An In Vitro Model of Avian Skin Reveals Evolutionarily Conserved Transcriptional Regulation of Epidermal Barrier Formation

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The function of the skin as a barrier against a dry environment evolved in a common ancestor of terrestrial vertebrates such as mammals and birds. However, it is unknown which elements of the genetic program of skin barrier formation are evolutionarily ancient and conserved. In this study, we determined the transcriptomes of chicken keratinocytes (KCs) grown in monolayer culture and in an organotypic model of avian skin. The differentiation-associated changes in global gene expression were compared with previously published transcriptome changes of human KCs cultured under equivalent conditions. We found that specific keratins and genes of the epidermal differentiation complex were upregulated during the differentiation of both chicken and human KCs. Likewise, the transcriptional upregulation of genes that control the synthesis and transport of lipids, anti-inflammatory cytokines of the IL-1 family, protease inhibitors, and other regulators of tissue homeostasis was conserved in the KCs of both species. However, some avian KC differentiation-associated transcripts lack homologs in mammals and vice versa, indicating a genetic basis for taxon-specific skin features. The results of this study reveal an evolutionarily ancient program in which dynamic gene transcription controls the metabolism and transport of lipids as well as other core processes during terrestrial skin barrier formation.

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INTRODUCTION

Adaptations of the epidermis played key roles in the evolution of vertebrates that colonized the land (Alibardi, 2003; Chuong et al., 2002; Matsui and Amagai, 2015). In particular, the evolution of an efficient protection against cutaneous water loss was a crucial event in the transition of amniotes to a fully terrestrial lifestyle (Alibardi, 2003). The main cell type present in the epidermis, the keratinocyte (KC), forms dynamically renewing but mechanically stable cell layers. KCS proliferate in the innermost (basal) layer of the epidermis and differentiate in the suprabasal layers. The outermost compartment of the epidermis, that is, the stratum corneum, consists of cornified KCS (corneocytes) and extracellular lipids. Corneocytes are the end product of terminal differentiation, which culminates in the loss of organelles and the covalent cross-linking of structural proteins through transglutamination, commonly referred to as cornification (Alibardi, 2006; Candi et al., 2005; Eckhart et al., 2013). The most abundant proteins in corneocytes are keratins and proteins encoded by genes of the epidermal differentiation complex (EDC) (Henry et al., 2012; Mischke et al., 1996). The latter include loricrin in all amniotes; involucrin, small proline-rich proteins and S100A proteins in mammals; and CBPs, also known as beta-keratins, and many as yet uncharacterized EDC proteins in reptiles and birds (Kalinin et al., 2002; Rice and Green, 1979, 1977; Robinson et al., 1997; Strasser et al., 2014; Steinert and Marekov, 1995). Keratins are present in the epidermis of all vertebrates, whereas EDC proteins have evolved in amniotes (Mlitz et al., 2014; Strasser et al., 2014; Vanhoutteghem et al., 2008). A further critical step of skin barrier formation is the production of ceramides and other lipids in KCS (Feingold and Elias, 2014). Glycosylceramides, also known as cerebrosides, accumulate in vesicles that fuse with the cell membrane to release the lipid cargo into the intercellular space before cornification. The lipids block the passage of water out of the skin and thereby prevent transepidermal water loss. Although the mechanisms of skin barrier formation have not been characterized in detail in each group of vertebrates, a general path of skin barrier evolution can be proposed on the basis of currently available data (Figure 1).

The differentiation process of mammalian epidermal KCS can be replicated in vitro by culturing KCS at an air-liquid interface (Oh et al., 2013). Conventional culture of KCS in the form of a cell monolayer facilitates proliferation but does not allow full differentiation of KCS. However, when KCS are transferred onto an appropriate matrix and exposed to the air, they form a stratified epidermis and activate the expression of terminal differentiation-associated genes such as they do in vivo (Arnette et al., 2016; Kobayashi et al., 2013; Oh et al., 2013; Shamir and Ewald, 2014). Three-dimensional (3D) skin
models have been generated with KCs from various mammalian species, such as human, dog, and horse (Cerrato et al., 2014; Sharma et al., 2016), but have not yet been reported for avian skin.

In this study, we established an organotypic model of chicken skin that is equivalent to human 3D skin models and determined the gene expression of chicken KCs before and after terminal differentiation in this model. The differentiation-associated transcriptome of chicken KCs was compared with that of human KCs to identify the evolutionarily ancient core program of dynamic gene expression that controls terrestrial skin barrier formation.

RESULTS

Establishment of an organotypic skin model facilitates the transcriptomic analysis of chicken KC differentiation

To facilitate the investigation of nonmammalian skin barrier formation under controlled conditions, we established a 3D model of chicken skin in vitro (Figure 2a). Primary KCs and fibroblasts were isolated from the skin of chicks aged 1 day and maintained in conventional monolayer culture that allowed cell proliferation but lacked stimuli for cell differentiation. KC differentiation was induced by seeding KCs on top of a collagen matrix seeded with fibroblasts and by lifting the culture to the air–liquid interface. Under these conditions, KCs formed a stratified epidermis in which an eosinophilic cornified layer equivalent to that of normal skin could be demonstrated by H&E staining (Figure 2b and c). Moreover, the chicken 3D skin model showed an organization similar to that of an in vitro model of human skin (Supplementary Figure S1) (Mildner et al., 2010; Rendl et al., 2002). Importantly, reconstructed chicken skin established a functional barrier that stopped the diffusion of the marker biotin at the border of the viable epidermis to the stratum corneum (Figure 2d and e).

To identify the alterations of gene expression that are associated with differentiation of chicken KCs and skin barrier formation, we compared the transcriptomes of KCs in conventional undifferentiated monolayer cell culture with those of KCs in stratified barrier-proficient 3D skin models. RNA from KC monolayer cultures (n = 3, cells prepared from different animals) and that from the epidermal compartments of organotypic skin models (n = 3, cells prepared from different animals) was subjected to RNA sequencing on an Illumina NextSeq500 platform (Illumina, San Diego, CA).

The RNA sequence reads were aligned to the chicken genome sequence assembly Galgal5.0 with annotations of genes according to the Ensembl database and additional gene annotations in the EDC (Strasser et al., 2014) (Supplementary Table S1), the CBP gene cluster within the EDC (Holthaus et al., 2018) (Supplementary Table S2), keratin gene clusters (Ehrlich et al., 2020) (Supplementary Table S3), and other gene loci associated with KC differentiation (Supplementary Table S4) whereby double annotations of genes were avoided (Supplementary Table S5). The expression levels of all genes were compared with appropriate corrections for multiple testing between KCs in monolayer and organotypic cultures.

RNAs corresponding to 19,227 genes were detected in chicken KCs cultured in monolayer or 3D skin models (Supplementary Table S6). In skin models relative to monolayer cultures, the abundance of 889 transcripts was significantly increased (log2 fold change > 2, adjusted P < 0.001), and 1,454 transcripts were significantly decreased in abundance (log2 fold change < −2, adjusted P < 0.001) (Figure 3a). RT-PCR analysis confirmed differential expression of a selected set of genes in monolayer and organotypic skin cultures (Supplementary Figure S2). Among the top 50 upregulated genes in the 3D skin models, there were 29 genes located in the EDC and five keratins homologous to differentiation-associated human keratins (Ehrlich et al., 2020) (Supplementary Figure S3). Gene ontology term enrichment analysis of the transcriptomic changes indicated the upregulation of lipid metabolic processes (Figure 3b). These data showed that the switch from monolayer to an organotypic culture of chicken KCs induced genome-wide changes in gene expression and upregulation of genes located in classical KC differentiation-associated gene clusters.

Specific keratins and EDC genes are upregulated in an organotypic chicken skin culture

EDC and keratin gene clusters are present in humans, chicken, and all other amniotes (Ehrlich et al., 2020; Henry et al., 2014; Sharma et al., 2016). The chicken 3D skin model showed an organization similar to that of an in vitro model of human skin (Supplementary Figure S1) (Mildner et al., 2010; Rendl et al., 2002). Importantly, reconstructed chicken skin established a functional barrier that stopped the diffusion of the marker biotin at the border of the viable epidermis to the stratum corneum (Figure 2d and e).
et al., 2012; Strasser et al., 2014), but it is not known which of the individual EDC and keratin genes are expressed during skin barrier formation in nonmammalian epidermal KCs. We therefore utilized the RNA-sequencing data of differentiating chicken KCs to determine the skin barrier–associated regulation of gene expression across the EDC and keratin gene loci of the chicken.

In the EDC, >50% of all genes were significantly upregulated in 3D skin model culture (Figure 4a). These included LOR1-3 and EDQrep, which are homologous to the mammalian epidermal differentiation markers loricrin and involucrin, respectively (Davis et al., 2019; Strasser et al., 2014; Vanhousetteghem et al., 2008). By contrast, genes previously reported to be expressed specifically in feathers, such as EDCRP, EDMTFH, EDDM, and TCHHUScaffoldin (Alibardi et al., 2016; Lachner et al., 2019; Mlitz et al., 2014; Strasser et al., 2015), were not transcriptionally upregulated (Figure 4a). Likewise, none of the feather CBP genes was induced by epidermal stratification in vitro, whereas the so-called KC CBPs were increased in their mRNA abundance during barrier formation in the 3D model of chicken skin (Supplementary Figure S4).

Keratins are classified as type-I and type-II proteins that are encoded by genes located in separate clusters (Figure 4b and c). In monolayer cultures of chicken KCs, type-I (KRT14L1, KRT14L2, KRT15, and KRT19) and type-II (KRT5L2, KRT5L3, KRT7) keratins homologous to mammalian keratins associated with stem cells and epithelial proliferation were expressed (Figure 4b and c). mRNAs of these genes remained to be expressed or declined in abundance after the switch to 3D skin model culture of KCs (Figure 4b and c), reflecting the presence of undifferentiated KCs in the basal layer of the epidermis. By contrast, KRT9-like genes homologous to type-I keratin of the suprabasal epidermis of human palmoplantar skin and KRT78-like type-II keratin genes were not expressed in monolayer cultures and upregulated in 3D model cultures of chicken KCs (Figure 4b and c). Other keratins, including the feather keratin KRT78L1C1, also known as HBST1 keratin (Ehrlich et al., 2020), were expressed at low levels in both culture states of chicken epidermal KCs.

The KC differentiation program of chicken KCs was compared with that of human KCs (Affymetrix GeneChip analysis, GenBank accession number GSE145059) cultured under equivalent conditions as those described earlier for chicken KCs (Beer et al., 2020). As in chicken models, there was a broad upregulation of gene expression in the EDC and selective upregulation of a subset of keratin genes in human skin models (Supplementary Figure S5). In line with the concept of diversifying evolution of EDC and keratin genes in amniotes (Ehrlich et al., 2020; Holthaus et al., 2016; Strasser et al., 2014), only these general patterns of regulation of the gene clusters but not the regulation of individual EDC and keratin genes were conserved in both species.

In vitro differentiation of chicken KCs allows the identification of genes with evolutionarily conserved transcriptional skin barrier formation or homeostasis

Next, we utilized the transcriptomes of organotypic chicken skin to identify the skin barrier genes that in contrast to EDC and keratin genes (Ehrlich et al., 2020; Strasser et al., 2014) are dispersed across the genome and only share the feature of transcriptional upregulation during KC differentiation. We
identified differentiation-associated genes with diverse functions ranging from antimicrobial defense (e.g., *AvBD14/GAL14*) (Harder et al., 1997) to the control of proteolysis (e.g., *LOC101749502* encoding a SPINK5-like serine protease inhibitor) and WNT signaling (e.g., *WNT5A, APCDD1*) (Shimomura et al., 2010) (Supplementary Table S7). Many of the genes highly expressed during differentiation of chicken KCs have homologs that are transcriptionally upregulated during differentiation of human KCs (Supplementary Table S7) (Mattiuzzo et al., 2011; Toulza et al., 2007).

Figure 3. Transcriptome analysis of differentiated chicken keratinocytes. (a) Volcano plot of chicken keratinocyte differentiation. RNA-seq analysis was performed on chicken keratinocytes from a monolayer (n = 3) and 3D skin model cultures (n = 3). Significance (–log_{10} of adjusted P-value) is plotted against FC. Red dots indicate genes with –log_{10}P > 3 and log_{2}FC > 2 or log_{2}FC < 2. The font colors indicate the different types of genes (magenta for EDC, blue for keratins; orange for lipid metabolism and transport; and black for others). (b) Gene ontology term analysis. 3D, three-dimensional; EDC, epidermal differentiation complex; FC, fold change; GO, gene ontology; RNA-seq, RNA sequencing.
Examples include transcription factors such as EGR3 (Kim et al., 2019); the keratin-interacting protein EPPK1 (Spazierer et al., 2003); anti-inflammatory cytokines of the IL-1 family, namely IL-1RN and IL-36RN (Gibson et al., 2012a, 2012b; Lachner et al., 2017) (Supplementary Figure S6); and genes, such as poly-U-specific endonuclease (ENDOU) (Edqvist et al., 2015), which have not been functionally characterized in the skin yet.

The most outstanding group of differentiation-associated genes encodes proteins that control the metabolism and transport of lipids (Supplementary Table S7 and Figures 3 and 5). In differentiated KCs of mammals, acyl-ceramides are synthesized, glycosylated, and secreted to seal the intercellular space at the skin surface (Feingold and Elias, 2014). We synthesized, glycosylated, and secreted to seal the intercellular space at the skin surface (Feingold and Elias, 2014). We found that genes regulating key steps of ceramide synthesis are transcriptionally upregulated in differentiating KCs of both chicken and human (Figure 5a). An evolutionarily conserved transcriptional regulation was evident for CYP4F genes, ELOVL4, DGA2, SPTSSB, DEGS1, ABHD5, and PNPLA1 (Figure 5a). Furthermore, lipid-binding and -transporting proteins such as ABCA12, Fabp5, NIPAL4, and GLTP were transcriptionally upregulated during KC differentiation (Figure 5b). ABCA12, Fabp5, and NIPAL4 are important for mammalian skin barrier function and mutations in ABCA12 and NIPAL4 lead to ichthyoses (Kelsell et al., 2005; Mauldin et al., 2018; Wajid et al., 2010), but GLTP has not yet been characterized in mammals. RT-PCR and immunoblot analysis showed an induction of GLTP expression in differentiated human KCs (Figure 5c and d), and immunohistochemistry showed an increase of GLTP abundance as KCs move toward the skin surface in vivo (Figure 5e and f). Together, these data suggested an evolutionarily conserved upregulation of genes that establish lipid-dependent barrier functions of mammalian and avian skin.

**DISCUSSION**

The results of this study suggest that the transcriptional upregulation of a diverse set of genes is evolutionarily conserved and associated with epidermal barrier formation in human and chicken. Genes that control mechanical resilience of epithelia (e.g., DSC1, EPPK-like 1), proteases and protease inhibitors (CTSK, CSTA), antimicrobial proteins (AvBD14/gallinacin-14), anti-inflammatory cytokines of the IL-1 family (IL36RN), transcription factors (EGR3), and multiple lipid metabolism–associated genes (see in the following paragraphs) (Supplementary Table S7) were expressed at high levels in stratified epidermal models but not or only at low levels in nondifferentiated KCs in monolayer cultures. We
Figure 5. Differentiation of chicken keratinocytes is associated with the transcriptional upregulation of genes that control the synthesis and transport of skin barrier lipids. (a) Schematic diagram of ceramide synthesis and transcriptional regulation of enzymes (orange fonts). The Log₂ FCs of 3D skin model versus that of monolayer (mono) cultures of chicken (accession number GSE162385) and human (accession number GSE145059) keratinocytes under equivalent conditions are shown on the right. Asterisks mark statistical significance (chicken keratinocytes: adjusted \( P < 0.001 \), human keratinocytes: \( P < 0.05 \), t-test). (b) Lipid-binding proteins that are transcriptionally upregulated during keratinocyte differentiation in 3D models of chicken and human skin. (c) QRT-PCR analysis of GLTP expression in monolayer and 3D skin model cultures of human keratinocytes. Bars and error bars indicate means (\( n = 3 \)) and SD. *\( P < 0.05 \) (t-test). (d) Western blot analysis of GLTP and GAPDH (housekeeping gene) in monolayer and 3D skin model cultures of human keratinocytes. Molecular mass markers are shown on the right. Bands corresponding to the predicted molecular mass of two GLTP splice variants (23.8 and 19.4 kD) are indicated by arrows. (e, f) Immunohistochemical analysis of GLTP in human skin. Skin sections were immunostained (red) for GLTP in f. In neg. con., experiments, the rabbit anti-GLTP antibody was replaced by a nonspecific rabbit immunoglobulin. Bars = 20 \( \mu \)m. 3D, three-dimensional; der, dermis; epi, epidermis; FC, fold change; neg. con., negative control; QRT-PCR, quantitative real-time reverse transcriptase–PCR; sc, stratum corneum; ULC-FA, ultra-long-chain fatty acid.

Conclude that the association between skin barrier formation and upregulation of most or all of these genes has been inherited from the last common ancestor of chickens and humans, that is, a stem amniote that lived \( > 300 \) million years ago (Kumar et al., 2017).

The dissection of the avian KC differentiation process was based on the development of monolayer and 3D cultures of chicken skin cells. The in vitro model of chicken skin replicates important features of the epithelium such as expression of keratins and cornification proteins such as loricrin, a barrier against intercellular diffusion of the small molecules, and the presence of a protective layer of cornified dead cells on the surface. Therefore, the 3D model of chicken skin is a major advance compared with conventional KC cultures in which only incomplete differentiation can be induced (Vanhouteghem et al., 2004). Morphological variation between experiments and the thickening of the epidermal compartment in 3D skin models relative to that in the skin may be reduced by further optimization of the culture conditions. Transcriptome analysis confirmed the expression of epidermal differentiation genes, including those located in the EDC (Strasser et al., 2014) and type-I keratin and type-II keratin gene clusters (Ehrlich et al., 2020), whereas the expression of feather-associated genes, such as \( KRT78LC1 \) \( \text{HBS1, EDCRP, and EDMTH4 (Alibardi et al., 2016; Ehrlich et al., 2020; Strasser et al., 2015)} \) were not induced in the skin model. Comparative analysis of the chicken skin model transcriptome and published chicken skin transctromes (Zhang et al., 2015) confirmed that genes associated with the de novo formation of an epidermal barrier in vitro are also expressed under conditions of cutaneous homeostasis in vivo (Supplementary Table S8). Moreover, an exploratory...
transcriptomic study of chicken skin at the single-cell level suggested that mRNAs, such as KRT9L4, LOR1, SPTSSB, ELOVL4, and EPPKL1, which are induced during differentiation in 3D skin models, are also enriched in differentiated KCs within homeostatic chicken skin (accession number GSE169274, Supplementary Table S9 and Supplementary Figure S7). Thus, chicken skin models are valid experimental systems for the identification of KC differentiation genes. Nevertheless, additional investigations of freshly isolated KCs from the various epidermal layers are necessary to comprehensively define the transcriptional control of the avian epidermis during long-term maintenance and renewal.

In line with the notion that the barrier function of the skin depends not only on proteins but also on lipids, a large group of genes controlling lipid metabolism and transport, such as ELOVL4, CERS3, SPTSSB, ABCA12, and GLTP, was strongly upregulated after induction of skin barrier formation by chicken KCs in vitro. An essential role of lipids in the avian skin barrier was proposed in previous studies (Elias et al., 1987; Menon et al., 1986), but the genetic regulation of their synthesis has remained elusive. Homologs of most of the genes related to the synthesis and transport of lipids in differentiated chicken KCs are also upregulated during differentiation of human KCs, suggesting that not only their coding sequences but also the differentiation-dependent expression has been inherited from the last common ancestor of chicken and humans. Notably, CERS3 and ELOVL4 are predominantly expressed in the skin, whereas other genes, such SPTSSB and DGAT2, are broadly expressed in human tissues. Our identification of GLTP as an evolutionarily ancient protein of the human skin barrier will be the basis for further studies of its functions in normal and diseased human skin.

It is important to note that our study, besides identifying conserved epidermal differentiation genes, also suggests molecular skin barrier features that are specific for either birds or mammals. For example, CBPs, also known as beta-keratins (Holthaus et al., 2018), are expressed in chicken skin models, whereas CBPs do not exist in humans. Conversely, kallikreins KLK5, 6, 7, 10, 11, and 13 are upregulated in cornifying human KCs, whereas the only chicken kallikrein homolog of the mammalian epidermal kallikreins, that is, KLK7 (Lundwall, 2013), was not expressed in chicken skin models. These and other differences between the transcriptomes of chicken and human KCs indicate that some aspects of skin barrier formation evolved divergently after the phylogenetic separation of the major lineages of terrestrial vertebrates.

In conclusion, this study extends the characterization of KC differentiation to nonmammalian vertebrates and defines the core components of the molecular machinery that protects terrestrial vertebrates against a dry environment during life on land.

MATERIALS AND METHODS

Isolation and culturing of chicken skin cells
A detailed protocol for the isolation and culturing of chicken KCs and fibroblasts is provided in the Supplementary Materials and Methods. In brief, the skin from chicks aged 1 day was digested with thermolysin to separate epidermis from the dermis. The epidermis was incubated in 0.05% trypsin/EDTA to dissociate KCs that were filtered and subsequently seeded in culture flasks precoated with collagen. The dermis was incubated with collagenase and hyaluronidase to isolate fibroblasts. KCs and fibroblasts were cultured in DMEM/Ham’s F-12 and DMEM, respectively, with supplements as described in Supplementary Materials and Methods.

Preparation of chicken 3D skin models
Chicken fibroblasts were detached by trypsin treatment from a confluent monolayer culture, suspended at a density of 1 × 10⁶ cells per milliliter in Hank’s Balanced Salt Solution (Life Technologies, Carlsbad, CA) (pH 7.5) containing 2.4 mg/ml bovine collagen (PureCol, Advanced BioMatrix, San Diego, CA) and 10% chelated chicken serum (Life Technologies). The suspension was incubated in cell culture inserts (Falcon) in six-well plates without elevation of ambient carbon dioxide at 37 °C for 2 hours, allowing the mixture to form a gel. For further 2 hours, the fibroblast–collagen gel was equilibrated with KC growth medium 2 (PromoCell, Heidelberg, Germany) supplemented with 0.004 ml/ml bovine pituitary extract, 0.125 ng/ml epidermal GF, 5 μg/ml insulin, 0.33 μg/ml hydrocortisone, 0.39 μg/ml epinephrine, 10 μg/ml transferrin, and 0.06 mM calcium chloride according to the manufacturer’s instructions. Chicken KCs from an 80% confluent monolayer culture with a maximum of two previously splitting events were suspended in KC growth medium 2 and seeded onto two inserts with fibroblast–collagen matrix. On the following day, the medium was changed to supplemented KC-defined medium, corresponding to KC growth medium 2 supplemented with 0.125 ng/ml epidermal GF, 5 μg/ml insulin, 0.33 μg/ml hydrocortisone, 10 μg/ml transferrin, and 0.1% BSA (PAA Laboratories, Pasching, Austria); 21.3 mM calcium chloride (Merck, Darmstadt, Germany); and 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), and the culture was moved to the air–liquid interface to initiate KC differentiation (Mildner et al., 2010). The 3D skin models were harvested after 7 days. For histological analysis, the tissue was fixed in 3.7% formaldehyde. For the analysis of gene expression in KCs, the epidermal compartment was peeled off and subjected to RNA extraction.

RNA sequencing and data analysis
RNA was isolated and purified with TriFast (VWR International, Radnor, PA) according to a published protocol (Lachner et al., 2017). Sequencing libraries were prepared using the NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA). Libraries were quality control checked on a Bioanalyzer 2100 (Agilent, Santa Clara, CA) using a High Sensitivity DNA Kit for correcting insert size and quantified using Qubit dsDNA HS Assay (Invitrogen, Waltham, MA). Pooled libraries were sequenced on a NextSeq500 instrument (Illumina) in 2 × 75 base pairs paired-end sequencing mode. Approximately 40 million read pairs per sample were generated. Reads in fastq format were aligned to the chicken reference genome, version gga5 (ggal5 chicken reference genome downloaded from Ensembl on April 11, 2019: ftp://ftp.ensembl.org/pub/release-94/fasta/gallus_gallus/dna/Gallus_gallus.Gallus_gallus-5.0.dna_sm.toplevel.fa.gz) with Ensembl 91 annotations (chicken genome annotations downloaded from ensemble on April 11, 2019: ftp://ftp.ensembl.org/pub/release-91/gtf/gallus_gallus/Gallus_gallus.Gallus_gallus5.0.91.gtf.gz) using STAR aligner (Dobin et al., 2013), version 2.6.1a, in two-pass mode. Reads per gene were counted by STAR, and differential gene expression was
calculated using DESeq2 (Love et al., 2014), version 1.20.0 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html). Results are available at the National Center for Biotechnology Information Gene Expression Omnibus (Edgar et al., 2002) under accession number GSE162385. Gene ontology analysis and comparisons with transcriptome data from total chicken skin and from single cells isolated from chicken skin were performed as described in the Supplementary Materials and Methods.

Analysis of specific mRNAs and proteins
Quantitative real-time reverse transcriptase–PCRs and western blots were performed according to published protocols (Lachner et al., 2017). For details, see Supplementary Materials and Methods.

Histology and immunohistochemistry
H&E staining and immunohistochemistry were performed according to published protocols with modifications (Mlitz et al., 2014). For details, see Supplementary Materials and Methods.

Analysis of skin barrier permeability
A barrier diffusion assay was performed according to a published protocol (Gschwandtner et al., 2013). For details, see Supplementary Materials and Methods.

Ethics
No experiments on live animals were performed. The ethics committee of the Medical University of Vienna decided that in agreement with the national laws, a permission for killing animals for organ preparation was not required. The investigation of human KCs and skin biopsies was approved by the ethics committee of the Medical University of Vienna (approval number 2011/273) and was performed according to the Declaration of Helsinki after patients had given their written informed consent.

Data availability statement
The data underlying this article are available in National Center for Biotechnology Information’s Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/ and can be accessed with Gene Expression Omnibus Series accession numbers GSE162385 and GSE169274.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: JL; LE; Data Curation: JL; SD; Formal Analysis: JL; SD; Funding Acquisition: LE; Investigation: JL, VM, TW, KBH, FE, MM; Methodology: JL, SD, MM; Project Administration: LE; Resources: MM, ET; Software: JL, DD; Supervision: LE, Validation: JL, SD, KBH, FE; Visualization: JL, LE; Writing - Original Draft Preparation: JL, LE; Writing - Review and Editing: JL, SD, VM, TW, KBH, FE, MM, ET, LE

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.04.029.

REFERENCES
Aliabardi L. Adaptation to the land: the skin of reptiles in comparison to that of amphibians and endotherm amniotes. J Exp Zool B Mol Dev Evol 2003;298:12–41.
Aliabardi L. Structural and immunocytochemical characterization of keratinization in vertebrate epidermis and epidermal derivatives. Int Rev Cytol 2006;253:177–259.
Aliabardi L, Holthaus KB, Sukseere S, Hermann M, Tschachler E, Eckhart L. Immunolocalization of a histidine-rich epidermal differentiation protein in the chicken supports the hypothesis of an evolutionary developmental link between the embryonic subperiderm and feather barbs and barbules. pLoS One 2016;11:e0167789.
Arnette C, Koetsier JL, Hoover P, Getisio S, Green KL. In vitro model of the epidermis: connecting protein function to 3D structure. Methods Enzymol 2016;569:287–308.
Beer L, Kalinina P, Köcher M, Laggner M, Jeitler M, Abbas Zadeh S, et al. miR-155 contributes to normal keratinocyte differentiation and is upregulated in the epidermis of psoriatic skin lesions. Int J Mol Sci 2020;21:9288.
Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 2005;6:328–40.
Cerrato S, Ramió-Lluch L, Brazis P, Rabanal RM, Fondevilla D, Puigdemont A. Development and characterization of an equine skin-equivalent model. Vet Dermatol 2015;24:475–e77.
Chuong CM, Nickoloff BJ, Elias PM, Goldsmith LA, Macher E, MADERSON PA, et al. What is the ‘true’ function of skin? Exp Dermatol 2002;11:159–67.
Davis AC, Greenwold MJ, Sawyer RH. Complex gene loss and duplication events have facilitated the evolution of multiple loricrin genes in diverse bird species. Genome Biol Evol 2019;11:984–1001.
Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
Eckhart L, Lippens S, Tschachler E, Declercq W. Cell death by cornification. Biochim Biophys Acta 2014;1841:280–308.
Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30:207–10.
Edqvist PH, Fagerberg L, Hallström BM, Danielsson A, Edlund K, Uhlen M, et al. Expression of human skin-specific genes defined by transcriptomics and antibody-based profiling. J Histochem Cytochem 2015;63:129–41.
Ehrlich F, Lachner J, Hermann M, Tschachler E, Eckhart L. Convergent evolution of cysteine-rich keratins in hard skin appendages of terrestrial vertebrates. Mol Biol Evol 2020;37:982–93.
Elías PM, Menon GK, Grayson S, Brown BE, Rehfeld SJ. Avian sebokeratocytosis: a model of human skin barrier disease. Vet Dermatol 2006;17:77–87.
Feingold KR, Elías PM. Role of lipids in the formation and maintenance of the cutaneous permeability barrier. Biochim Biophys Acta 2014;1841:280–94.
Gibson MS, Fife M, Bird S, Salmon N, Kaiser P. Identification, cloning, and functional characterization of the II-1 receptor antagonist in the chicken reveal important differences between the chicken and mammals. J Immunol 2012a;188:539–50.
Gibson MS, Salmon N, Bird S, Kaiser P, Fife M. Identification, cloning and characterisation of interleukin-1F5 (IL-36RN) in the chicken. Dev Comp Immunol 2011b;35:136–47.
Gschwandtner M, Mildner M, Mlitz V, Gruber F, Eckhart L, Werfel T et al. Histamine suppresses epidermal keratinocyte differentiation and impairs skin barrier function in a human skin model. Allergy 2013;68:37–47.
Harder J, Bartels J, Christophers E, Schröder JM. A peptide antibiotic from human skin. Nature 1997;387:861.
Henry J, Toulza E, Hsu CY, Pellerin L, Balica S, Mazereeuw-Hautier J et al. Update on the epidermal differentiation complex. Front Biosci (Landmark Ed) 2012;17:1517–32.
Holthaus KB, Eckhart L, Dalla Valle L, Alibardi L. Review: evolution and diversification of corneous beta-proteins, the characteristic epidermal proteins of reptiles and birds. J Exp Zool B Mol Dev Evol 2018;330:438–53.

Holthaus KB, Strasser B, Sipos W, Schmidt HA, Mlitz V, Sukereee S, et al. Comparative genomics identifies epidermal proteins associated with the evolution of the turtle shell. Mol Biol Evol 2016;33:726–37.

Kalinin AE, Kajava AV, Steinert PM. Epithelial barrier function: assembly and structural features of the cornified cell envelope. BioEssays 2002;24:789–800.

Kellsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, et al. Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. Am J Hum Genet 2005;76:794–803.

Kim KH, Son ED, Kim HJ, Lee SH, Bae IH, Lee TR. EGR3 is a late epidermal differentiation regulator that establishes the skin-specific gene network. J Invest Dermatol 2019;139:615–25.

Kobayashi T, Enomoto K, Wang YH, Yoon JS, Okamura R, Ide K, et al. Epidermal structure created by canine hair follicle keratinocytes enriched with bulge cells in a three-dimensional skin equivalent model in vitro: implications for regenerative therapy of canine epidermis. Vet Dermatol 2013;24:77–83.e19–20.

Kumar S, Stecher G, Suleski M, Hedges SB. TimeTree: a resource for timelines, timetrees, and divergence times. Mol Biol Evol 2017;34:1812–9.

Lachner J, Mlitz V, Hermann M, Tschachler E, Eckhart L. Convergent evolution and phylogenetic profiling of the feather protein with the highest cysteine content. Protoplasma 2019;256:1257–65.

Lachner J, Mlitz V, Tschachler E, Eckhart L. Epidermal cornification is preceded by the expression of a keratinocyte-specific set of pyroptosis-related genes. Sci Rep 2017;7:17446.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

Lundwall A. Old genes and new genes: the evolution of the kallikrein locus. J Invest Dermatol 2014;138:2685–92.

Mildner M, Ban J, Mrass P, Mayer C, Lengauer B, Eckhart L, et al. Caspase-14 expression by epidermal keratinocytes is regulated by retinoids in a differentiation-associated manner. J Invest Dermatol 2002;119:1150–5.

Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum [published correction appears in Int Immunol 2019;31:243]. J Invest Dermatol 2019;139:611–5.

Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum [published correction appears in Int Immunol 2019;31:243]. J Investig Dermatol 2019;139:611–5.

Mlitz V, Strasser B, Eckhart L, Dalla Valle L, Alibardi L. Review: evolution and morphogenesis of skin appendages. J Invest Dermatol 2019;133:1–4.