Supplementary Information

Supplementary Materials and Methods

Cells

Primary fibroblasts and keratinocytes < passage 6 were used. All surplus material was obtained in accordance with the Dutch Law on Medical Treatment Agreement. All 3 donors used in this study were <50 years of age, female and Caucasian.Keratinocytes were immortalized using the HPV16 E6E7 virus as previously described (Spörrer et al. 2019).

WT and Δ73 collagen VII expression in HEK293 cells and purification

Generation of *COL7A1* cDNA lacking exon 73 was generated from *WT COL7A1* cDNA using Gibson assembly strategy as previously described (Bornert and Nyström 2019). Next, HEK-293 cells were transfected with 28 μg of cDNA per 10-cm dish using Lipofectamine 2000 (SigmaAldrich, St. Louis, MO) for *COL7A1* WT and lacking exon 73 in pcDNA3.1. Cells containing the transfected plasmid were selected with phleomycin (InvivoGen, San Diego, CA) for at least 72 hours. Expression of WT collagen VII or Δ73 collagen VII was performed in serum-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 50 μg/ml ascorbic acid added fresh daily (Fluka, Buchs, Switzerland). Medium was collected after 48 hours and cleared by centrifugation at 3,000 rpm for 10 minutes. Next, ethylenediaminetetraacetic acid (Serva, Heidelberg, Germany) at a concentration of 10 mmol/l and 1 mmol/l Pefabloc (Sigma-Aldrich, St. Louis, MO) was added before precipitation of high molecular weight proteins with 25% ammonium sulphate (Roth, Karlsruhe, Germany) at 4 °C

overnight. Precipitated proteins were collected by high speed centrifugation at 14,000 rpm for 30 minutes at 4 °C and resuspended in trisbuffered saline (TBS) buffer (50 mol/l Tris-HCl (Roth) pH 7.4 and 150 mmol/l NaCl (Roth)) supplemented with 10 mmol/l ethylenediaminetetraacetic acid and 1 mmol/l Pefabloc (Sigma-Aldrich). Proteins were used immediately or stored at −70°C for long term.

Limited trypsin digestion

To assess thermal stability of collagen VII using trypsin digestion, 1µg of purified WT collagen VII or Δ73 collagen VII in 10µl TBS was used. Samples were first incubated for 10 minutes with 1µl of 0.5% trypsin on ice. The samples were then incubated at various temperatures (30, 38, 40, 42, 44 or 46°C) for 1 minute. The reaction was stopped by adding 5µl of pre-heated SDS-PAGE sample buffer and incubated at 98°C for 5 minutes. For denatured control collagen VII was heated to 98°C for 5 minutes prior to exposure to trypsin. The samples were analyzed by Western blotting using the NC2 and P1 domain specific antibody (NC2.10) (Kühl et al. 2015).

Solid phase binding to collagen IV and laminin-332

500 ng of human collagen IV (Sigma-Aldrich) or 100 ng of rat laminin-332 (Millipore, Burlington, MA) was immobilized on Nunc MaxiSorp 96-well plates overnight at 4 °C. The next morning, wells were blocked with 1% BSA in PBS for 1 hour and various concentrations of denaturated and non-denaturated WT collagen VII and Δ73 collagen VII in TBS were then added for 1 hour. Denatured collagen VII was obtained by heating to 98°C for 5 minutes. After extensive wash with PBS, 1:1,000 diluted rabbit polyclonal collagen VII antibody directed against the NC1 domain

(Kühl et al. 2015) was added to the wells during 1 hour at room temperature. The wells were washed three times with PBS and HRP-conjugated goat anti-rabbit antibody diluted 1:10,000 was added. Finally, SigmaFast OPD tablet (Sigma-Aldrich) was used for detection of peroxidase activity and plate was read at 450 nm on infinite M200 plate reader (Tecan, Männedorf, Switzerland).

Cell adhesion and cell migration assays

Cell adhesion and migration assays were performed as previously described (Bornert et al. 2016). Briefly, Nunc F Untreated 96 wells plates (Thermo Fisher Scientific, Waltham, MA) were coated at 4°C overnight with 100 ng of BSA, fibronectin, WT collagen VII or Δ73 collagen VII in PBS. Next, 10,000 keratinocytes or fibroblasts were seeded per well and the plate was incubated for 2 h at 37°C. After washing with PBS, the cells were fixed with 100% methanol and stained with 0.1% crystal violet. Photos were taken and subsequently cell lysis was performed with 1% acetic acid. The wells were quantified by reading the absorbance at 550 nm. Cell migration assays were performed by using silicone inserts (ibidi, Martinsried, Germany). The dishes were first coated with 100 ng of BSA, fibronectin, WT collagen VII and Δ73 collagen VII in PBS. After seeding of 100,000 fibroblasts or keratinocytes, were grown to confluence. The inserts were then removed to initiate migration, and pictures were taken at different time points to follow cell migration. Quantification was made using Image J.

QR-313 AONs and gel formulation

All AONs for screening were ordered at 40 nM scale and were HPLC purified (ITD technologies). QR-313 used in the HSE experiments was manufactured under GMP

by Avecia (Blackley, United Kingdom). All sequences are listed in Table S1. The carbomer gel composed of 0.75% carbomerum 980 (w/w), 0.75% trometamol (w/w), 10% propylene glycol (w/w), 1% EDTA (w/w) and water (q.s.). The QR-313 containing gel additionally contained 1% QR-313 (w/w), corrected for water and purity. After weighing all excipients, they were combined into one solution under vigorous mixing to obtain a homogenous gel. The carbomer gel (with or without QR-313) was subsequently filled in type 1 glass vials closed with a rubber stopper and cap, or in polypropylene syringes closed with wingcaps. Vials and syringes were autoclaved for at least 15 minutes at 121°C.

In vitro transfection of cell cultures

Exon skipping efficacy of AONs, including QR-313, was assessed in fibroblasts control (NHF-1) RDEBF-muE73-1 and in immortalized keratinocytes control (NHK-1) and RDEB (RDEBK-muE73-1 and RDEBK-muE73-3) cells. For this purposed, fibroblasts were seeded 100,000 into 6 wells plates and subsequently transfected with 3,12- 200 nM QR-313 with polyethylenimine (maxPEI mw 40.000, Polysciences Inc. (Warrington, PA)) for 24 hours. Keratinocytes were seeded into 6-well plates and were transfected with 0-100 nM of QR-313 using INTERFERin (Polyplus Transfection, Illkirch, France) or lipofectamine-2000 (Thermo Fisher Scientific) as transfection agents according to the manufacturers' protocols.

HSE generation

For Figure 3a, 3b, 4f, 4g and S9 HSEs were assembled as previously described (Spörrer et al. 2019). For Figure 3d, 3e and S5 dermal equivalents were generated by seeding rat-tail collagen ~4 mg/mL (Advanced Biomatrix, San Diego, CA) with

80,000 primary fibroblasts and incubated for a period of one week under submerged conditions in fibroblast medium (Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific), supplemented with 5% fetal bovine serum (BioWest, Nuaillé, France) and 1% penicillin/streptomycin (10.000U / 10mg/ml) (Sigma-Aldrich)). Subsequently, the dermal equivalents were seeded with 300,000 keratinocytes. HSEs were incubated under submerged conditions at 37°C and 7.3% CO_2 in standard HSE medium (DMEM and Ham's F12 medium (Thermo Fisher Scientific) in a 3:1 ratio, supplemented with 5% FBS, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin (Sigma-Aldrich) and 1% penicillin/ streptomycin). After 2 days, FBS was reduced to 1%. Two days later, FBS was omitted and the HSEs were cultured at the air–liquid interface for a maximum of 40 days. During this period, HSEs were cultured in keratinocyte medium as described above but without serum and supplemented with 10 mM l-serine, 10 μM l-carnitine. Medium was refreshed twice a week; at that time the following supplements were freshly added; 100 μg/ml ascorbic acid, 1 μM DL-α-tocopherolacetate in β-dextrine, 24 μM bovine serum albumin and a free fatty acid supplement that contained 25 μM palmitic acid, 15 μM linoleic acid and 7 μM arachidonic acid.

For treatment with QR-313, 100 μl, 100 nM QR-313 in PBS was added drop wise and evenly distributed over the keratinocyte layer. The treatment was repeated every other day. After 21 days in culture the HSEs were either snap frozen in OCT for cryosectioning or fixed in formalin and processed for paraffin sectioning and H&E staining. Processing-induced epidermal separation was analyzed by H&E staining and quantified by ImageJ as the percentage of epidermal separation along the total epidermal-dermal interphase.

HSE wounding and treatment

In HSEs, superficial wounds were made by removing part of the epidermis with the use of a surgical scalpel (Swann-Morton, Sheffield, UK). From the moment of wounding, HSEs were treated three times per week; 50 mg/cm^2 QR-313 formulation (10 mg/g in 0.75% carbomer gel) or placebo gel was topically applied to the wound bed. (RDEB-) HSEs were harvested after 1 week or 2 weeks of AON treatment. At this time, several samples were taken from HSE: a small stroke strip (2-3 mm) was taken for formalin fixation (Klinipath, 4186) and paraffin embedding (FFPE). From the remaining parts four different samples were collected in RNAlater (Thermo Fisher AM7021) for RNA isolation; a sample containing epidermis and dermis, a part with only the epidermis and two parts samples containing only the dermis; one directly underneath the wound obtained from the wound-bed and the second from or underneath the intact epidermis.

Histological analysis

Global histological analysis was performed on FFPE HSE sections. Models were fixed in 4% formaldehyde for 24-48 hours and subsequently processed in the Excelsior™ AS Tissue Processor (Thermo Fisher). Haematoxylin phloxine saffron (HPS) stain was done on 4 µm sections using Mayers Haematoxylin (Sigma-Aldrich), 0.25% Phloxin B (VWR, Radnor, PA) and 0.3% Saffron (Sigma-Aldrich) Digital micrographs were obtained using the Zeiss Axio microscope (Zeiss, Jena, Germany).

Immunofluorescence and histology

IF analysis was performed on 6 µm cryosections or 4 µm FFPE sections on Superfrost+ slides (Thermo Fisher Scientific). For FFPE, enzymatic antigen retrieval of the sections was done by Proteinase K (DAKO, Agilent, Santa Clara, CA) treatment, 15 minutes at 37°C. Subsequently the sections were blocked in blocking buffer (1% BSA, 10% goat serum) for 1 hour at RT. Anti-collagen VII rabbit polyclonal, (Kühl et al. 2015)) was diluted in blocking buffer and incubated overnight at 4°C. The next day the sections were incubated with Alexa568 labeled secondary antibody (Thermo Fischer Scientific) for 1 hour at RT. To visualize cell nuclei and ensure right orientation, Hoechst 3342 (Life Technologies) was used as nuclear counterstain. Finally, sections were mounted with Vectashield (H-1000, Vectorlabs, Burlingame, CA). H&E staining was performed using standard protocols as previously described (Kühl et al. 2015). Digital micrographs were made using the Zeiss Axio D1 observer microscope (Zeiss).

RNA/cDNA

RNA was isolated from either in vitro cultures using Reliaprep RNA isolation kit from Promega or human skin equivalents using RNeasy plus universal mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Subsequently, cDNA was produced using the Verso cDNA synthesis kit (Applied Biosystems, Foster City, CA).

PCR

Products were analysed using polymerase chain reaction (PCR) for the exon 73 region. FW primer (5'-GCTGGCATCAAGGCATCT-3) and RV primer (5'-

TCCTTTCTCTCCCCGTTCTC-3) (Expected products: Not skipped: 357 bp, Skipped: 156bp). PCR was performed for 35 cycles using an annealing temperature of 56°C and 1 µl (15 µg) of cDNA in a total volume of 25 µL (end concentrations 0.2 mM dNTPs; 3 mM $MgCl_2$; 0.4 mM of each primer, 1 U Amplitaq). PCR products were visualized on the Bioanalyzer 2100 from (Agilent). For Bioanalyzer products semi-quantification was performed on the WT *COL7A1* transcripts and Δ73 *COL7A1* transcripts to estimate the skipping efficiency. Exon skipping efficacy was calculated with the following formula:

$$\text{Exon 73 skip \%} = \frac{\text{Exon 73 skipped PCR product (nmol/L)}}{\text{Total PCR products (nmol/L)}} \text{ X 100\%}$$

ddPCR

The exon73 skip % was quantified using 2 duplex assays. In assay 1 the total amount of *COL7A1* in the samples was measured (*COL7A1* reference assay with a FAM label) and in the assay shown in Figure S2a primer and probe set specific for the skipped exon 73 (*COL7A1* exon73 skip assay with a FAM label) were used. In order to normalize the samples both assays were duplexed using GusB as reference gene with a HEX label. See table 2 for primers and probe sequences. Duplex ddPCR was performed using the ddPCR supermix for probes (no dUTP) (Bio-Rad, Carlsbad, CA) with 4µL cDNA template and primers and probes (end concentration of 0.2 µM) in a total volume of 20µL. Droplets were generated using the QX200 droplet generator (Bio-Rad) and PCR was performed for 40 cycles using an annealing temperature of 62°C using the T100 thermal cycler (Bio-Rad). Following PCR, droplets were analyzed with the QX200 droplet reader (Bio-Rad), counting fluorescent signals from

single labeled, double labeled, and negative droplets. Exon 73 skip % was calculated using the following formula:

$$\text{Exon 73 skip \%} = \frac{\left(\frac{\text{Copies exon 73 skip/reaction}}{\text{Copies GusB/reaction}}\right)}{\left(\frac{\text{Copies C7/reaction}}{\text{Copies GusB/reaction}}\right)} \text{ X 100}$$

Cell staining of collagen VII

RDEB keratinocytes were transfected with 100 nM QR-313 or scrambled QR-313 as described above. 72 hours after transfection cells were fixed with 4% formaldehyde. Staining for Collagen VII was performed with rabbit polyclonal antibody directed against the NC1 domain, LH 7.2 (Kühl et al., 2015). Nuclei were stained with DAPI.

Immunoelectron microscopy

Immunoelectron microscopy analyses were preformed as previously described (Keene and Tufa 2018). Shortly, 2-week-treated HSEs. A 0.5mm x 0.5mm part of HSE, containing both dermis and epidermis, was locked in a mesh cassette, collected in Michel's Transport Medium (American Mastertech, Lodi, CA) and shipped at 4°C to Shriner's Hospital (Portland, Oregon) were it was processed immediately. Strong fix was performed for general morphology and immune gold labeling performed with ab185 (for NC1) and LH24 (for NC2).

FISH

To visualize QR-313 in HSEs, FFPE cross sections were made and prepared for fluorescent *in situ* hybridization (FISH) analysis. Sections were first deparaffinized, then incubated for 60 min with a pre-hybridization buffer. This buffer contains 10% formamide (VWR) and 2x saline-sodium citrate buffer (Sigma-Aldrich). Next, sections are incubated with hybridization buffer at 45°C overnight. Hybridization buffer is similar to the pre-hybridization buffer but additionally contains 1 mg/ml yeast tRNA (Sigma-Aldrich), 10 % (w/v) dextran sulfate (Sigma-Alrdich) and 100nM of Alexa555 labeled QR-313 probe ({C}GG{C}TT{T}C{T}GG{A}GA{A}CG (Eurogentec, Seraing, Belgium)). Nuclei were stained with Hoechst 33342 (Life Technologies) to ensure visualization of the location and orientation of cells in the skin. Digital micrographs were obtained using the Zeiss Axio Z1 Observer and analyzed using ZEN software (Zeiss).

Studies in murine skin

Skin of adult collagen VII-hypomorphic (RDEB) mice (Fritsch et al. 2008) was intradermally injected with 10 μg WT collagen VII or Δ73 collagen VII in PBS. The injections were repeated after one week. One month after the last injection the skin samples were harvested and embedded in optimal cutting temperature compound (OCT) (Sakura, AJ Alphen aan den Rijn, The Netherlands) and either fixed in formalin for histological analysis or fixed in 3% glutaraldehyde for transmission electron microscopy (TEM) analysis. Tissue was processed for immunostaining and histological analysis as described above and TEM was performed as previously described (Kühl et al. 2015).

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Supplementary tables

Table S1 AON sequences and parameters

AON name	Mw	Sequence 5’ – 3’	Modifications	Bp	Tm (°C)
QR-313	7326	CGUUCUCCAGGAAAGCCGAUG	2'-O-ME-PS	21	53.2
EB-73-AON 1	8411	UCUCCAGGAAAGCCGAUGGGGCCC	2'-O-ME-PS	24	70.2
EB-73-AON 2	8731	AGCCCGCGUUCUCCAGGAAAGCCGA	2'-O-ME-PS	25	63.8
EB-73-AON 3	8557	GUCGCCCUUCAGCCCGCGUUCUCCA	2'-O-ME-PS	25	58.5
EB-73-AON 4	7926	ACGGUCGCCCUUCAGCCCGCGUU	2'-O-ME-PS	23	59.8

EB-73-AON 5	**8739**	**CCCCUGAGGGCCAGGGUCUCCACGG**	**2'-O-ME-PS**	**25**	**72.4**
EB-73-AON 6	**8786**	**CAGACCAGGUGGCCCCUGAGGGCCA**	**2'-O-ME-PS**	**25**	**74.8**
EB-73-AON 7	**8409**	**CCAAGGGCCAGACCAGGUGGCCCC**	**2'-O-ME-PS**	**24**	**75.5**
EB-73-AON 8	**8762**	**CCAGACCAGGUGGCCCCUGAGGGCC**	**2'-O-ME-PS**	**25**	**77.2**
EB-73-AON 9	**7660**	**UCUCCCCAAGGGCCAGACCAGG**	**2'-O-ME-PS**	**22**	**69.4**
EB-73-AON 10	**8459**	**GGAAGGCCCGGGGGGGCCCCUCUC**	**2'-O-ME-PS**	**24**	**79.2**
EB-73-AON 11	**8545**	**CCGGCAAGGCCGGAAGGCCCGGGG**	**2'-O-ME-PS**	**24**	**75.5**
EB-73-AON 12	**8,348**	**AGGCUUUCCAGGCUCCCCGGCAAG**	**2'-O-ME-PS**	**24**	**64.8**
EB-73-AON 13	**8373**	**CGGGAAUACCAGGCUUUCCAGGCU**	**2'-O-ME-PS**	**24**	**68.3**
EB-73-AON 14	**8077**	**UGCCUGGGAGCCCGGGAAUACCA**	**2'-O-ME-PS**	**23**	**70.0**
EB-73-AON 15	**8243**	**CCCACACCCCCAGCCCUGCCUGGG**	**2'-O-ME-PS**	**24**	**75.6**
EB-73-AON 16	**7413**	**CCUCUCCCACACCCCCAGCCCU**	**2'-O-ME-PS**	**23**	**68.3**
EB-73-AON 17	**7762**	**UCUCUCCUGGCCUUCCUGCCUCU**	**2'-O-ME-PS**	**24**	**60.0**
EB-73-AON 18	**7408**	**CACCCUCUCUCCUGGCCUUCCU**	**2'-O-ME-PS**	**22**	**59.3**
EB-73-AON 19	**7471**	**CCAGCCUCACCCUCUCUCCUGG**	**2'-O-ME-PS**	**22**	**62.9**
EB-73-AON 20	**8075**	**CUCCAGGAAAGCCGAUGGGGCCC**	**2'-O-ME-PS**	**23**	**69.9**
EB-73-AON 21	**7740**	**UCCAGGAAAGCCGAUGGGGCCC**	**2'-O-ME-PS**	**22**	**70.1**
EB-73-AON 22	**7404**	**CCAGGAAAGCCGAUGGGGCCC**	**2'-O-ME-PS**	**21**	**69.8**

EB-73-AON 23	**8340**	**CTCCAGGAAAUCCGAUGGGGCCCU**	**2'-O-ME-PS**	**24**	**65.7**
EB-73-AON 24	**8451**	**UCCAGGAAAGCCGAUGGGGCCCUG**	**2'-O-ME-PS**	**24**	**69.9**
EB-73-AON 25	**8451**	**CCAGGAAAGCCGAUGGGGCCCUGC**	**2'-O-ME-PS**	**24**	**72.4**
EB-73-AON 26	**8515**	**AGGAAAGCCGAUGGGGCCCUGCAG**	**2'-O-ME-PS**	**24**	**71.7**
EB-73-AON 27	**8515**	**GAAAGCCGAUGGGGCCCUGCAGGA**	**2'-O-ME-PS**	**24**	**71.8**
EB-73-AON 28	**8492**	**AAGCCGAUGGGGCCCUGCAGGAGU**	**2'-O-ME-PS**	**24**	**71.9**
EB-73-AON 29	**8524**	**GCCGAUGGGGCCCUGCAGGAGUGG**	**2'-O-ME-PS**	**24**	**73.7**
EB-73-AON 30	**8197**	**GAUGGGGCCCUGCAGGAGUGGAA**	**2'-O-ME-PS**	**23**	**71.0**

Table S2 Primers and probes

Gene	FW 5'-3'	RV 5'-3'	Probe	Assay
GusB	GTTTTTGATCCAGACCCAGATG	GCCCATTATTCAGAGCGAGTA	TGCAGGGTTTCACCAGGATCCAC (HEX)	ddPCR
COL7A1	TCGGTTGCTGGAAACTGC	CACAGGCAGGAAGCTACC	ATCAAGGCATCTGCCCTGCGGGAG (FAM)	ddPCR
Δ73 COL7A1	GGTAGCTTCCTGCCTGTG	GCCATCTCTGCCCTGTTC	TCCCCGTTCTCCCTCCTTGCCTGG (FAM)	ddPCR
COL7A1	GCTGGCATCAAGGCATCT	TCCTTTCTCTCCCCGTTCTC	Na	PCR