Appendix S1

SUPPLEMENTARY METHODS

Hair samples and extraction of low-molecular-weight proteins

Human hair shafts derived from the distal parts (usually representing 10–30% of total hair length) used in this study were obtained from 5 healthy donors (mean ± standard deviation age: 24.2 ± 0.44 years). The collected hair was free of dandruff, scalp diseases and without prior chemical treatments, such as hair dyes, bleach, or perms. Written informed consent was taken from donors to collect hair samples for analysis. This study was approved by Institutional Review Board of Nanyang Technological University (IRB-2016-11-042). This study followed the principles of the Declaration of Helsinki. Before extraction, the human hair shafts were washed twice with 70% ethanol, followed by 3 washes with MilliQ water for 2 min. The hair shafts were then vacuum-dried and low-molecular-weight (LMW) proteins were extracted from hair as described earlier (S1, 5). In brief, human hair (100 mg) was cut into fragments and incubated in 3 ml of "Shindai solution" consisting of 25 mM Tris-HCl (pH 9.5), 25% ethanol, 200 mM dithiothreitol and 8 M urea for 72 h at 50°C. The solution was centrifuged at 17,000×g for 15 min at 25°C and the supernatant comprising of LMW proteins was stored at – 80°C until further use.

Separation of antimicrobial proteins on reverse-phase high-performance liquid chromatography

Antimicrobial proteins in the LMW extracts were separated by reverse-phase high-performance liquid chromatography (RP-HPLC), as described earlier, with slight modifications (S2). Ten µl LMW protein extract was loaded onto a C18 column (Delta PAK WAT011793 Waters column, 5 µm, 150×3.9 mm) and separated using an RP-HPLC system (Agilent Technologies, 1290 Infinity Series) at 24°C after column equilibration in 0.1% trifluroacetic at a flow rate of 0.5 ml/min, using a gradient of acetonitrile in 0.1% formic acid. Protein separation was monitored at wavelengths of 214, 254 and 280 nm. The eluted fractions were collected at 1-min intervals, frozen and then lyophilized. Protein concentration was determined using the Micro BCA protein Assay Kit (23235#, Pierce Company).

Antimicrobial activity assay of high-performance liquid chromatography fractions

For determination of potential antimicrobial activity, a radial diffusion assay (RDA) method was used essentially as described previously (S3), with minor modifications. Lyophilized fractions were re-suspended in distilled water and tested against *Escherichia coli*. In brief, *E. coli* was grown overnight at 37°C in 20 ml full-strength trypticase (3% w/v) soy broth (TSB). To obtain mid-log bacteria, 20 µl culture was inoculated into 20 ml fresh TSB and incubated until OD 600 of 0.5 was reached. Bacterial CFU (4×10^6) was added to 10 ml sterile 1% (w/v) low electroendosmosis (EEO) agarose gel containing 1% (v/v) TSB, 10 mM Tris Buffer (pH 7.4) and 0.02 % (v/v) Tween-20 in a Petri dish. Samples were added to punched 3-mm wells. The plate was incubated for 3 h at 37°C to enable diffusion and was subsequently overlaid with 10 ml sterile 1% (w/v) low EEO agarose gel containing 6% (w/v) TSB. The diameter of the bacterial inhibition zone was measured after 18 h of incubation at 37ºC.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis

Dialysed LMW protein extract and HPLC fractions were analysed for protein/peptides by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Ten µg of samples were separated on 10–20% Tris-tricine gels (Novex™ EC6625, Novex Life Technologies) for 2 h at 90 V. Protein bands were visualized using a silver staining kit (Thermo Fisher Scientific, Cat#24612) according to the manufacturer's instructions.

Mass spectrometry analyses

HPLC fractions (18–23) were lyophilized and 40 µg protein were reduced with dithiothreitol (10 mM), alkylated using iodoacetamide (55 mM) and then subjected to overnight digestion in sequencing-grade modified trypsin at 37°C. The resultant peptides were separated and analysed on a Dionex Ultimate 3000 RSLC nanoLC system coupled to a Q-Exactive apparatus (Thermo Fisher, Waltham, MA, USA). Approximately 5 µl sample was injected into an acclaim peptide trap column via the autosampler of the Dionex RSLC nano LC system. The flow rate was set at 300 nl/min. Mobile phase A (0.1% formic acid in 5% acetonitrile) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish a 60-min gradient. Peptides were then analysed on a Dionex EASY-spray column (PepMap® C18, 3 um, 100 A) using an EASY nanospray source at an electrospray potential of 1.5 kV. A full MS scan (350−1,600 m/z range) was acquired at a resolution of 70,000 at m/z 200, with a maximum ion accumulation time of 100 ms. Dynamic exclusion was set to 30 s. Resolution for MS/MS spectra was set to 35,000 at m/z 200. The AGC setting was 1E6 for the full MS scan and 2E5 for the MS2 scan. The 10 most intense ions above a 1000-count threshold were selected for high-energy collision dissociation (HCD) fragmentation, with a maximum ion accumulation time of 120 ms. An isolation width of 2 Da was used for the MS2 scan. Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 28%. The under-fill ratio was defined as 0.1%.

Data analysis

Raw data files were converted into the mascot generic file (mgf) format using Proteome Discoverer version 1.4 (Thermo Electron, Bremen, Germany) with the MS2 spectrum processor for de-isotoping the MS/MS spectra. The concatenated target-decoy UniProt human database (sequence 92867, downloaded on 25 July 2016) was used for data searches. In-house Mascot server (version 2.4.1, Matrix Science, Boston, MA, USA) with MS tolerance of 5.1 ppm and MS/MS tolerance of 0.02 Da was used for database search. Two missed trypsin cleavage sites per peptide were tolerated. Carbamidomethylation (C) was set as a fixed modification, while oxidation (M) and deamidation (N and Q) were variable modifications. The peptide/protein lists obtained were exported to Microsoft Excel and analysed further. Gene ontological analysis was performed by adopting the online tool PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system (http://www.pantherdb.org/).

Western blot analysis

Rabbit polyclonal antibodies against histone H1.0 (Cat#ab83058, Abcam, Cambridge, UK), histone H2A (Cat# 2578S, Cell Signalling Technology, Danvers, MA, USA), histone H2B (Cat#ab1790, Abcam), histone H3 (Cat#9715S, Cell Signalling Technology) and histone H4 (Cat#2592S, Cell Signalling Technology) were used. Twenty µg samples from the LMW protein extract and HPLC fractions were loaded on a 4–20% Tris-Glycine gel (Mini-PROTEAN® TGX™, Cat#4561091). Electrophoresis was carried out as described above and gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Danvers, MA, USA). The membrane was blocked with 5% BSA dissolved in Tris-buffered

saline containing 0.1% Tween 20 (Tween 20) and incubated with primary antibodies (1:1000) overnight at 4°C. The membranes were probed with a secondary antibody (Polyclonal Goat Anti-Rabbit Immunoglobulins, Cat#D0487, Dako) and the bands were detected by the Chemiluminescence method (SuperSignal® West Dura Extended Duration substrate, Cat #34076, Thermo Scientific, Waltham, MA, USA).

Immunohistochemistry

Formalin-fixed, wax-embedded sections of hair biopsies were stained by immunoperoxidase after epitope heat retrieval at pH 6. After 1 h incubation with a primary antibody (Histone 3, ab8898 Abcam), sections were washed in tap water and incubated for 30 min with HRP-labelled polymer conjugated to goat anti–rabbit immunoglobulins (Dako). Sections were developed with 3,3'Diaminobenzidine substrate (Abcam) and counterstained with haematoxylin before dehydrating and mounting in a mixture of distyrene plasticiser xylene (CellPath). Photographs were taken with a microscope (Axio Imager Z1; Carl Zeiss) using a 40×, NA 0.60 Ph2 Corr long distance Plan-Neofluar objective (S4).

REFERENCES

S1. Fujii. T, Shunsuke Takayama, Ito Y. A novel purification procedure for keratin-associated proteins and keratin from human hair. Biol Macromol J 2013; 13: 92–106.
S2. Lee DY, Huang CM, Nakatsuji T, Thiboutot D, Kang SA, Monestier M, et al. Histone H4 is a major component of the antimicrobial action of human sebocytes. J Invest Dermatol 2009; 129: 2489–2496.
S3. Lehrer RI, Rosenman M, Harwig SSSL, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. J Immunol Methods 1991; 137: 167–173.
S4. Dreesen O, Chojnowski A, Ong PF, Zhao TY, Common JE, Lunny D, et al. Lamin B1 fluctuations have differential effects on cellular proliferation and senescence. J Cell Biol 2013; 200: 605–617.