Single-cell transcriptomics defines keratinocyte differentiation in avian scutate scales

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The growth of skin appendages, such as hair, feathers and scales, depends on terminal differentiation of epidermal keratinocytes. Here, we investigated keratinocyte differentiation in avian scutate scales. Cells were isolated from the skin on the legs of 1-day old chicks and subjected to single-cell transcriptomics. We identified two distinct populations of differentiated keratinocytes. The first population was characterized by mRNAs encoding cysteine-rich keratins and corneous beta-proteins (CBPs), also known as beta-keratins, of the scale type, indicating that these cells form hard scales. The second population of differentiated keratinocytes contained mRNAs encoding cysteine-poor keratins and keratinocyte-type CBPs, suggesting that these cells form the soft interscale epidermis. We raised an antibody against keratin 9-like cysteine-rich 2 (KRT9LC2), which is encoded by an mRNA enriched in the first keratinocyte population. Immunostaining confirmed expression of KRT9LC2 in the suprabasal epidermal layers of scutate scales but not in interscale epidermis. Keratinocyte differentiation in chicken leg skin resembled that in human skin with regard to the transcriptional upregulation of epidermal differentiation complex genes and genes involved in lipid metabolism and transport. In conclusion, this study defines gene expression programs that build scutate scales and interscale epidermises of birds and reveals evolutionarily conserved keratinocyte differentiation genes.

Keratinocytes of the epidermis form a cornified cell layer at the body surface which protects against water loss and insults from the environment. In coordination with mesenchymal cells, epidermal keratinocytes also form skin appendages, such as claws, hair, feathers and scales, which have important functions in defense, capture of prey, thermoregulation and locomotion1–4. In birds most parts of the body surface are covered by a soft epidermis which suppresses water loss whereas hard skin appendages, such as the beak, feathers, and claws are used for interactions with the environment that require mechanical resilience5. The lower legs and the toes of birds are covered by scales which can be distinguished into scutate and reticulate scales6,7.

Scutate scales are located on the tarsometatarsus and on the dorsal side of the toes. They consist of overlapping hard scales that are separated by interscale or hinge regions. The structure of scutate scales resembles that of overlapping scales of reptiles. However, the hypothesis that avian scutate scales are homologous to reptilian scales, meaning that they have been inherited from a common ancestor of archosaurs (birds and crocodilians)8–10, has been challenged. The alternative hypothesis holds that avian scutate scales are secondarily derived from feathers6,11,12. Like feathers and scales of squamates, avian scutate scales develop from an anatomical placode13–15. The epidermal compartment of scutate scale placodes is characterized by the expression of beta-catenin (CTNNB1)16. A patterning mechanism distinct from that of avian scutate scales leads to the development of non-overlapping reticulate scales17.

The protective function of scutate scales depends on their structure and molecular composition. Corneous beta-proteins (CBPs), also known as beta-keratins18, keratins and other proteins were shown to be expressed in mature scutate scales17–26. A transcriptome analysis of embryonic scutate scales provided information on genome-wide gene expression, but only a subset of selected keratin intermediate filament and CBP genes were localized by mRNA in situ hybridization in hard scales and soft interscale regions27. To the best of our knowledge, a comprehensive gene expression catalog resolving the alternating pattern of soft and hard cornification has not been reported yet. In a recent study, we isolated keratinocytes from chicken leg skin, cultured them in an in vitro model of avian skin and determined their transcriptome28. Differentiation of keratinocytes in this culture system induced the expression of many genes, including members of the keratin family29, that were not
expressed in monolayer cultures. However, it remained elusive which genes are expressed in the hard and soft segments of scutate scales in vivo.

Here, we report single-cell RNA-sequencing (scRNA-seq) of chicken leg skin and the characterization of two distinct types of differentiated keratinocytes of scutate scales.

**Results**

**Keratin KRT9LC2 is a marker of differentiated keratinocytes in scutate scales of chickens.** The chicken has a diversified set of keratins which are hypothesized to mark specific states of epithelial cell differentiation. Keratin 9-like cysteine-rich 2 (KRT9LC2), also referred to as Hard Acid Sauropsid-specific 2 (HAS2), was detected by RT-PCR in scutate scales and analysis of transcriptome data suggested that it is transcriptionally upregulated during in vitro differentiation of keratinocytes isolated from chicken leg skin (Fig. 1A). An antibody raised against a carboxy-terminal peptide of the KRT9LC2 protein (Supplementary Fig. S1) detected a prominent band at the predicted size of 51 kD in protein extracts from the stratified epidermis of an in vitro skin model but not in extracts from monolayer cultures of undifferentiated chicken keratinocytes (Fig. 1B). The KRT9LC2 protein was also detected in extracts from scutate scales but not in back skin or reticulate scales of chickens (Fig. 1C).

Immunohistochemical staining yielded a strong KRT9LC2 signal in the suprabasal keratinocytes of scutate scales whereas interscale epidermis was not immunostained (Fig. 1D,E). When the primary antibody was replaced by preimmune serum, there was no immunostaining, confirming the absence of unspecific staining (Fig. 1F). The reticulate scales were immunonegative (Fig. 1G). These results demonstrated that KRT9LC2 is a marker of differentiated keratinocytes that form the hard outer surface of scutate scales.

**scRNA-seq analysis reveals two distinct populations of differentiated keratinocytes in chicken scutate scales.** To characterize gene expression during keratinocyte differentiation in chicken scutate scales, we isolated cells from the legs of 1-day old chicks (n = 3) and subjected them to single-cell RNA-sequencing (scRNA-seq). The protocol was designed to enrich for epidermal keratinocytes but smaller populations of fibroblasts, smooth muscle cells, endothelial cells, Schwann cells and erythrocytes were also detected (Fig. 2A, Supplementary Fig. S2).

According to nearest neighbor clustering as implemented in Seurat, keratinocytes were divided into 5 clusters (Supplementary Table S1). Clusters KC1, KC2 and KC3 (Fig. 2A) represented non-differentiated cells, characterized by high expression levels of KRT14L1 (Fig. 2B) which is an avian homolog of KRT14, a marker of the basal epidermal layer in mammals. KRT9L3 (Fig. 2C), a cysteine-poor keratin upregulated during differentiation of chicken keratinocytes in in vitro skin models, was expressed at high levels in cluster KC4 (Fig. 2A), for which CBP63 was defined as another marker gene (Supplementary Table S1). Expression of CBP63 (GenBank Gene ID: 101751614), previously referred to as "β-keratin, Chr25, Ktn13" 27, was demonstrated by mRNA in situ hybridization in the interscale epidermis of scutate scales 27. Therefore, cluster KC4 contained keratinocytes of the soft interscale epidermis. Cluster KC5 (Fig. 2A) was defined by marker genes such as KRT9LC2 (Supplementary Table S1; Fig. 2D). From the immunolocalization of KRT9LC2 (Fig. 1D) we inferred that KRT9LC2-positive cells represented the hard scales. The clustering of cells was reproduced in the 3 biological replicates investigated in this study (Supplementary Fig. S3).

**Keratinocyte differentiation is associated with the expression of distinct genes in scale and interscale segments of chicken scutate scales.** To determine genes that are upregulated during keratinocyte differentiation in scutate scales, we compared gene expression in cells containing KRT14L1 transcripts, marking the non-differentiated state of keratinocytes, versus gene expression in cells positive for one or both of the differentiation markers defined above, i.e. KRT9L3 and KRT9LC2. In KRT9L3-positive cells 219 genes were expressed at higher levels than in KRT14L1-positive cells (Fig. 3A, Supplementary Table S2), and in KRT9LC2-positive cells 213 genes were upregulated (P > 0.25 Log2-fold average upregulation, P < 0.001) (Fig. 3B, Supplementary Table S3). The majority of these genes (n = 133), including the type II keratin, KRT78L2, the epidermal differentiation complex gene EDQL (Supplementary Fig. S4), homologs of mammalian keratinocyte differentiation-associated genes, such as DSP, FABP5, POF1B, and others (Supplementary Tables S2 and S3) were upregulated both in KRT9LC2-positive and in KRT9LC2-positive cells relative to KRT14L1-positive cells.

To identify specific markers of hard and soft epidermal differentiation in scutate scales, we compared gene expression levels in KRT9LC2-positive versus KRT9L3-positive cells and determined the genes that differed most strongly with regard to their expression in these cells (Tables 1 and 2, Supplementary Fig. S4). KRT9LC2-positive cells accumulated, amongst others, the cysteine-rich keratin KRT9LC1 (Supplementary Fig. S4B), scale-associated CBPs such as CBP53 (Supplementary Fig. S4F), the lectin LGA5L1, and HOPX, whose mammalian ortholog regulates keratinocyte differentiation 31 (Table 1). Likewise, CTNNBI, previously reported as a regulator of scutate scale development, was found enriched in differentiated keratinocytes of hard scales (Table 1). KRT9LC3-positive cells accumulated cysteine-poor keratin KRT9L4 (Supplementary Fig. S4A), keratinocyte-associated CBPs such as CBP62 and CBP63 (Supplementary Fig. S4E), the lectin LGA5L1, and PRDX1, an antioxidant enzyme 32 (Table 2).

Thus, the results of this study suggest catalogues of genes associated with keratinocyte differentiation in hard epidermal segments (Table 1) and genes associated with keratinocyte differentiation in soft interscale segments (Table 2) of chicken scutate scales.
Discussion

Differentiation of keratinocytes underlies the growth of epithelial skin structures, such as claws, hair, feathers and scales of mammals, reptiles and birds. The molecular control of keratinocyte differentiation is well characterized for mammalian interfollicular epidermis and skin appendages, whereas little is known about the genetic regulation of keratinocyte differentiation in sauropsids. The results of the present study shed light into keratinocyte differentiation in scutate scales and provide a basis for the comparative analysis of further epithelial cell differentiation processes in avian claws, beak and feathers.
We have used scRNA-seq to characterize two types of keratinocyte differentiation, leading to the hard outer surface and the soft interscale epidermis of scutate scales. The methodology was adapted from successful scRNA-seq analyses of human and mouse skin. scRNA-seq of mouse tail skin revealed two paths of keratinocyte differentiation into scale and interscale epidermis. Of note, hard scales of the mouse tail were found to contain transcripts of cysteine-rich keratins such as KRT31 whereas the soft interscale regions contained epidermal keratins such as KRT10 and epidermal differentiation complex (EDC) genes such as involucrin. In contrast to the availability of many antibodies against mouse keratinocyte proteins, we had only one antibody, anti-KRT9LC2, specific for a keratin expressed in chicken scutate scales. This was a significant limitation of the present study. We were able to ascertain the expression of KRT9LC2 in differentiated keratinocytes of hard scales, and mRNA in situ hybridization data published by Wu et al. 2015 supported the expression of CBP63 in interscale epidermis. However, other putative differentiation markers, that are suggested by our results, remain to be localized in situ in future studies.

Gene expression in interscale epidermis of chicken leg skin showed several similarities to gene expression in two models of chicken epidermis with a soft cornified layer. scRNA-seq analysis of chicken back skin and bulk RNA-seq analysis of an organotypic model of chicken skin revealed expression of EDC genes and cysteine-poor but not cysteine-rich keratins. Many of the genes upregulated during differentiation of back skin keratinocytes, such as KRT9L4, LOR1, KRT9L3, BDH1L, EDQM2, SPTSSB, EDQM1, AADACL2, LIPML2, and ELOVL4 (Table 2) were enriched in interscale versus scale epidermis. Conversely, genes enriched in hard scale epidermis, such as KRT9LC2, KRT9LC1, CBP53-S, CBP54-S, CBP55-S, EDMTF1, and MT4 (Table 1) were not upregulated during differentiation of back skin keratinocytes. Therefore, we conclude that the genetic program of keratinocyte differentiation in the soft interscale epidermis of scutate scales is similar to the keratinocyte differentiation program in the soft back epidermis.
Keratinocyte differentiation in the hard outer surface of scutate scales differs substantially from that in the interscale regions. The results of the present study establish KRT9LC2, also referred to as HAS2 keratin29, as a protein marker of the hard scutate scales and identify other genes that are co-regulated with KRT9LC2. Among these scale-associated genes was KRT9LC1 (GenBank Gene ID:772,080) (Supplementary Fig. S3B), also referred to as Hard Acidic Sauropsid-specific 1 (HAS1) keratin29. In situ hybridization of transcripts corresponding to this gene (then named KRT13A) demonstrated predominant expression in the outer surface epithelium of scutate scales27, thus validating our scRNA-seq data. Another gene co-expressed with KRT9LC2 was CBP55-S, a scale CBP (beta-keratin). In the aforementioned study of Wu and colleagues27, expression of this gene (then named β-keratin, Chr25, Scale18) was detected by in situ hybridization specifically in the outer surface epithelium of scutate scales, further validating our scRNA-seq data.

An important result of this study is the genome-wide gene expression catalog of scutate scale epidermis resolved at the single-cell level. In addition to the genes discussed above, many more genes with differentiation-dependent expression were identified both in soft interscale epidermis (Supplementary Table S2) and hard scales (Supplementary Table S3). With regard to ongoing efforts to characterize evolutionarily ancient and derived patterns of gene expression during epidermal keratinocyte differentiation28,29,42–48, the results of the present study support the hypothesis that EDC genes, anti-inflammatory interleukin 1 family cytokines (IL-36RN and IL-1RN) (Supplementary Tables S2 and S3; Supplementary Fig. S5) and lipid metabolism and lipid transport-related genes, such as FABP5 and GLTP28 belong to the common keratinocyte differentiation program of amniotes. The transcriptome data generated in this study will be particularly useful for characterizing the process of hard cornification in a non-mammalian model species. The single-cell transcriptomes of chicken leg skin are now accessible for data searches according to criteria not limited to keratinocyte differentiation, so that new research questions pertaining to avian skin biology can be addressed in future studies.

Methods

Tissue preparation and scRNA-seq analysis. One day old chicks (strain Lohmann) were obtained from Schropper GmbH, Gloggnitz, Austria. Skin was excised from the leg of sacrificed animals and incubated in thermolysin (0.5 mg/ml) (Sigma-Aldrich) at 37 °C for 45 min. The lower dermis was removed using forceps and the remaining tissue, including the epidermis and parts of the upper dermis, was processed further according to protocols established for human skin39,40. For the isolation of cells, the tissue was split into two fractions that were incubated either in buffer-enzyme mix of the Whole Skin Dissociation Kit human (MACS Milteny Biotec) for 2.5 h at 37 °C or with 0.05% trypsin/EDTA (Thermo Fisher Scientific) and DNase 1 (10 µg/ml) (Roche Diagnostics) at 37 °C for 15 min. Afterwards the samples were combined and processed according to the manufacturer's protocol (Whole Skin Dissociation Kit human, MACS Milteny Biotec). In brief, the epidermis-
| Rank | Gene symbol | Gene name | P value (adjusted) | Average Log(e) FC | Percentage (KRT9L3 + cells) | Percentage (KRT9LC2 + cells) |
|------|-------------|-----------|-------------------|-------------------|-----------------------------|-----------------------------|
| 1    | KRT9LC2     | Keratin 9-like cysteine-rich 2 | 1.50E−130         | −1.76             | 14                          | 100                         |
| 2    | KRT9LC1     | Keratin 9-like cysteine-rich 1 | 1.76E−102         | −1.50             | 11                          | 90                          |
| 3    | ENSGALG0000036110 | Serine/threonine-protein kinase ULK4 | 4.12E−46         | −0.57             | 4                           | 49                          |
| 4    | ENSGALG00000046632 | Ly6/PLAUR domain-containing protein 2-like | 2.17E−42         | −0.74             | 16                          | 65                          |
| 5    | CBP53-S     | Corneous beta-protein 53 scale type | 9.59E−32          | −0.52             | 3                           | 37                          |
| 6    | MT4         | Metallothionein 4 | 1.81E−26         | −0.82             | 21                          | 57                          |
| 7    | LGALS       | Galectin like | 1.04E−21         | −0.45             | 33                          | 65                          |
| 8    | LM07        | LIM domain 7 | 6.79E−21         | −0.37             | 22                          | 56                          |
| 9    | ENSGALG0000010979 | Hydroxysteroid 17-beta dehydrogenase 11 | 2.24E−20         | −0.31             | 14                          | 46                          |
| 10   | ENSGALG0000027083 | Pancreatic lipase-related protein 2 | 4.60E−19         | −0.28             | 4                           | 28                          |
| 11   | ENSGALG0000027207 | PERP2, TP53 apoptosis effector | 1.72E−18         | −0.41             | 98                          | 99                          |
| 12   | ENSGALG0000039470 | 60S ribosomal protein L10-like 1 | 1.47E−17         | −0.23             | 100                         | 100                         |
| 13   | CDK2AP1     | Cyclin dependent kinase 2 associated protein 1 | 4.98E−17         | −0.28             | 14                          | 44                          |
| 14   | RPS12       | Ribosomal protein S12 | 4.77E−16         | −0.38             | 96                          | 99                          |
| 15   | PSMD10      | Proteasome 26S subunit, non-ATPase 10 | 1.03E−15         | −0.24             | 7                           | 31                          |
| 16   | CTNBN1      | Catenin beta 1 | 6.79E−15         | −0.34             | 43                          | 72                          |
| 17   | RORA        | RAR related orphan receptor A | 1.22E−14         | −0.31             | 22                          | 52                          |
| 18   | ENSGALG0000028451 | Metallothionein 4-like | 2.83E−14         | −0.56             | 72                          | 84                          |
| 19   | KRT14L2     | Keratin 14-like 2 | 5.95E−13         | −0.35             | 7                           | 29                          |
| 20   | ENSGALG0000029833 | Digestive cysteine proteinase 2-like | 1.16E−12         | −0.37             | 62                          | 80                          |
| 21   | EXOC6       | Exocyst complex component 6 | 4.07E−12         | −0.16             | 2                           | 19                          |
| 22   | CBP55-S     | Corneous beta-protein 55 scale type | 8.40E−12         | −0.21             | 1                           | 15                          |
| 23   | TFP12       | Tissue factor pathway inhibitor 2 | 3.27E−11         | −0.22             | 7                           | 27                          |
| 24   | HOPX        | HOP homeobox | 6.03E−11         | −0.29             | 35                          | 60                          |
| 25   | ENSGALG0000023818 | Heat shock protein family B (small) member 9 | 1.70E−10         | −0.51             | 48                          | 72                          |
| 26   | ENSGALG0000020078 | H3 histone, family 3C | 3.34E−10         | −0.26             | 83                          | 91                          |
| 27   | CBP52L-S    | Corneous beta-protein 52-like scale type | 4.33E−09         | −0.18             | 0                           | 12                          |
| 28   | ENSGALG0000004026 | Tubulin alpha 1c | 1.02E−08         | −0.18             | 8                           | 27                          |
| 29   | BOK         | BCL2 family apoptosis regulator BOK | 1.07E−08         | −0.20             | 10                          | 30                          |
| 30   | RPS15       | Ribosomal protein S15 | 1.66E−08         | −0.19             | 100                         | 100                         |
| 31   | METAP2      | Methionyl aminopeptidase 2 | 4.60E−08         | −0.24             | 33                          | 55                          |
| 32   | TPT1        | Tumor protein, translationally-controlled 1 | 4.84E−08         | −0.21             | 99                          | 99                          |
| 33   | ENSGALG00000027536 | PERP1, TP53 apoptosis effector | 6.27E−08         | −0.25             | 84                          | 90                          |
| 34   | SEPP1       | Selenoprotein P | 6.59E−08         | −0.20             | 9                           | 27                          |
| 35   | TUBB3       | Tubulin beta 3 class III | 6.60E−08         | −0.16             | 5                           | 22                          |
| 36   | RPL4        | Ribosomal protein L4 | 9.82E−08         | −0.24             | 94                          | 99                          |
| 37   | RPL15       | Ribosomal protein L15 | 1.04E−07         | −0.21             | 97                          | 99                          |
| 38   | CRIP2       | Cysteine rich protein 2 | 1.18E−07         | −0.17             | 10                          | 28                          |
| 39   | CBP54-S     | Corneous beta-protein 54 scale type | 1.49E−07         | −0.38             | 1                           | 11                          |
| 40   | PAK1        | p21 (RAC1) activated kinase 1 | 2.46E−07         | −0.16             | 7                           | 24                          |
| 41   | SERPINB2    | Serpin family B member 2 | 2.57E−07         | −0.22             | 10                          | 29                          |
| 42   | PDE6D       | Phosphodiesterase 6D | 2.82E−07         | −0.13             | 3                           | 16                          |
| 43   | EMDTF1-EDC  | Epidermal differentiation protein MTFL1, EDC | 3.00E−07         | −0.58             | 1                           | 12                          |
| 44   | ENSGALG0000036099 | Eukaryotic translation elongation factor 1 delta | 3.62E−07         | −0.21             | 93                          | 95                          |

Continued
enzyme mix was diluted in 0.5 ml medium and dissociated with the gentleMACS Dissociator. The ground tissue was filtered through 100-micron (Falcon) and 40-micron (Falcon) meshes. Subsequently, cells were stained with DAPI dye for 10s and viable cells were sorted via an Aria Fusion high-speed cell sorting device (BD Biosciences, San Jose, CA, USA). Single cell RNA sequencing was performed according to a published protocol. In brief, a 10× Genomics Chromium instrument (10× Genomics, Pleasanton, CA) was used for single cell partitioning and barcoding and Illumina HiSeq 3000/4000 (Illumina, San Diego, CA) was used for sequencing (Center for Molecular Medicine, Vienna, Austria). Using the Cell Ranger Fastq pipeline (10X Genomics) the demultiplexed raw sequencing data were aligned to the chicken reference genome Gallus_gallus-5.0.

**Analysis of scRNA-sequencing data.** We distinguished between background noise and droplets containing cells using emptyDrops. Briefly, this method models ambient RNA background in the data set and tests for deviations from the background RNA. We used a false discovery rate of 0.05 to call cells to be included into further analysis. On the other end of the spectrum we used scan package to remove droplets containing more than one cell. The applied approach simulates thousands of doublets by adding together two randomly chosen single cell profiles. For the doublet score calculation, cell clustering including the set randomly generated doublets is performed. Then for each cell of the original dataset, the number of simulated doublets in their neighbourhood is recoded and used as input for score calculation. We used 200 nearest neighbours for each cell and applied a threshold of doublet score > 4 to identify doublets in each dataset separately. Doublet score was log10 of the ratio between simulated doublet cells and total number of neighbours taken into consideration for each cell. The data obtained from 3 biological replicates were submitted to the NCBI Gene Expression Omnibus (GEO) database under accession numbers GSE179690 (BioProject PRJNA74554). The individual samples were referred to as "leg skin 1" (BioSample: SAMN20109848, SRA: SRX11375855), "leg skin 2" (BioSample: SAMN20109847, SRA: SRX11375856) and "leg skin 3" (BioSample: SAMN20109846, SRA: SRX11375857).

**Table 1.** Gene expression levels in KRT9L3 + versus KRT9LC2 + cells: Genes upregulated in KRT9LC2 + keratinocytes (hard scales).

| Rank | Gene symbol | Gene name | P value (adjusted) | Average Log(e) FC | Percentage (KRT9L3 + cells) | Percentage (KRT9LC2 + cells) |
|------|-------------|-----------|-------------------|-------------------|-----------------------------|-----------------------------|
| 45   | GCAT        | Glycine C-acetyltransferase [Homo sapiens] | 3.71E–07            | -0.23             | 43                          | 63                          |
| 46   | PTTG1IP     | PTTG1 interacting protein                   | 4.62E–07            | -0.25             | 21                          | 40                          |
| 47   | MSX2        | Msh homeobox type 6                         | 5.15E–07            | -0.53             | 3                           | 16                          |
| 48   | SMX2        | Msh homeobox 2                              | 5.63E–07            | -0.12             | 2                           | 13                          |
| 51   | LGR4        | Leucine-rich repeat G protein-coupled receptor 4 | 1.52E–06          | -0.16             | 6                           | 22                          |
| 57   | ENSGALG00000045796 | Cytosolic phospholipase A2 epsilon-like | 2.61E–06            | -0.12             | 1                           | 12                          |

**Quantitative reverse-transcription polymerase chain reaction.** RNA was isolated from chicken tissues and skin models and purified with TRIzol according to a published protocol and reverse-transcribed with the iScriptTM cDNA synthesis kit (Biorad, Hercules, CA). Polymerase chain reactions (PCRs) were performed with primer pairs specific for KRT9L2 (KRT9L2-s, 5′-GAAGCGGCTACAAACCCAC-3′ and KRT9L2-a, 5′-TGCTTCAAGGATCTCTTCAATT-3′), IL1RN (IL1RN-s, 5′-GAGAAGGGTGTGGGTTGGGC-3′ and IL1RN-a, 5′-TAGGTGGGAAAGGGTGA-3′), IL36RN (IL36RN-s, 5′-GAGTTCACCCTTGATCC-3′ and IL36RN-a, 5′-AACAGCTTGTCCTCCCATCC-3′), and the housekeeping gene Hydroxymethylbilane synthase (HMBS) (HMBS-s, 5′-AAPGTGGGGAAAACGCAT-3′ and HMBS-a, 5′-TTTCCTTCTGATCCAGCAGA-3′) on a Roche LightCycler with LC480 SYBR Green I Master Kit according to the manufacturer's instructions.
| Rank | Gene symbol | Gene name | P-value (adjusted) | Average Log(e) FC | Percentage (KRT9L3 + cells) | Percentage (KRT9LC2 + cells) |
|------|-------------|-----------|-------------------|------------------|----------------------------|----------------------------|
| 1    | KRT9L3      | Keratin 9-like 3 | 1.50E−129        | 1.76             | 100                        | 46                         |
| 2    | ENSGALG0000007127 | Fatty acid desaturase 1 (FADS1) | 5.13E−38        | 0.69             | 57                         | 12                         |
| 3    | CBP63-K     | Corneous beta-protein 63 keratinocyte type | 1.89E−34        | 2.47             | 63                         | 26                         |
| 4    | ENSGALG00000045042 | D-beta-hydroxybutyrate dehydrogenase | 4.14E−33        | 0.64             | 63                         | 22                         |
| 5    | CBP62-K     | Corneous beta-protein 62 keratinocyte type | 8.51E−29        | 2.02             | 52                         | 16                         |
| 6    | GAPDH       | Glyceraldehyde-3-phosphate dehydrogenase | 4.79E−27        | 0.48             | 95                         | 82                         |
| 7    | KRT9L4      | Keratin 9-like 4 | 9.55E−27        | 1.08             | 40                         | 6                          |
| 8    | LGALS1      | Galectin 1     | 2.21E−26        | 0.46             | 100                        | 99                         |
| 9    | PRDX1       | Peroxiredoxin 1 | 4.20E−26        | 0.54             | 58                         | 21                         |
| 10   | S100A6      | S100 calcium binding protein A6 | 5.34E−24        | 0.60             | 97                         | 88                         |
| 11   | GPX1        | Glutathione peroxidase 1 | 5.47E−24        | 0.38             | 100                        | 94                         |
| 12   | IL13RA2     | Interleukin 13 receptor subunit alpha 2 | 1.01E−20        | 0.28             | 28                         | 1                          |
| 13   | SCCPDH      | Saccharopine dehydrogenase (putative) | 2.76E−20        | 0.43             | 53                         | 21                         |
| 14   | ENSGALG00000021451 | Uncharacterized oxidoreductase-like | 4.21E−20        | 0.39             | 58                         | 25                         |
| 15   | ENSGALG00000045989 | Trypsin II-P29-like, lincRNA | 5.95E−19        | 0.41             | 49                         | 18                         |
| 16   | S100A11     | S100 calcium binding protein A11 | 2.50E−18        | 0.40             | 91                         | 81                         |
| 17   | ENSGALG00000007220 | Ferritin heavy chain 1 | 1.84E−17        | 0.41             | 56                         | 23                         |
| 18   | ST13        | ST13 Hsp70 interacting protein | 8.37E−16        | 0.29             | 45                         | 15                         |
| 19   | ACAT2       | Acetyl-CoA acetyltransferase 2 | 9.12E−14        | 0.29             | 35                         | 10                         |
| 20   | BARX2B      | BARX homeobox 2B | 1.42E−13        | 0.25             | 20                         | 1                          |
| 21   | YBX1        | Y-box binding protein 1 | 1.84E−13        | 0.35             | 86                         | 62                         |
| 22   | PPA1        | Inorganic pyrophosphatase 1 | 6.03E−12        | 0.22             | 30                         | 7                          |
| 23   | MOGAT1      | Monoacylglycerol O-acyltransferase 1 | 5.43E−11        | 0.19             | 19                         | 2                          |
| 24   | OLAH        | Oleoyl-ACP hydrolase | 7.13E−11        | 0.18             | 19                         | 1                          |
| 25   | ATP5G3      | ATP synthase, mitochondrial F0 complex, subunit C3 | 3.76E−10        | 0.37             | 60                         | 38                         |
| 26   | ANXA1       | Annexin A1     | 4.20E−10        | 0.34             | 46                         | 20                         |
| 27   | NAP1L1      | Nucleosome assembly protein 1 like 1 | 4.79E−10        | 0.26             | 52                         | 26                         |
| 28   | TKT         | Transketolase   | 9.13E−10        | 0.21             | 31                         | 10                         |
| 29   | DUSP14      | Dual specificity phosphatase 14 | 1.55E−09        | 0.27             | 41                         | 18                         |
| 30   | EDQM2-EDC   | Epidermal differentiation protein Q motif 2, EDC | 1.61E−09        | 0.39             | 35                         | 12                         |
| 31   | ENSGALG00000045170 | Lymphocyte antigen 6E-like | 2.82E−09        | 0.31             | 20                         | 3                          |
| 32   | CHCHD2      | Coiled-coil-helix-coiled-helix domain containing 2 | 3.27E−09        | 0.31             | 76                         | 62                         |
| 33   | FDPS        | Farnesyl dipiphosphate synthase | 5.30E−09        | 0.45             | 40                         | 18                         |
| 34   | ENSGALG00000006723 | Isopentenyl-diphosphate delta isomerase 1 | 5.48E−09        | 0.28             | 34                         | 13                         |
| 35   | IL20RA      | Interleukin 20 receptor subunit alpha | 6.03E−09        | 0.16             | 19                         | 3                          |
| 36   | PPDFF       | Pancreatic progenitor cell diff. proliferation factor | 6.45E−09        | 0.29             | 65                         | 46                         |
| 37   | ENSGALG00000008439 | CD36 | 9.64E−09        | 0.20             | 23                         | 5                          |
| 38   | HOMER2      | Homer scaffold protein 2 | 2.80E−08        | 0.19             | 28                         | 8                          |
| 39   | ACLY        | ATP citrate lyase | 3.09E−08        | 0.26             | 38                         | 17                         |
| 40   | ATP5G1      | ATP synthase, H + transporting, mito. F0 compl. sub. C1 | 4.27E−08        | 0.29             | 57                         | 35                         |
| 41   | HACD3       | 3-hydroxyacyl-CoA dehydratase 3 | 5.08E−08        | 0.20             | 32                         | 12                         |
| 42   | EDQM1-EDC   | Epidermal differentiation protein Q motif 1, EDC | 8.39E−08        | 0.47             | 39                         | 18                         |
| 43   | KRT9L1      | Keratin 9-like 1 | 1.14E−07        | 0.17             | 15                         | 1                          |

Continued
| Rank | Gene symbol | Gene name                                                                 | P-value (adjusted) | Average Log(e) FC | Percentage (KRT9L3 + cells) | Percentage (KRT9LC2 + cells) |
|------|-------------|---------------------------------------------------------------------------|--------------------|--------------------|-------------------------------|-------------------------------|
| 52   | HSD17B12    | Hydroxysteroid 17-beta dehydrogenase 12                                   | 4.27E−07           | 0.21               | 35                            | 15                            |
| 54   | ELOVL4      | ELOVL fatty acid elongase 4                                                | 6.89E−07           | 0.41               | 76                            | 62                            |
| 74   | FASN        | Fatty acid synthase                                                        | 8.33E−05           | 0.17               | 26                            | 10                            |
| 75   | ENSGALG00000027494 | Serine palmitoyltransferase small subunit B (SPTSSB)                   | 1.05E−04           | 0.19               | 40                            | 21                            |
| 79   | LOR1-EDC    | Loricrin, EDC 2                                                           | 2.53E−04           | 0.17               | 10                            | 1                             |
| 80   | ENSGALG00000045194 | Lipase member M-like 2                                                    | 2.53E−04           | 0.10               | 10                            | 1                             |
| 92   | ENSGALG00000044962 | Arylacetamide deacetylase-like 4-like (AADAC4L)                  | 2.55E−03           | 0.21               | 21                            | 8                             |

Table 2. Gene expression in KRT9L3 + versus KRT9LC2 + cells: Genes upregulated in KRT9L3 + keratinocytes (interscale epidermis).

Facteur’s protocol. Quantitative analysis of IL1RN and IL36RN expression in chicken tissues was performed according to a published method. The expression levels of these genes were compared between scutate scales and other tissues, considering differences with a P value of < 0.05 significant (two-sided t-test).

Western blot analysis. Proteins were prepared from chicken skin and scales by treatment with the PreCelssys system (VWR, International, Radnor, PA) and from chicken keratinocytes cultured in vitro by sonication in Laemmli buffer containing 2% SDS. Thirty µg of protein per lane were electrophoresed through an ExcelGel SDS 8–18% polyacrylamide gel (GE Healthcare Life Sciences) and afterwards blotted onto a nitrocellulose membrane (GVS Life Sciences). Subsequently, the membrane was blocked with phosphate-buffered saline containing 5% milk powder (Sigma-Aldrich), 2% bovine serum albumin (Sigma-Aldrich) and 0.1% Tween (Sigma-Aldrich) at room temperature for one hour, and incubated with mouse anti-KRT9LC2 antibody (1:500) that was raised in mice by immunization with a synthetic peptide CAAAEIQVPCRRICD, corresponding to the carboxy-terminus of the protein (GenBank accession number XP_418162.6, GenBank definition: keratin, type I cytoskeletal 19) (Supplementary Fig. S1) conjugated to keyhole limpet hemocyanin, according to a published protocol. After overnight incubation at 4 °C, the membrane was washed and sheep anti-mouse immunoglobulin G (1:10,000, GE Healthcare UK Limited) coupled with horseradish peroxidase used as secondary antibody at room temperature for one hour. The chemiluminescence system (Clarity Western ECL Substrate, BioRad) served for the protein detection. For loading control, the membrane was reincubated with anti-mouse GAPDH (1:5000, HyTest) and sheep anti-mouse immunoglobulin G (1:10,000, GE Healthcare UK Limited), coupled with horseradish peroxidase. The recordings of the chemiluminescence signal over the entire blots are shown in Supplementary Fig. S6 and the relevant portions thereof are depicted in Fig. 1C, D.

Immunohistochemistry. Immunohistochemistry was performed according to published protocols with modifications. In brief, chicken tissue samples were fixed in 7.5% formaldehyde and embedded in paraffin. Citrate buffer pH6 (DAKO) was used to retrieve the antigens and mouse anti-KRT9LC2 antibody (1:500) as primary antibody. To block unspecific binding, 10% sheep serum was added to secondary sheep anti-mouse immunoglobulin G (GE Healthcare), and further the nuclei were counterstained with haematoxylin. For control experiments, the primary antibody was replaced by the pre-immune serum.

Ethics statement. All animal procedures were approved by the Animal Care and Use Committee of the Medical University of Vienna. All procedures were performed in accordance with the guidelines established by this committee and in adherence to the ARRIVE guidelines.

Data availability. Single-cell transcriptomes generated in this study are available at GEO under accession number GSE179690. All other data generated or analysed during this study are included in this published article and its Supplementary Information files.

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J.L., E.T. and L.E. conceived the study, J.L., F.E. and M.H. performed experiments, J.L., F.E., M.W., M.F. and L.E. analyzed the results, J.L. and L.E. wrote the manuscript. All authors reviewed the manuscript.

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