Methods

Patients

The study, being part of the Network for Ichthyosis and Related Keratinization disorders (NIRK), was approved by the institutional review board of the University Hospital of Münster (2013-573-f-S). Written informed consent was obtained from the patient and parents. A detailed medical and dermatological history was obtained. Clinical diagnosis was made by experienced dermatologist (PT). Punch biopsies (4 mm) for histology, cell culture or ultrastrucure were taken.

Ultrastructure

Specimens of approximately 1 mm³ were fixed for at least 2 h at room temperature in 3% glutaraldehyde solution in 0.1M cacodylate buffer pH 7.4, post-fixed for 1 h at 4oC in 1% osmium tetroxide, rinsed in water, dehydrated through graded ethanol solutions, transferred into propylene oxide, and embedded in epoxy resin (glycidether 100, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Semi-thin and ultra-thin sections were cut with an ultramicrotome (Reichert Ultracut E, C. Reichert AG, Vienna, Austria). Ultra-thin sections were treated with uranyl acetate and lead citrate, and examined with an electron microscope (Zeiss EM900, Carl Zeiss AG, Oberkochen, Germany) equipped with a 1K wide-angle dual speed CCD camera (TRS – Tröndle Restlichtverstärker Systeme, Moorwein, Germany).

Primary culture of human keratinocytes and fibroblasts

Primary keratinocytes and fibroblasts were obtained by enzymatic digestion of skin biopsies according to a standard protocol. Biopsies were incubated in 0.5 mg/ml protease X (Sigma, Munich, Germany) overnight at 4°C. The epidermis was subsequently peeled off the dermis and incubated in 0.25% trypsin/0.02% EDTA (PAA, Pasching, Austria) for 15 min at 37°C to achieve single-cell suspension. Trypsin activity was stopped by medium containing foetal calf serum (FCS) and 10 ng/ml EGF, 50 mg/ml BPE, 0.5 mM CaCl₂, 4 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.4 µg/ml hydrocortisone, 48 µg/ml ascorbic acid) medium for 2 h and 2 ml keratinocytes were seeded at a density of 1.5×10⁶ cells/ml KSFM2 medium. After 24 h the skin equivalents were raised to the air-liquid interface and medium was changed to specific 3D culture medium. The models were grown up for 9 days and every second day the medium was renewed.

RNA isolation and reverse transcriptase PCR of keratinocytes

For RNA isolation differentiated keratinocytes, cultured as described above, were used. Cells on a 10-cm Petri dish were washed once with PBS, then harvested with 1 ml TRIzol® (Invitrogen, Darmstadt, Germany). Cell lysis was performed mechanically using a 0.9-mm injection needle. Total RNA was isolated by phenol chloroform extraction and RNA quality was analysed on a 1% agarose gel.

RNA was reverse transcribed to cDNA using the First Strand cDNA Synthesis Kit from Thermo Scientific (Schwerte, Germany) according to the manufacturer’s protocol.

Quantitative real-time PCR

Quantitative real-time PCR was performed with an iCycler iQ™ Real-Time PCR Detection System (BioRad, Munich, Germany) using iQ™ SYBR® Green Supermix (BioRad, Munich, Germany) and gene-specific primers. All primers were designed by the program PerlPrimer and obtained from Eurofins MWG Operon (Ebersberg, Germany). The experiments were performed in triplicates and RPL13A (ribosomal protein L13A) serves as internal standard for normalization. Relative gene expression was analysed by the 2−ΔΔCT method.

Western blot analysis

For Western blot analysis 3D human skin equivalents were generated as described. Skin equivalents of healthy skin and skin affected with p.Ile345Serfs*121/p.Lys59* were generated. Proteins were extracted by boiling biopsies of the skin equivalents in extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 20 mM DTT, 2% SDS, pH 8.5) under continuous shaking. The same amounts of proteins were separated by a 12.5% SDS-PAGE under reducing conditions and proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by semi-dry blotting. Immunoblotting was performed with specific primary antibodies and alkaline phosphatase (AP)-conjugated secondary antibodies. Chromogenic detection was performed using NBT/BCIP substrate (AppliChem, Darmstadt, Germany). For specific detection of CDSN the monoclonal mouse antibody G36-19 (10, S1) was used. Actin serves as loading control and transglutaminase 1 (TGase1) as late differentiation control of keratinocytes.

Generation of three-dimensional human skin equivalents

To generate three-dimensional (3D) skin equivalents, keratinocytes and fibroblasts from passage 2 were taken. The generation of skin equivalents was performed in 3-µm filter tissue culture inserts (BD Biosciences, Heidelberg, Germany). Cells were cultured as described in the section above and incubation was performed at 37°C and 5% CO₂ in humidified conditions. For model generation 1 × 10⁵ fibroblasts per ml collagen (PureCol) and 3 × 10⁶ keratinocytes per insert were used. 2.5 ml of a fibroblast collagen mixture (2.4 ml PureCol, 0.3 ml 10× HBSS, 1 × 10⁵ fibroblasts in 0.3 ml FCS) were poured into each culture insert and were incubated for 2 h. The fibroblast/collagen matrix was subsequently equilibrated in KSFM2 (KSFM supplemented with 10 ng/ml EGF, 50 mg/ml BPE, 0.5 mM CaCl₂, 4 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.4 µg/ml hydrocortisone, 48 µg/ml ascorbic acid) medium for 2 h and 2 ml keratinocytes were seeded at a density of 1.5×10⁶ cells/ml KSFM2 medium. After 24 h the skin equivalents were raised to the air-liquid interface and medium was changed to specific 3D culture medium. The models were grown up for 9 days and every second day the medium was renewed.

Reference