJ strain, suggesting that identification of the gene(s) accounting for this phenomenon would be extremely useful biomarkers for disease prognosis if the discovery is transferable to human skin disease. As an indication of the profound effects on proteomic profiles, a single gene mutation on one background may give an outcome quite distinct from a second allelic mutation in the same gene on a different

inbred mouse background. Such effects are magnified for humans (outbred) where each individual is unique. In combination with transcriptomics, metabolomics, lipidomics and other evolving and emerging technologies, including genetically modified organisms (e.g., CRISPR), proteomic approaches can help pinpoint the primary modifier gene(s) and protein(s) involved or elaborate on the molecular networks that direct the variety of clinical outcomes that patients experience. Newer technologies will increase the complexity of our understanding of pathophysiology. However, as profound changes or subtle differential expression variations correlate better with specific disease diagnoses, diagnostic and therapeutic accuracy will improve along with the prognostic abilities of the dermatologist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

***CASP14**
caspase 14

CRISP1
cysteine-rich secretory protein 1

CRISPR
clustered regularly interspaced short palindromic repeats

CSTA
cystatin A1

DSG1
desmoglein 1

EPS8L1
epidermal growth factor receptor kinase substrate 8-like protein 1

FLG
filaggrin

EVPL
envoplakin

FOXN1

*Gene symbols are in italics, capital letters for human genes, first letter capitalized, remainder in lower case for mouse genes. Proteins are all in capital letters, not i

forkhead box N1

GABPA (formerly NRF2)
GA repeat binding protein alpha

GJB2
gap junction protein beta 2

HRNR
hornerin

IVL
involucrin

KPRP
keratinocyte expressed proline-rich

KRT
keratin

JUN
jun proto-oncogene

LCE
late cornified envelope protein

LOR
loricrin

NFE2L2
nuclear factor erythroid derived 2 like 2

PPL
periplakin

SERPINB2/3
serine endopeptidase inhibitor clade B member 2/3

SOAT1
sterol O-acyltransferase 1

STS
steroid sulfatase

SDS
sodium dodecyl sulfate

TGM1
transglutaminase 1

TMEM79

transmembrane protein 79.

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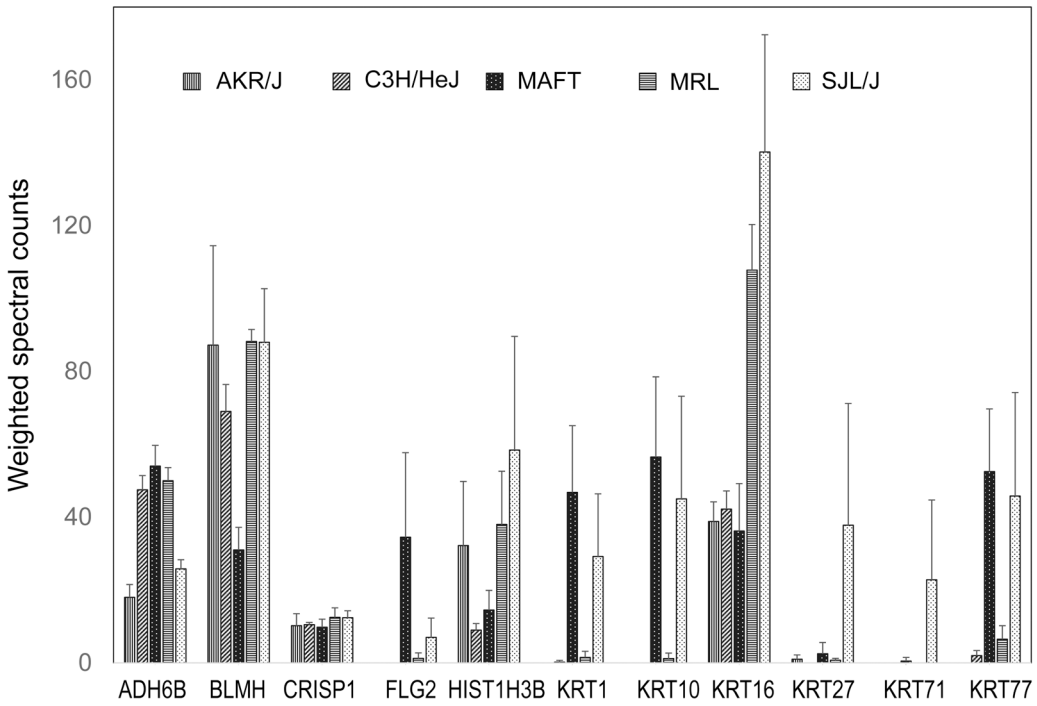

Figure 1. Protein expression levels in 5 inbred mouse strains.

A sample from each of four mice per strain was processed essentially as previously described.[8] Numbers are weighted spectral counts of peptides, averages ± standard deviations. ADH6B, aldehyde dehydrogenase 6b; BLMH, bleomycin hydrolase; CRISP1, cysteine rich secretory protein 1; FLG2, filaggrin 2; HIST1H3B, histone 1H3B; KRT1, keratin 1; KRT10, keratin 10; KRT16, keratin 16; KRT27, keratin 27; KRT71, keratin 71; KRT77, keratin 77.

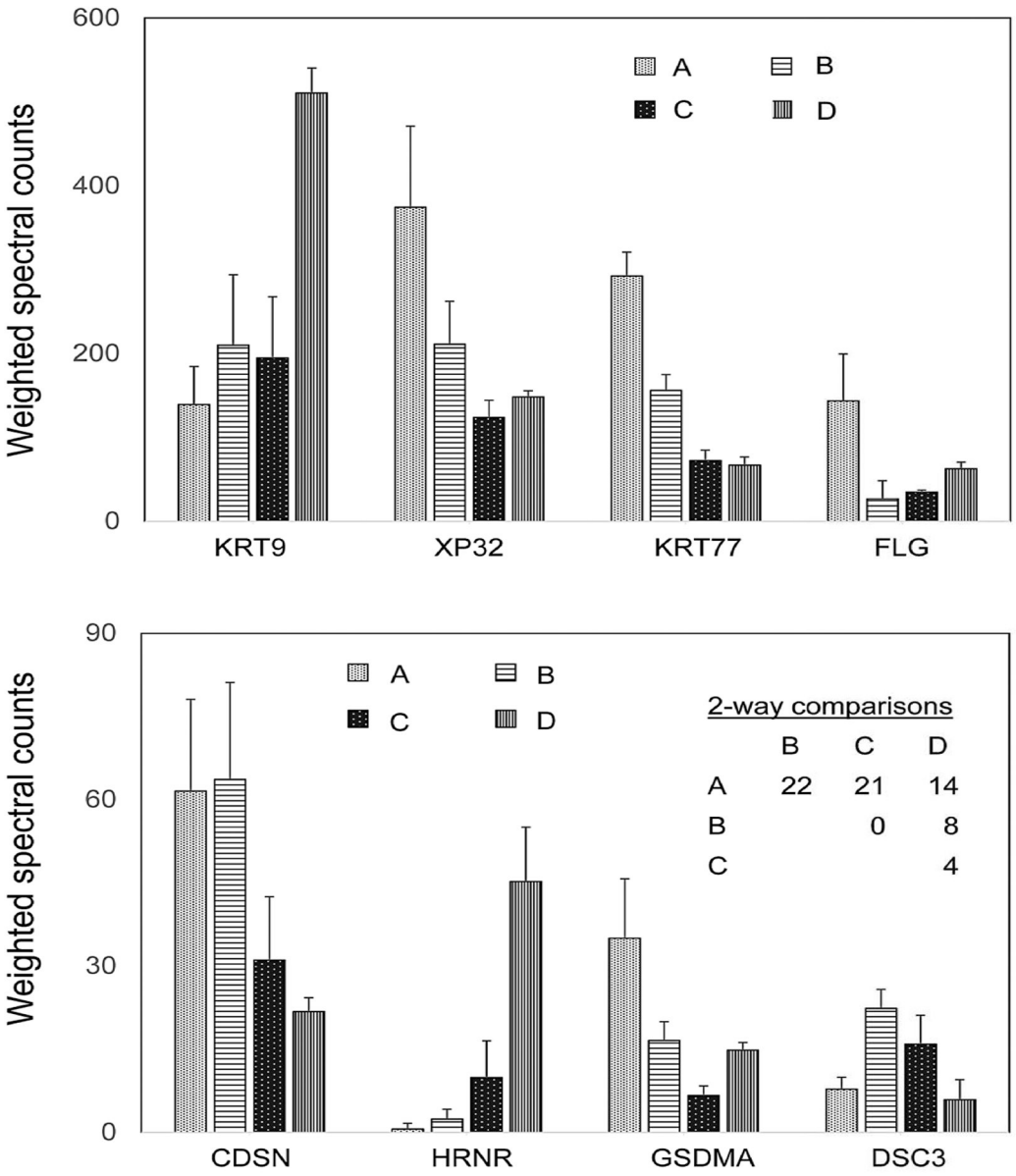

Figure 2. Variation among individuals in expression of human epidermal callus proteins.

The data show the mean and standard deviation of weighted spectral counts from triplicate samples of each subject (A, B, C, D). The table of 2-way comparisons shows the numbers of proteins with significantly different weighted spectral counts. Protein expression was compared between subjects using a one-way ANOVA model in limma. KRT9, keratin 9; XP32, skin specific protein 32; KRT77, keratin 77; FLG, filaggrin; CDSN, corneodesmosin; HRNR, hornerin; GSDMA, gasdermin; DSC3, desmocollin 3.

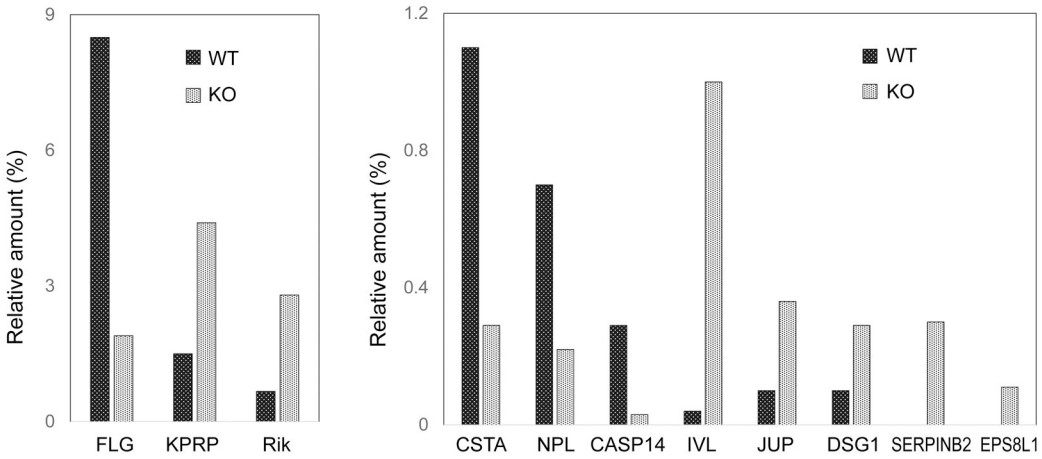

Figure 3. Change in envelope protein profile in loricrin null mice.

The epidermis of mouse skin, collected on day 4 after birth, was obtained by heat separation, extracted 4 times with SDS in the presence of dithioerythritol, processed for mass spectrometry and analyzed as described.[43] Relative protein amounts were estimated by iBAQ calculations. FLG, filaggrin; KPRP, keratinocyte proline rich protein; Rik, 2310050C09Rik; CSTA, cystatin A; NPL, N-acetylneuraminate pyruvate lyase; CASP14, caspase 14; IVL, involucrin; JUP, junctional plakoglobin; DSG1, desmoglein 1; SERPINB2, serine endopeptidase inhibitor family B member 2; EPS8L1, epidermal growth factor pathway substrate 8 like 1.

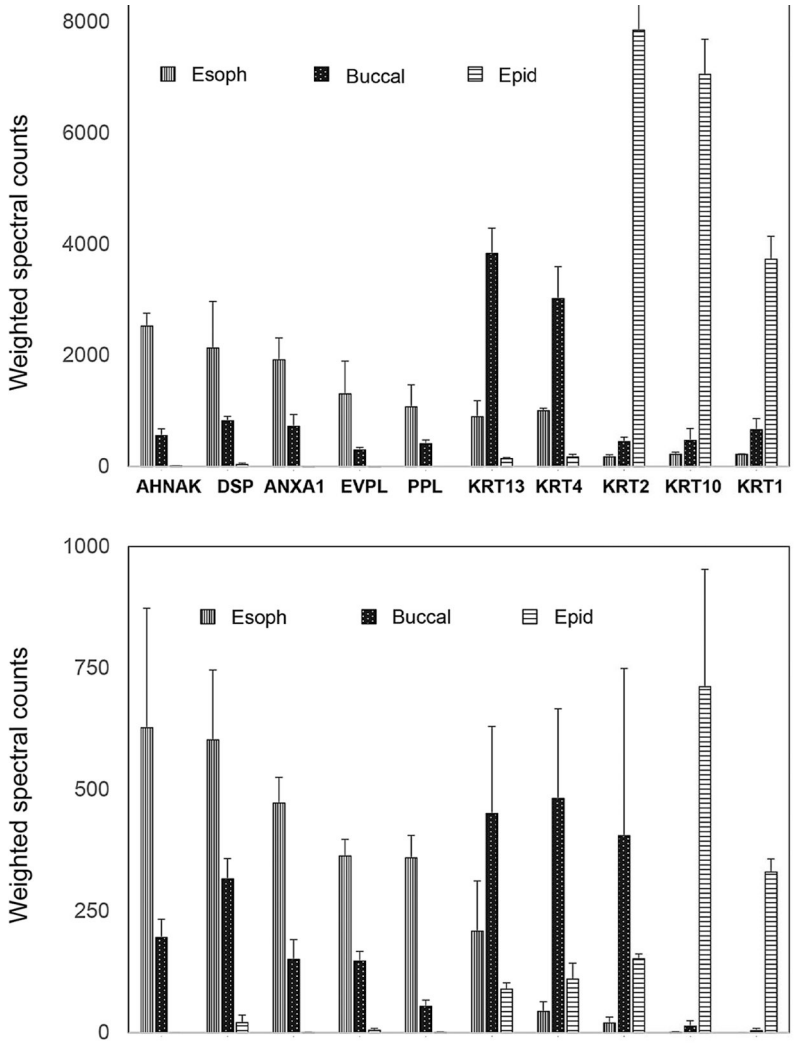

Figure 4. Distinct protein profiles from human epidermal callus, esophageal epithelium and buccal swabs.

Shown are averages ± standard deviations of samples from three (esophageal), four (buccal) and 12 (epidermal) subjects. AHNAK, neuroblast differentiation associated protein; DSP, desmoplakin; ANXA1, annexin A1, EVPL, envoplakin; PPL, periplakin; KRT13, keratin 13; KRT4, keratin 4; KRT2, keratin 2; KRT10, keratin 10; KRT1, keratin 1; SPRR3, small proline rich protein 3; JUP, junctional plakoglobin; S100A11, soluble in 100% ammonium sulfate family A, number 11; PKP1, plakophilin 1; PLEC, pleckstrin; KRT6A, keratin 6A; KRT16, keratin 16; KRT76, keratin 76; KPRP, keratinocyte proline rich protein; FLG2, filaggrin 2.

Table 1.

Protein components of cross-linked envelopes in wild type (WT) and *Lor* knockout (KO) mice. Amounts were estimated by iBAQ calculations. Clusters of proteins (†) in some cases were used due to their shared peptides.

%(WT)	Gene name	%(KO)	Gene name
20.9	Krt10	16.3	Krt1
19.2	Krt1	15.3	Krt10
11.5	Lor	5.5	Krt71
8.5	Flg	4.4	Kprp
3.2	Flg2	4.1	Hrnr
3.0	Krt28	3.8	Flg2
2.8	Hrnr	3.2	Krt27
2.2†	Krt13,14,15,16,17	2.8	2310050C09Rik
2.1	Krt27	2.8	Sprr1a
2.0	Krt25	2.8†	Krt2,5,6a,6b,8,73,75,76,77,79