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Identification of autophagy-regulated proteins by proteomic analysis of tape-stripped stratum corneum

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Autophagy is one of the main mechanisms for the degradation of organelles and intracellular proteins. The substrates of autophagy are enclosed in vesicles which fuse with lysosomes. Autophagy is active during stress responses and terminal differentiation of keratinocytes forming the stratum corneum (SC) and skin appendages. Dysfunctions of autophagy are implicated in the etiology of skin diseases such as psoriasis [1, 2]. Methods and markers for the detection of impairments of autophagy are required to further validate the clinical significance of autophagy.

Here, we investigated whether the lack of autophagy in keratinocytes can be detected by the proteomic analysis of the SC. As a model, we utilized *Atg7^{fl/fl} Krt14-Cre* mice (*Atg7-EKO* for *Atg7* epidermal knockout, n=6), in which the essential autophagy gene *Atg7* is deleted specifically in keratinocytes, in comparison to fully autophagy-competent mice (*Atg7^{fl/fl}*, denoted wild-type, WT, n=5) [3]. SC was sampled by tape-stripping from the soles of sacrificed mice and subjected to label-free proteomics as previously described [4] (Supplementary Methods) (Fig. 1a). Keratins Krt2 and Krt10 were identified as the quantitatively dominant proteins in the SC of both WT and *Atg7-EKO* mice (Fig. 1b). Among more than 170 proteins detected (Suppl. Table S1), 10 proteins were significantly increased in abundance when autophagy was suppressed (adjusted P-value <0.05) (Fig. 1b, c).

Five of the proteins elevated in the absence of autophagy, namely pyruvate kinase (Pkm), elongation factor 2 (Eef2), malate dehydrogenase 2 (Mdh2), valosin-containing protein (Vcp) and galectin 7 (Lgals7), have recently been found to be elevated in autophagy-deficient hair shafts, whereas the five others are not significantly elevated or have not been quantified in hair shafts [5]. Interestingly, the increase of lamin A/C (Lmna) in *Atg7-EKO* SC is in line with the proposed role of autophagy in degrading components of the nucleus during the cornification of epidermal keratinocytes [1]. Furthermore, the skin barrier-associated protein filaggrin (Flg) and two desmosomal proteins, junction plakoglobin (Jup) and desmoplakin (Dsp), are elevated in *Atg7-EKO* samples (Fig. 1a).

These data indicate that the impairment of keratinocyte autophagy manifests in alteration of the SC that can be detected by proteomic analysis of tape-strip samples. Notably, the autophagy-dependent changes in the protein composition of the SC are less pronounced than those of hair shafts in which a broad range of proteins are significantly increased upon suppression of autophagy [5]. The lower number of autophagy-related marker proteins in the SC is likely caused by additional autophagy-independent modifications of the proteome, such as proteolysis by endogenous enzymes and proteases of commensal microbes. This study had limitations such as the incomplete sampling of the SC and the lack of a comparison with

interfollicular epidermis. We conclude that proteomics of the SC is a promising method for the analysis of autophagic activity in the epidermis, with candidate markers being provided by the present study in a genetically well-defined mouse model. Further studies on human SC and cornified skin appendages are required to determine the most useful approach for the detection of autophagy-dependent aberrations of keratinocyte differentiation in skin diseases.

Ethics statement

No experiments on live animals were performed in this study. In agreement with the national laws of Austria, a permission for killing animals for tissue preparation was not required.

Data availability statement

The raw data are available at the MassIVE Proteomics repository (<https://massive.ucsd.edu/>) with MassIVE id number MSV000095353.

Author Contributions

S.S., R.H.R. and L.E. designed the study. S.S., N.K., I.M.N., R.H.R. and L.E. collected and analyzed the data. S.S. and L.E. wrote the original draft of the manuscript. N.K., I.M.N. and R.H.R. reviewed and edited the manuscript. R.H.R. and L.E. supervised the study. All authors have read and approved the final manuscript.

Competing Interests

The authors have no competing interests.

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Figure legend

Figure 1. Proteomic analysis of sole skin stratum corneum (SC) from wildtype (WT) and *Atg7* epidermal knockout (EKO) mice. (a) Schematic depiction of the study design. **(b)** The normalized protein abundance is shown for the predominant keratins of sole skin and ten proteins that differed significantly (adjusted *P*-values <0.05, * and <0.01, **) in abundance in WT and EKO SC. Bars and error bars show means and standard deviations, respectively. **(c)** Fold changes (EKO/WT) of the proteins shown in panel **b**. Note that the vertical axes in both panels show dimensionless quantities. ns, not significant.

Supplementary methods

Mice

Atg7^{fl/fl} K14-Cre (EKO) and *Atg7^{fl/fl}* (WT) mice were maintained under standard housing conditions [3, 5]. Male mice were sacrificed at an age of 9-10 weeks immediately before samples were prepared.

Tape-stripping of stratum corneum

Stratum corneum was prepared from the soles of hind limb feet by repeated application of D-Squame discs (Cuderm, Dallas, TX, USA). The first adhesive disc containing the most superficial layer of the stratum corneum was excluded from further analysis. The next three discs containing deeper layers of the stratum corneum were collected and processed for subsequent proteomic analysis as previously described [4].

Mass spectrometry and protein identification

The trypsin-digested samples were analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) on a Thermo Scientific Dionex UltiMate 3000 RSLC system according to a published method [5]. The raw data were analyzed using PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, ON, Canada) as previously described [5].

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA). Two-tailed t-tests with a correction for multiple comparisons using the Holm-Šidák method ($\alpha = 0.05$) were performed to determine the significance of differences.

