QR-313, an Antisense Oligonucleotide, Shows Therapeutic Efficacy for Treatment of Dominant and Recessive Dystrophic Epidermolysis Bullosa: A Preclinical Study

JID Open

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Dystrophic epidermolysis bullosa (DEB) is a blistering skin disease caused by mutations in the gene *COL7A1* encoding collagen VII. DEB can be inherited as recessive DEB (RDEB) or dominant DEB (DDEB) and is associated with a high wound burden. Perpetual cycles of wounding and healing drive fibrosis in DDEB and RDEB, as well as the formation of a tumor-permissive microenvironment. Prolonging wound-free episodes by improving the quality of wound healing would therefore confer substantial benefit for individuals with DEB. The collagenous domain of collagen VII is encoded by 82 in-frame exons, which makes splice-modulation therapies attractive for DEB. Indeed, antisense oligonucleotide–based exon skipping has shown promise for RDEB. However, the suitability of antisense oligonucleotides for treatment of DDEB remains unexplored. Here, we developed QR-313, a clinically applicable, potent antisense oligonucleotide specifically targeting exon 73. We show the feasibility of topical delivery of QR-313 in a carbomer-composed gel for treatment of wounds to restore collagen VII abundance in human RDEB skin. Our data reveal that QR-313 also shows direct benefit for DDEB caused by exon 73 mutations. Thus, the same topically applied therapeutic could be used to improve the wound healing quality in RDEB and DDEB.

Journal of Investigative Dermatology (2021) **141,** 883–893; doi:10.1016/j.jid.2020.08.018

INTRODUCTION

Dystrophic epidermolysis bullosa (DEB) is a genetic blistering disorder, caused by mutations in the *COL7A1* gene encoding collagen VII. DEB can be inherited either recessively (RDEB) or dominantly (DDEB). In normal human skin, collagen VII forms anchoring fibrils that link the dermis to the epidermal basement membrane (Keene et al., 1987). Absence of functional collagen VII leads to impaired anchoring fibril formation, causing fragility of the skin and mucosal membranes. Apart from skin blistering, patients also suffer from mucosal erosions, esophageal strictures, progressive fibrosis, and ultimately aggressive squamous cell carcinoma (Fine, 2010). Currently, patient care is limited to palliative measures (El Hachem et al., 2014). However, several curative approaches are being evaluated in clinical trials, including autologous gene therapy with epidermal grafts of gene-corrected keratinocytes (KCs) (Eichstadt et al., 2019; Siprashvili et al., 2016) and injections of gene-corrected fibroblasts (Lwin et al., 2019). However, despite these positive advances, it is clear that complementary treatment options are urgently required (Bruckner-Tuderman, 2019).

Although DEB-causing mutations are spread rather evenly over the *COL7A1* gene, there are certain mutational hotspots, of which exon 73 is one. Mutations in this exon can cause DDEB or RDEB (Varki et al., 2007). RNA exon skipping is the modulation of splicing of a pre-mRNA and can be employed to omit specific exons from the mRNA. The *COL7A1* gene is an attractive candidate for exon-skipping therapies as the majority of its exons are short and can be individually removed without disturbing the open reading frame (Bornert et al., 2016). Exon skipping in *COL7A1* by the use of antisense oligonucleotides (AONs) has been successfully shown

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Abbreviations: Δ73 collagen VII, collagen VII lacking the amino acids encoded by exon 73; AON, antisense oligonucleotide; DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; DEB, Dystrophic epidermolysis bullosa; DEJ, dermal-epidermal junction; HSE, human skin equivalent; KC, keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; WT, wild type

Received 7 November 2019; revised 21 July 2020; accepted 12 August 2020; accepted manuscript published online 16 September 2020; corrected proof published online 17 November 2020

for exons 13, 70, 73, 80, and 105 (Bornert et al., 2016; Bremer et al., 2016; Goto et al., 2006; Turczynski et al., 2016). Thus far, work has focused on RDEB and little is known about the therapeutic potential of AONs for treatment of DDEB, although it represents around 40% of DEB cases (Fine, 2016). Recent natural history studies suggested no to even negative effects of exon skipping for DDEB (Bremer et al., 2019a). However, direct experimental assessment in a disease-relevant setting has not been performed. Specifically for RDEB, it has been shown that collagen VII synthesis and anchoring fibril formation can be restored following intradermal injections of nonclinical grade AONs targeting exon 73 in skin equivalents composed of compound heterozygous exon 73–mutated RDEB cells grafted onto mice (Turczynski et al., 2016).

Chronically injured and inflamed skin in DEB is associated with extreme pain after injections. Thus, injectable drugs that require repeated administrations are unfavorable treatment candidates. To allow for clinical translation of an AON as topical treatment for a substantial number of patients with DEB, we focused on developing an efficient topical delivery of therapeutically optimized AONs targeting exon 73. After reconfirming that collagen VII lacking the amino acids encoded by exon 73 remains functional, we designed QR-313, a therapeutically and manufacturability optimized AON targeting exon 73. To allow for safe topical delivery of clinically relevant concentrations of QR-313, the AON was formulated in a carbomer-based hydrogel. Topical application restored collagen VII abundance and epidermal-dermal cohesion, led to efficient distribution of QR-313 in the wound bed of wounded skin equivalents, and promoted specific skipping of exon 73. QR-313 was subsequently tested in exon 73–mutated DDEB KCs (DDEBKs), where it also showed evidence of therapeutic efficacy in ex vivo models. Collectively, these data provide a rationale for a clinical trial (NCT 03605069) evaluating topical delivery of QR-313 to wounds in people with mutations in *COL7A1* exon 73.

RESULTS

Preserved functional properties of homotrimeric collagen VII lacking amino acids encoded by exon 73

This study aimed to develop a clinically applicable AON for treatment of DEB. Toward this end, we focused on exon 73, which is the most frequently mutated in-frame exon in *COL7A1* (Mecklenbeck et al., 1999). Because the evaluation of potential therapeutics is more direct in the recessive setting, we first focused on RDEB. To mimic restoration of collagen VII production after targeting mutation-carrying exon 73 by AONs in a biallelic null mutation situation, we expressed homotrimeric collagen VII lacking the amino acids encoded by exon 73 (Δ73 collagen VII). Previous studies have indicated that Δ73 collagen VII remains functional (Turczynski et al., 2016). To support these previous observations, as subtle changes in stability and functionality of collagens can confer pathological activity (Zou et al., 2017), we performed careful biochemical assessment of Δ73 collagen VII. Δ73 collagen VII and wild-type (WT) collagen VII were secreted at similar levels (Figure 1a). Correctly folded collagen triple helices are resistant to trypsin proteolysis. Limited exposure of Δ73 vis-à-vis WT collagen VII to trypsin at 30 °C or higher indicated that the collagenous P1 domain of Δ73 collagen VII was folded into a thermostable triple helix (Figure 1a and b) (Bornert et al., 2016).

Changes in the collagen VII collagenous domain may alter binding to interaction partners (Bornert et al., 2016). Solid-phase binding assays to immobilized collagen IV or laminin-332 showed that Δ73 collagen VII bound both proteins with a similar affinity as WT collagen VII, whereas denatured collagen VII did not (Figure 1c). Additional support of retained functionality of Δ73 collagen VII came from its ability to support RDEB fibroblast (RDEBF) adhesion and RDEB KC (RDEBK) migration (Supplementary Figure S1), which can be used as a surrogate marker of its functionality (Bornert et al., 2016; Woodley et al., 2008).

To obtain in vivo support of functionality, 10 μg purified human WT or Δ73 collagen VII was injected twice intradermally into RDEB model mice. The skin was stained with antibodies specific for human collagen VII 30 days after the first injection (Kühl et al., 2015). WT and Δ73 collagen VII were both localized to the dermal-epidermal junction (DEJ) (Figure 1d). Transmission electron microscopy analyses revealed no clearly visible anchoring fibrils. However, both WT and Δ73 collagen VII partially rescued the width of the lamina densa (Figure 1e and f), which is known to be reduced in collagen VII hypomorphic mice (Nyström et al., 2013). Together, our analyses did not disclose any discernable differences in the level of functionality between homotrimeric WT and Δ73 collagen VII.

AON QR-313 can efficiently skip exon 73 and promote collagen VII resynthesis in vitro

Exon 73 covers 201 base pairs, making it the largest of the collagenous domain–encoding *COL7A1* exons, which could allow flexibility in the design of AONs targeting this exon. However, exon 73 is flanked by two short introns (86 and 96 base pairs, respectively) and is followed by the short exon 74 (36 base pairs). This arrangement is a challenge for AON design, as coskipping of exon 74 when targeting exon 73 has been observed before (Turczynski et al., 2016). Partial coremoval of exon 74 would create an additional collagen VII polypeptide that could confer negative effects and should thus be avoided. To develop clinically applicable AONs, we performed in silico analyses, taking into account the optimal placement, length, and sequence features of the AONs (Supplementary Figure S2 and Supplementary Table S1). The analysis yielded several candidates, which were synthesized and screened for exon 73 skipping efficacy and absence of exon 74 coskipping. Because of high exon skip efficiency and favorable chemical composition, QR-313 was selected for further development (Figure 2a).

Dose-dependent exon 73 skipping efficacy of QR-313 was observed in human WT fibroblasts (normal human fibroblast cells 1 and normal human fibroblast cells 2) and RDEBFs compound heterozygous for exon 73 mutations (RDEBF-muE73-1 and RDEBF-muE73-2) after transfection with 0 to 200 nM QR-313 (Figure 2a). QR-313 was slightly more potent in RDEBFs than in control fibroblasts (half maximal effective concentration, 9.23 ± 1.7 nM and 12.5 ± 1.4 nM,

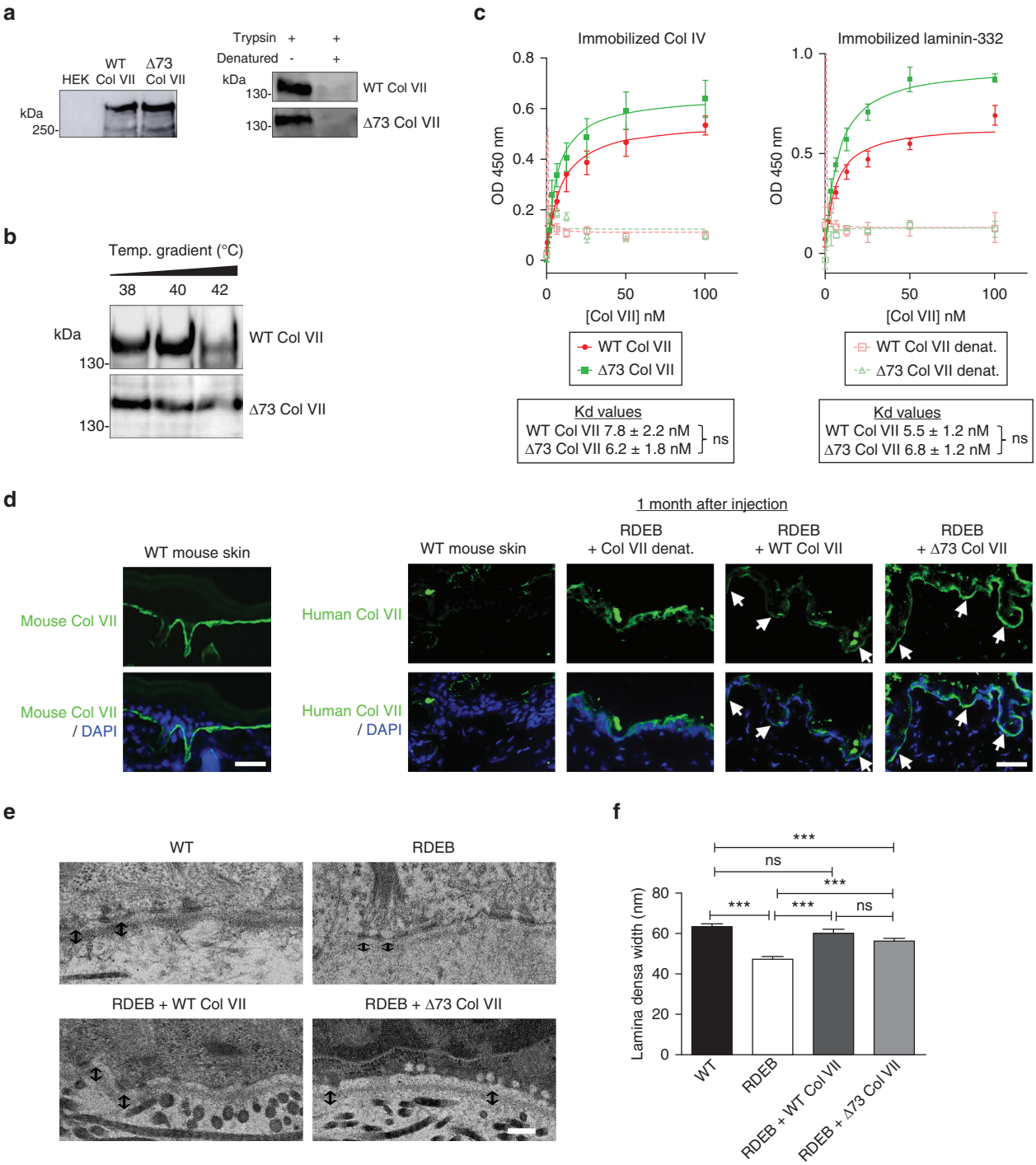

Figure 1. Deletion of exon 73 maintains the intrinsic properties of collagen VII. (**a**) Western blot on conditioned medium from control HEK293 cells or cells expressing WT or Δ73 Col VII shows that both proteins are secreted at the same level in transfected cells. The stability of the triple helical structure of Col VII was assessed by limited trypsin digestion of native or heat-denatured Col VII. Well-folded Col VII collagenous domain is resistant to trypsin proteolysis. Blots were probed with an antibody detecting the collagenous P1 domain. (**b**) Limited trypsin digestion using a temperature gradient demonstrates that Δ73 Col VII has a T_m of around 42 °C, which is similar to WT Col VII. (**c**) Solid phase binding experiments reveal that Δ73 Col VII shows similar affinity for Col IV and laminin-332 as WT Col VII. (**d**) Left, staining of WT mouse skin for Col VII. Right, skin section of WT and RDEB mouse skin 1 month after intradermal injections of PBS, WT Col VII, and Δ73 Col VII (10 μg) stained with an antibody specific for human Col VII (Kühl et al., 2015). Similar deposition of WT and Δ73 Col VII can be seen at the DEJ in injected skin. Nuclei were counterstained with DAPI. Bar = 100 μm. (**e**) Transmission electron microscopy analysis of skin as in (d), same section reveals a normalization of the lamina densa after injection of WT and Δ73 Col VII. Bar = 100 nm. (**f**) Bar graph showing quantification of the lamina densa in images as in (**e**), 10 images per condition. (**c**) and (**f**), values represent mean ± SEM, ***$P < 0.001$, Student's *t*-test. Δ73 collagen VII, collagen VII lacking the amino acids encoded by exon 73; Col, collagen; DEJ, dermal-epidermal junction; denat., denatured; ns, not significant; OD, optical density; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.

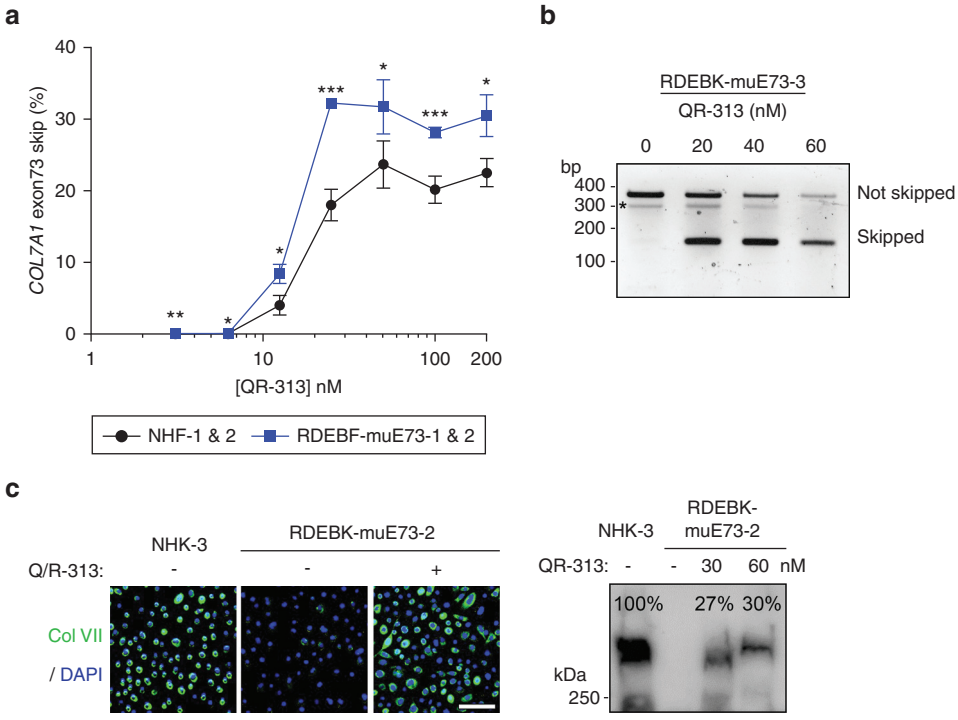

Figure 2. Efficacy of QR-313 for exon 73 targeting and Col VII reexpression. (**a**) ddPCR analysis of two WT fibroblasts and fibroblasts from RDEB donors with a compound heterozygous mutation in exon 73 transfected with QR-313 at concentrations ranging from 3.1 to 200 nM. (**b**) QR-313 is able to skip exon 73 carrying a homozygous frameshift mutation (c.6081delC; RDEBK-muE73-3) in a dose-dependent manner (20, 40, and 60 nM). Asterisk indicates unspecific product. (**c**) QR-313 is able to restore Col VII production in RDEBK-muE73-3. KCs stained for Col VII (green). Nuclei counterstained with DAPI. Bar = 100 μm. Western blot on RDEBK-muE73-3 conditioned medium (200 μl loaded per lane) revealed accurate secretion of Col VII in AON-treated RDEBK-muE73-3 KCs at 27% and 30% of WT KC (NHK) levels for 30 and 60 nM QR-313, respectively. (**a**) Values represent mean ± SEM, $*P < 0.05$, Student's *t*-test. AON, antisense oligonucleotide; Col, collagen; ddPCR, Droplet Digital PCR; KC, keratinocyte; NHF, normal human fibroblast; NHK, normal human keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; WT, wild type.

respectively) and reached higher exon 73 skipping levels at plateau in both tested donors (Figure 2a).

Next, the response to QR-313 was assessed in KCs homozygous for an exon 73 mutation (RDEBK-muE73-3). Correct exon 73 skipping was confirmed by PCR, followed by sequencing (Figure 2b and Supplementary Figure S3a). Immunofluorescence staining revealed a QR-313–mediated collagen VII restoration in RDEBK-muE73-2 cells (Figure 2c). Further, western blot analysis on AON-treated RDEBK-muE73-3 cell supernatants revealed restoration of collagen VII, at levels corresponding well to exon 73 skipping efficacy (27% and 30% of WT levels for 30 and 60 nM QR-313, respectively) (Figure 2c). Testing of KCs from one additional RDEB donor with a heterozygous mutation in exon 73 (RDEBK-muE73-1) indicated donor independency of QR-313–mediated collagen VII restoration (Supplementary Figure S3b). Altogether, our data supported QR-313 to be a good candidate for development of AON-based exon 73 skipping therapy.

Topically delivered QR-313 induces exon 73 skipping

To assess the ability of QR-313 to functionally restore skin cohesion in a tractable human model, we employed human skin equivalents (HSEs). The HSEs were composed of either RDEBK-muE73-2 together with completely collagen VII–deficient RDEBFs (RDEBF-1) (Table 1) or normal human KCs and normal human fibroblasts. At the start of coculture and every other day until the end of the experiment, 100 nM QR-313 was added dropwise to RDEBK-muE73-2. Analyses of HSEs after three weeks showed that QR-313 significantly restored collagen VII abundance at the DEJ (Figure 3a). QR-313 significantly reduced epidermal-dermal separation of the HSEs (Figure 3b), showing that QR-313 functionally promotes strengthening of skin integrity.

A prerequisite for a sustainable DEB therapy is efficient delivery of the drug candidate without frequent injections. Delivery through the epidermis remains a challenge. However, substantial benefits could be obtained without transepidermal delivery because DEB is associated with a high wound burden, and topical delivery to the healing edges of the wound epithelium is ideal in that this is where the new DEJ assembles and consequently constitutes an area of increased collagen VII synthesis (Nyström et al., 2013).

To enable topical application and rapid translation into the clinics, QR-313 was formulated into clinically approved gel formulations including hypromellose- and carbomer-based gels. Given close similarities in the barriers of porcine and human skin, ex vivo porcine skin can be used as a good model for evaluation of transdermal drug delivery (Abd et al., 2016). Based on the best diffusion into the dermis by following the dermal distribution of Cy-5–conjugated QR-313 after topical application onto porcine wounds (not shown), we selected a carbomer-based hydrogel. Next,

Table 1. Characteristics of Cells Used in This Study

Code	Donor Type	Mutations	Remarks
NHK-1, 2, 3, and 4	WT	—	Immortalized KCs
NHF-1, 2, 3, and 4	WT	—	Primary fibroblasts
RDEBK-muE73-1	RDEB, heterozygous recessive mutation in exon 73	c.425A>G, c.6022C>T	Primary and immortalized KCs
RDEBK- muE73-2	RDEB, homozygous recessive mutation in exon 73	c.6081 delC, c.6081 delC	Immortalized KCs
RDEBK-3	RDEB severe generalized, completely collagen VII deficient	c.425A>G, c.425A>G	Immortalized KCs
RDEBF- muE73-1	RDEB, heterozygous recessive mutation in exon 73	c.6176A>G, c.6501G>A	Primary fibroblasts
RDEBF- muE73-2	RDEB, heterozygous recessive mutation in exon 73	c.6176A>G, c.6501G>A	Primary fibroblasts
RDEBF-1	RDEB severe generalized, completely collagen VII deficient	c.425A>G, c.425A>G	Primary fibroblasts
DDEBK-muE73-1	DDEB, dominant mutation in exon 73	c.6118C>A	Immortalized KCs
DDEBK-muE73-2	DDEB, dominant mutation in exon 73	c. 6127 G>A	Immortalized KCs

Abbreviations: DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; KC, keratinocyte; NHF, normal human fibroblast; NHK, normal human keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; WT, wild type.

topical application of QR-313 in a carbomer-based hydrogel to unwounded porcine skin at a concentration of 50 mg/cm^2 yielded no overt distribution of QR-313 in the epidermis or dermis, as revealed by FISH (Figure 3c, upper panels). In contrast, application of an equal amount to wounded porcine skin resulted in efficient distribution and uptake in epidermal tongues, wound bed, and adjacent dermis (Figure 3c, lower panels). These data show efficient distribution in the main collagen VII–producing cells during regeneration and thus support the therapeutic applicability of topical delivery of the QR-313 formulation.

Next, QR-313 was evaluated in wounded HSEs, composed of WT or exon 73–mutated KCs and fibroblasts (Table 1). To mimic DEB blister wounds, the epidermis was partly stripped from the center of the HSE. Directly after wounding, 50 mg/cm^2 QR-313 formulation or placebo gel was topically applied to the wound bed three times a week until harvesting. FISH staining for QR-313 revealed distribution primarily in the wound bed and adjacent dermis (Figure 3d). To assess the skipping efficacy of QR-313, different regions of HSE were dissected and analyzed by Droplet Digital PCR after one or two weeks of treatment. Skipping efficacy strictly followed exposure to QR-313 with an increase in skipping with time of treatment, and cells in the wound bed or adjacent dermis showed significantly higher skipping of exon 73 than epidermis or cells close to the intact DEJ (Figure 3e).

In HSEs, fibroblasts contribute minimally to collagen VII deposition at the DEJ (Marinkovich et al., 1993) (Supplementary Figure S4). Thus, despite good skipping efficiency in fibroblasts, given the low uptake of QR-313 in KCs in unwounded and wound-adjacent epidermis (Figure 3b), high levels of collagen VII abundance restoration at the DEJ was not to be expected. Nevertheless, careful analyses of immunostained sections suggested de novo expression and deposition of collagen VII by wound-adjacent RDEB KCs (Supplementary Figure S5a). Immunogold-labeled NC1 and NC2 domain-specific collagen VII antibodies (NP185 and LH24) in transmission electron microscopy revealed some NC1 domain positivity in QR-313–treated RDEB HSEs, but in contrast to control HSEs, NC2 positivity or anchoring fibrils could not be proven in the analyzed RDEB HSE sections (Supplementary Figure S5b and data not shown).

Therapeutic efficacy of QR-313 for DDEB

As previously mentioned, exon 73 is the most frequently mutated exon in DEB. However, most disease-causing mutations in this exon result in DDEB (Varki et al., 2007). Topical application of AONs could hold great potential for the treatment of DDEB because of the generally more localized and milder disease in this form of DEB. Treatment of DDEB, in which individuals carry one mutated allele and one WT allele, raises concerns for reduced functionality and/or dominant, disease-aggravating negative effects from the formation of WT and shortened polypeptide heterotrimers (Bremer et al., 2019a). Thus, AONs were not considered initially as potential therapeutics for DDEB. Despite these potential challenges, we assessed the therapeutic potential of QR-313 for DDEB. We first confirmed that AON QR-313 could efficiently induce correct skipping of exon 73 in KCs from donors with DDEB carrying a missense mutation in exon 73 (Figure 4a and Supplementary Figure S6). There was no notable difference in the potency of QR-313 for control fibroblasts and DDEB fibroblasts (half maximal effective concentration, 12.5 ± 1.4 nM and 11.5 ± 1.6 nM, respectively). At 100 nM, QR-313 promoted a similar level of exon 73 skipping in exon 73–mutated DDEBK as in DDEB fibroblasts (Supplementary Figure S7).

A biochemical hallmark of DDEB is impaired triple helical formation and reduced thermostability of collagen VII. Analyses of thermostability by limited trypsin digestion revealed that treatment with QR-313 led to increased resistance of DDEBK-produced collagen VII to trypsin proteolysis (Figure 4b and c).

To assess if the improved stability of collagen VII could lead to a functional improvement, we used KC adhesion and migration as in vitro surrogate markers. On tissue culture, plastic KCs would primarily bind to laminin-332; however, we have previously shown that collagen VII in this setting also is crucial for its correct deposition (Nyström et al., 2013). QR-313–treated DDEBKs showed significantly improved migration and adhesion (Figure 4d and e). No difference was seen for control KCs after transfection with QR-313, indicating no dominant negative effect of exon 73 skipping (Figure 4d and e). Treatment of exon 73–mutated DDEBKs with 100 nM QR-313 every other day in HSEs significantly

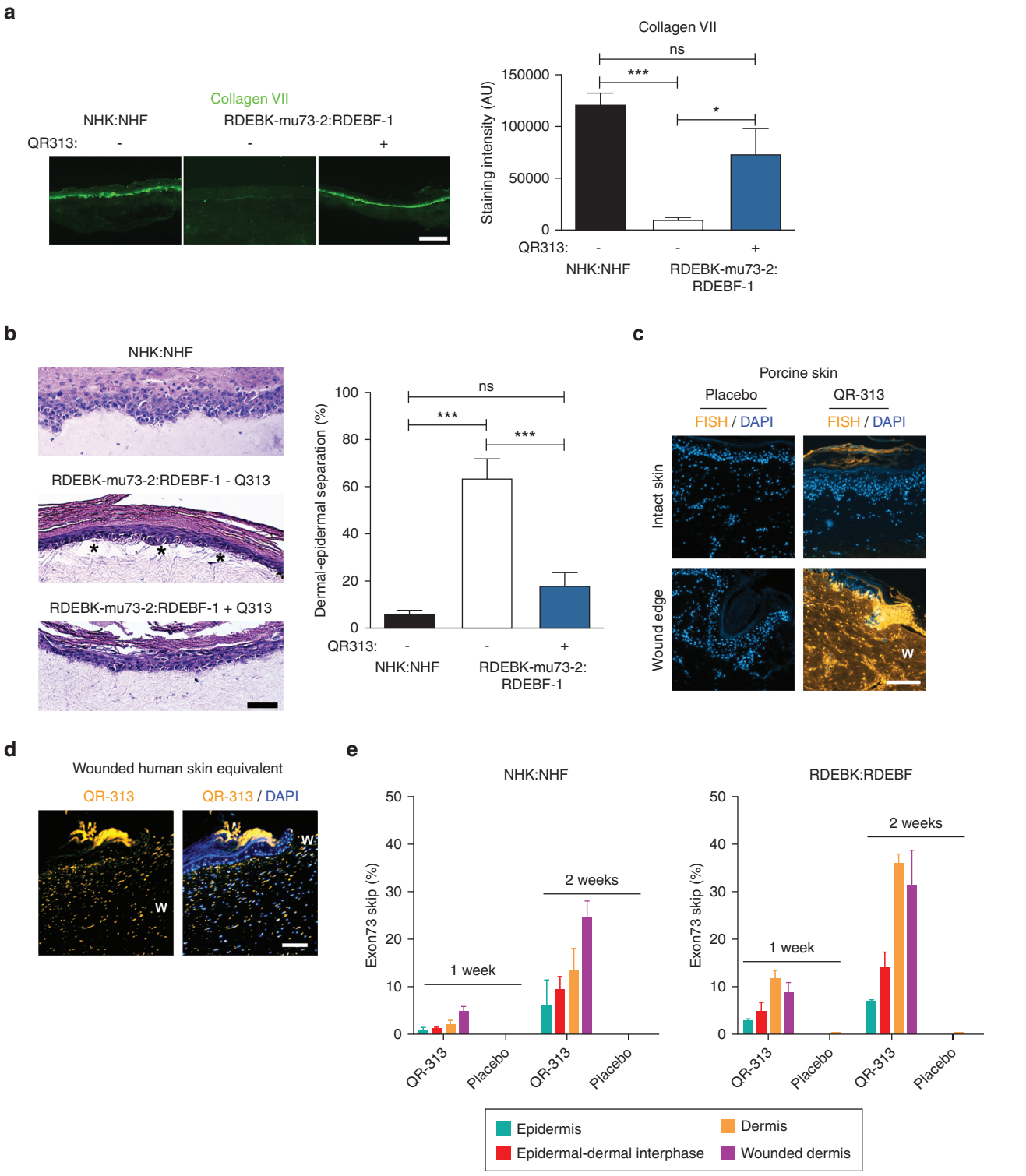

Figure 3. QR-313 restores skin cohesion and topical application of QR-313 to wounds is efficiently taken up by epidermal tongues and the wound bed and can promote exon 73 skipping. (**a**) HSEs composed of the indicated cell combinations, grown for 3 weeks ± 100 nM QR-313 every other day, stained for collagen VII (green). The bar graph shows quantification of collagen VII staining at the DEJ (n = 3 different replicates, 4 sections per replicate quantified). Bar = 100 μm. (**b**) H&E staining of HSEs as in (a). Note the clear epidermal separation (*) in the untreated and the increased epidermal attachment after QR-313 in RDEB HSEs. Quantification of the percentage of epidermal-dermal separation in HSEs (n = 3 different replicates, 4 sections per replicate quantified). Bar = 100 μm. (**c**) Ex vivo porcine skin was used to test the delivery of QR-313. After wounding of skin, QR-313 was applied to the wound in carbomer-based hydrogel and FISH was used to specifically detect QR-313. FISH reveals that QR-313 does not penetrate intact epidermis but is efficiently distributed in wounds. In wounds, QR-313 is mainly present in the epidermal tongues and the wound bed. In unwounded area, QR-313 can only be detected in the dermal area. Nuclei were counterstained with DAPI. Bar = 100 μm. (**d**) QR-313 delivered to wounded HSE reveals a limited uptake in epidermal KCs but good distribution in the dermis. Nuclei were counterstained with DAPI. Bar = 100 μm. (**e**) Percentage of exon 73 skipping detected after 1 and 2 weeks of treatment of wounded HSEs composed of WT KCs and fibroblasts or RDEBK and RDEBFs from a donor with a compound heterozygous mutation in exon73. Values represent mean ± SEM,

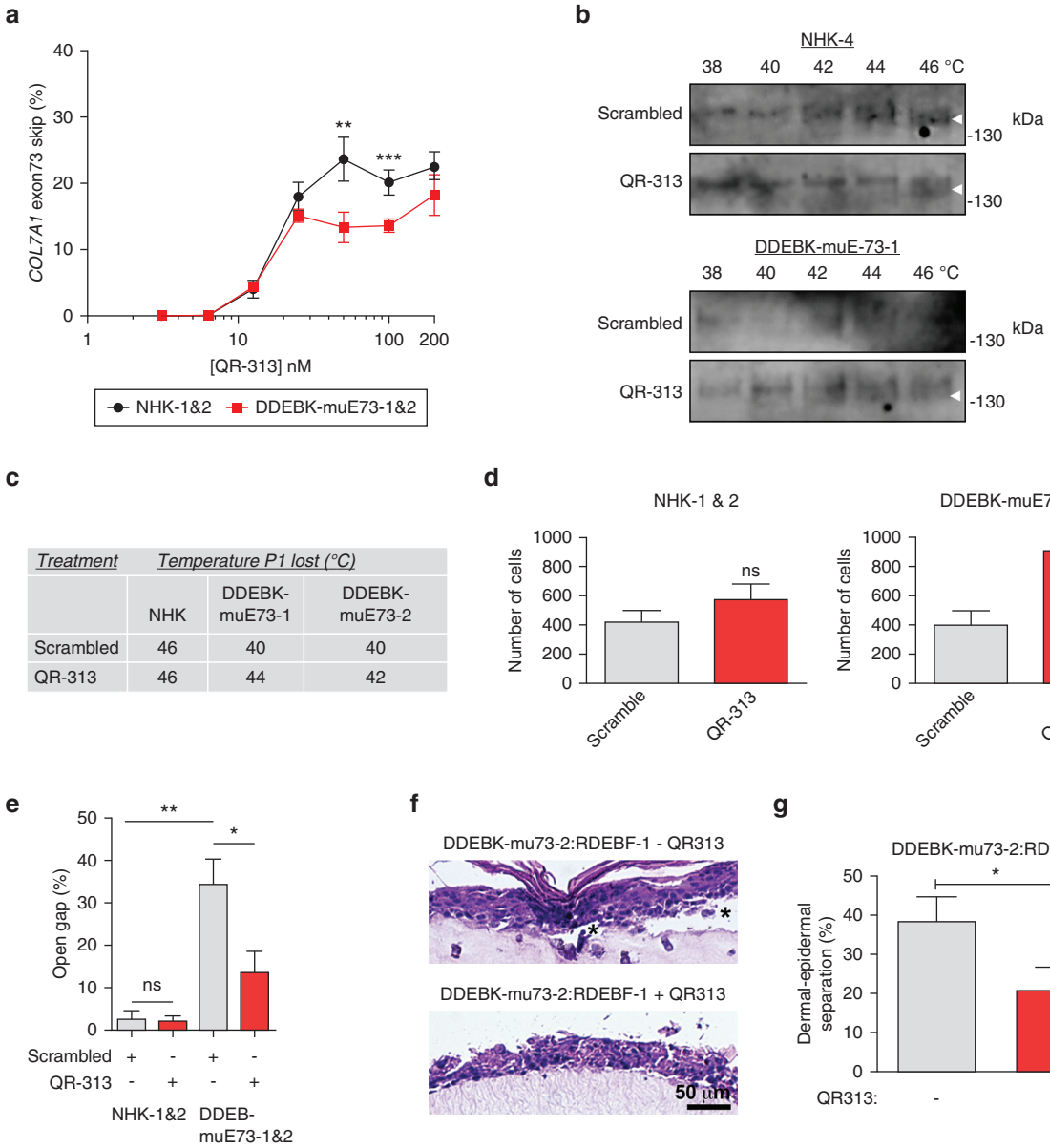

Figure 4. QR-313 shows benefit for DDEB. (**a**) QR-313 can efficiently skip exon 73 in DDEBKs from two donors with a mutation in exon 73 at the mRNA level in a dose-dependent manner. The graph shows that skipping efficacy in DDEB cells is around 20% at the concentration used (200 nM) in (b and c) limited trypsin digestion, (d) adhesion, and (e) migration assays. (**b**) Western blot of limited trypsin digestion assay of collagen VII of precipitated conditioned medium from scrambled or QR-313–treated control (NHK) or DDEBKs. Arrowheads show the collagenous P1 domain. Note the increased presence of P1 at higher temperature in DDEBKs treated with QR-313. (**c**) Table showing the temperature at which the P1 fragment completely disappears in KCs from one control (NHK) and two DDEB donors. (**d**) Adhesion of DDEBKs is increased after treatment with QR-313. In NHKs, the number of cells attached to the surface is similar in scramble and QR-313–treated cells. For DDEBKs, there are significantly more cells attached after QR-313 treatment than after scramble treatment. (**e**) Treatment with QR-313 improves migration of DDEBKs as compared with scramble treatment and, more importantly, has no negative effect on migration. KCs from two control and two DDEB donors were used. (**f**) H&E staining of HSEs composed of the indicated cells grown for 3 weeks ± 100 nM QR-313 every other day. Note the clear epidermal separation (*) in the untreated and the increased epidermal attachment after QR-313 in DDEB HSEs. Bar = 50 µm. (**g**) Quantification of the percentage of epidermal-dermal separation in HSEs (n = 3 different replicates, 4 sections per replicate quantified). (a), (d), (e), and (g) Values represent mean ± SEM. $*P < 0.05$, $**P < 0.01$, Student's *t*-test. DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; HSE, human skin equivalent; KC, keratinocyte; NHK, normal human keratinocyte; ns, not significant; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast.

reduced epidermal detachment (Figure 4f and g). Immunofluorescence staining showed a clear tendency to improved extracellular deposition of collagen VII in QR-313–treated HSEs (Supplementary Figure S8). Although the collagen VII deposition appeared rather broad and fuzzy, the data collectively indicate the improved epidermal-dermal

$***P < 0.001$, $*P < 0.05$, Student's *t*-test. AU, arbitrary unit; DEJ, dermal-epidermal junction; HSE, human skin equivalent; KC, keratinocyte; NHF, normal human fibroblast; NHK, normal human keratinocyte; ns, not significant; RDEB, recessive dystrophic epidermolysis bullosa; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; WT, wild type.

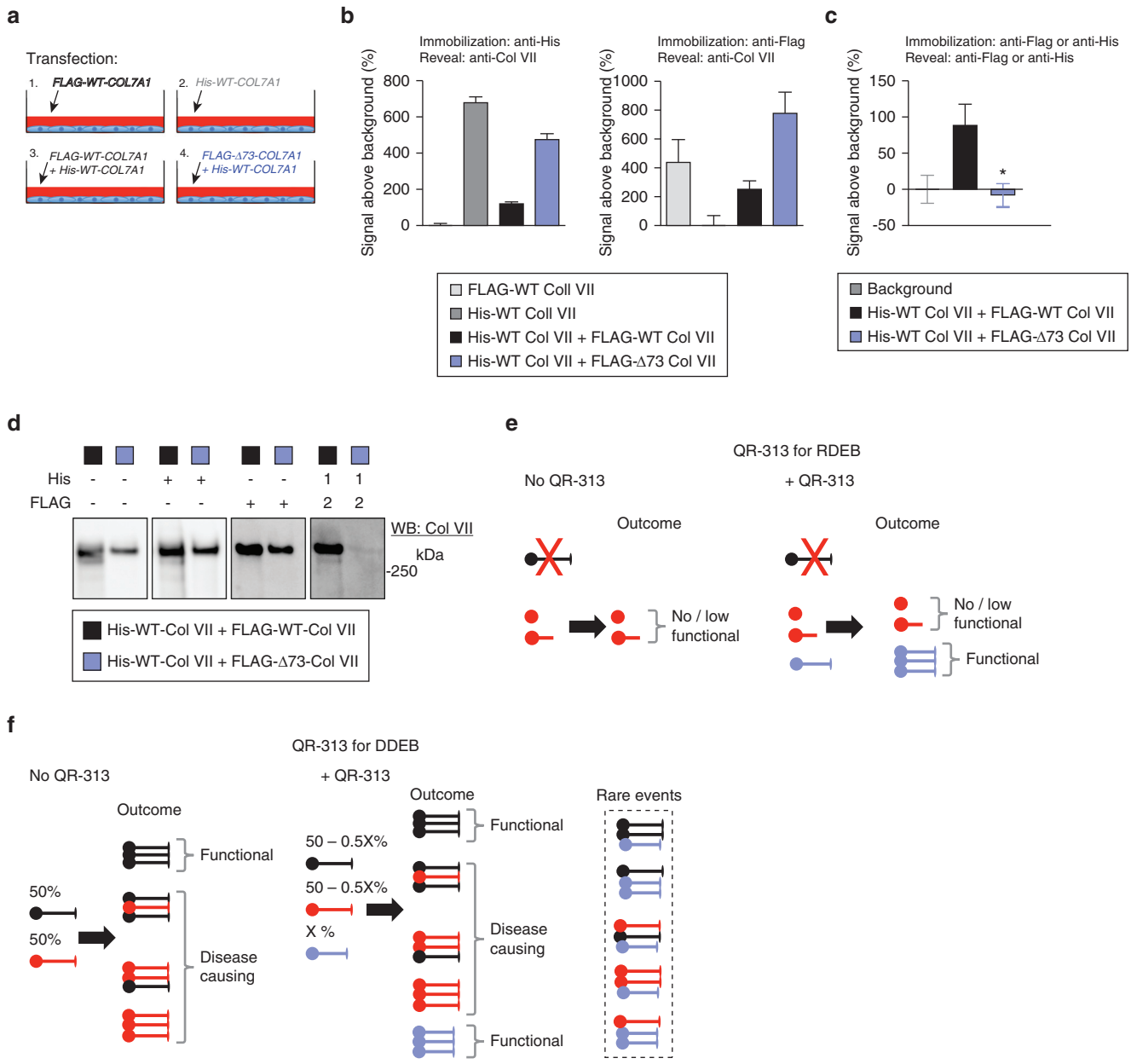

Figure 5. Limited interactions of Col VII α1 chains lacking amino acids encoded by exon 73 and WT Col α1 chains. (**a**) Schematic illustration of HEK-293 cell transfections. HEK-293 cells were either transfected with one *COL7A1* cDNA construct or two *COL7A1* cDNA constructs with different tags as shown. (**b**) Purified Col VII from cells as in (**a**) was trapped on microtiter plates by antibodies against His- or FLAG-tag. Trapped Col VII was revealed by Col VII antibodies. Results show specificity of His- or FLAG-antibody–mediated trapping of Col VII. Note the higher trapping of His-WT Col VII to FLAG-Δ73 Col VII. (**c**) Col VII combinations containing His- and FLAG-tagged α1 chains trapped with His or FLAG antibodies and then revealed by the opposite antibody, that is, after His trapping with FLAG antibodies and after FLAG trapping with His antibodies. Note the absence of specific signal for His-WT Col VII to FLAG-Δ73 Col VII despite higher abundance (**b**), indicating no or low presence of Col VII molecules composed of both WT and Δ73 Col VII α1 chains. Values in (**b**) and (**c**) represent mean ± SEM, $*P < 0.05$. (**d**) Col VII secreted from HEK-293 cells transfected with cDNA construct encoding the indicated Col VII combinations containing His- and FLAG-tagged α1 chains, as color coded, was affinity purified for either His- or FLAG-tag (− or +) or sequentially for His (1.) and then FLAG (2.). After purification, the products were analyzed by western blotting with a C-terminal Col VII antibody (NC2-10). (**e, f**) Schematic illustration of the disease-ameliorating actions of QR-313, that is, exon73 skipping, on the protein level for RDEB and DDEB, respectively. Δ73 collagen VII, collagen VII lacking the amino acids encoded by exon 73; Col, collagen; DDEB, dominant dystrophic epidermolysis bullosa; His, histidine; RDEB, recessive dystrophic epidermolysis bullosa; WB, western blot; WT, wild type.

attachment to be due to QR-313–evoked improvement of collagen VII functionality.

Finally, to elucidate a therapeutic course of action on the protein level after exon 73 skipping in DDEB, HEK-293 cells were singly or dually transfected with *WT-COL7A1* cDNA constructs carrying His- or FLAG-tags or with a FLAG-tagged *Δ73-COL7A1* cDNA construct (Figure 5a). ELISA for His- or FLAG-trapped collagen VII revealed that, whereas His- and FLAG-tagged WT collagen VII α1 chain polypeptides interacted, limited interaction occurred between WT and Δ73 collagen VII α1 chain polypeptides (Figure 5b and c). Single and sequential affinity purification further supported these

observations (Figure 5d). Furthermore, increased cellular stress, which could be a consequence of misfolded collagen molecules, did not occur in QR-313–treated control or DDEBKs (Supplementary Figure S9).

Taken together, our studies indicate efficacy and safety of QR-313 for treatment of DDEB caused by mutations in exon 73.

DISCUSSION

Here, we have developed a prospective topical wound healing therapeutic for treatment of exon 73–mutated DEB. By bolstering the functionality of the synthesized collagen VII, topical wound applications of the AON QR-313 should be able to improve wound healing significantly. QR-313 showed efficacy for both RDEB and DDEB. Using QR-313 for the treatment of RDEB and DDEB would meaningfully increase the patient population who could benefit from this therapy.

The potency of QR-313 was slightly higher in tested RDEB than DDEB and control cells. This could be a consequence of nonsense-mediated mRNA decay of the mutated transcript from the non–exon 73 mutation–carrying allele. In terms of maximal skipping efficacy, QR-313 reached 30–35% on mRNA level, resulting in a similar level of protein restoration. Previous studies on AON therapy for RDEB have reported protein restoration of 8–35% of WT levels (Bremer et al., 2016; Turczynski et al., 2016). This puts the effect of QR-313 on the higher end of AON studies for DEB. Even so, the level of collagen VII after QR-313 treatment would not be expected to reach that of unaffected heterozygous carriers. Nevertheless, reports indicate that a relatively modest increase in functional collagen VII has significant clinical benefit (Fritsch et al., 2008; Kern et al., 2009; Schwieger-Briel et al., 2015; Twaroski et al., 2019).

The data obtained herein reiterate and extend previous observations (Turczynski et al., 2016) showing that collagen VII lacking the amino acids contributed by exon 73 is functional in terms of retained folding, thermal stability, binding capacity, and DEJ incorporation. We did not address if the removal of these amino acids affected the flexibility of the collagen VII molecules. This could be a potential concern, as exon 73 encodes amino acids close to the hinge region that would provide flexibility to the molecule and could in future studies be addressed using, for example, rotary shadowing electron microscopy (Bächinger et al., 1990). Turczynski et al. (2016) showed that Δ73 collagen VII is capable of forming anchoring fibrils in vivo, suggesting that even if flexibility is reduced, anchoring fibril formation is still possible. However, these studies were performed in collagen VII–competent mice contribution and host-synthesized collagen VII could thus have influenced the results (Bremer et al., 2019b). QR-313–induced collagen VII expression promoted skin stability in exon 73–mutated RDEB and DDEB HSEs. However, we were not able to detect anchoring fibrils in our models. Reasons for this could relate to the fact that the timing may have been too short for anchoring fibrils to be formed under subphysiological collagen VII amounts. Studies from skin grafting of burn wounds have suggested that physiological anchoring fibril formation in the adult is a process that takes up to several months (Compton, 1992). In addition, the critical concentration of collagen VII needed to nucleate anchoring fibril formation may not have been reached. It has to be emphasized, though, that collagen VII may still support epidermal-to-dermal cohesion without assembling into well-formed anchoring fibrils (Bornert et al., 2016; Schwieger-Briel et al., 2015; Twaroski et al., 2019).

Topical application of AON-loaded gels efficiently delivered AONs to wounds but not intact skin. Given the high wound burden associated with DEB, this is nevertheless relevant for disease modulation. Restoration of collagen VII in healing wounds would stabilize healed skin, leading to higher protection from frictional damage and rewounding, and speed up wound healing (Nyström et al., 2013; Wang et al., 2013). Because collagen VII has an in vivo half-life of 1 to 2 months in homeostatic skin (Kühl et al., 2016), the skin-stabilizing benefits of AON treatment would ultimately wear off. However, the treatment would extend wound-free periods and, as such, reduce tissue damage and prolonged inflammation, which are risk factors for malignant transformation of KCs and progression to squamous cell carcinomas (Cho et al., 2018; Föll et al., 2018; Nyström and Bruckner-Tuderman, 2018).

Whereas therapy development for RDEB is seemingly straightforward by increasing the level of collagen VII (Figure 5e), treatment of DDEB has been considered more challenging because of dominant negative effects conferred by a mutated polypeptide. Our data indicate that Δ73 collagen VII and WT collagen VII polypeptides do not significantly interact (Figure 5), which could be due to the relatively large size of exon 73. The ultimate effect of QR-313 treatment would be depletion of disease-causing collagen VII molecules containing mutated polypeptides and thus reciprocal increase of functional collagen VII molecules—WT or Δ73 homotrimers (Figure 5f). This is in line with a previous study suggesting that increasing WT collagen VII polypeptide abundance could be effective as a treatment of DDEB (Fritsch et al., 2009). Future studies will have to determine if the benefit for exon 73–mutated DDEB is unique to the size and position of exon 73 or if exon skipping could also be effective for DDEB with other mutated exons.

To conclude, our results support the initiation of clinical trials with QR-313 as a topical therapeutic for the treatment of wounds in DEB, first for exon 73–mutated RDEB and subsequently for DDEB.

MATERIALS AND METHODS

For complete information, please see Supplementary Materials and Methods linked to the online version.

Ethics statement

Written informed consent was obtained before the use of healthy control and patient skin according to the Declaration of Helsinki. Breeding and experimentation of RDEB mice were approved by the regional ethics review board (Regierungspräsidium Freiburg, Germany), numbers G11/70 and G14/93.

Cells

Human cells used in this study are listed in Table 1. DEB donor cells were obtained from Dr Marinkovich (Stanford University) and from the EB House Austria in Salzburg. All cells were obtained after written informed consent.

WT and Δ73 collagen VII expression in HEK293 cells and purification

COL7A1 cDNA lacking exon 73 was generated from WT *COL7A1* cDNA using the Gibson assembly strategy as previously described (Bornert and Nyström, 2019) and expressed in HEK-293 cells.

Limited trypsin digestion

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

Solid-phase binding to collagen IV and laminin-332

Solid-phase binding assay was performed as previously described (Bornert et al., 2016) using increasing concentrations of recombinant collagen VII in solution and 500 ng of human collagen IV (Sigma-Aldrich, St. Louis, MO) or 100 ng of rat laminin-332 (Millipore, Burlington, MA) immobilized on Nunc Maxsorp 96-well plates (Thermo-Fisher Scientific, Waltham, MA).

Cell adhesion and migration assays

Cell adhesion and migration assays were performed as previously described (Bornert et al., 2016). A total of 10,000 KCs or fibroblasts per well were seeded for the adhesion assay and 100,000 KCs or fibroblasts per well were used for the migration assay.

HSE generation

HSEs were essentially assembled as previously described (Spörrer et al., 2019). For treatment with QR-313, 100 μl of 100 nM QR-313 in PBS was added dropwise 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 optimal cutting temperature compound 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.

Statistics

Data were analyzed with the GraphPad Prism software using Student's paired or unpaired *t*-test, with or without Welch's correction for unequal variances, when appropriate.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information file.

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CONFLICT OF INTEREST

JS, GP, and TR are employees of ProQR Therapeutics. DK has received consultancy fees from Armut Pharma Plc and Rheacell GmbH, which are unrelated to the manuscript content. The remaining authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by funds from Wings/ProQR grants from the German Research Foundation, (Deutsche Forschungsgemeinschaft [DFG]) (NY-90 2/1, NY-90 3/2, NY 90-5/1, SFB850 project B11, and SFB1160 project B03) to AN. DK was supported by funds from the EB research partnership. Further, this work was supported by DEBRA Austria. We want to thank Dr Hal Landy and Dr Deborah Ramsdell for corrections and input on the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization: UK, EMH, AN; Formal Analysis: OB, MH, JB, JS, SFT, DRK, SH, IH, VW, UK, EMH, AN; Funding Acquisition: DK, UK, EMH, AN; Investigation: OB, MH, PN, JB, JS, IA, SFT, DRK, EMM, SH, IH; Methodology: OB, EMM, JS, VW, UK, EMH, AN; Project Administration: GP, TR, UK, EMH, AN; Resources: DK, MPM, EMM, VW; Supervision: VW, UK, EMH, AN; Writing - Original Draft Preparation: OB, EMH, AN; Writing - Review and Editing: OB, MH, PN, JB, JS, SFT, DRK, DK, SH, MPM, IH, VW, UK, EMH, AN

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.08.018.

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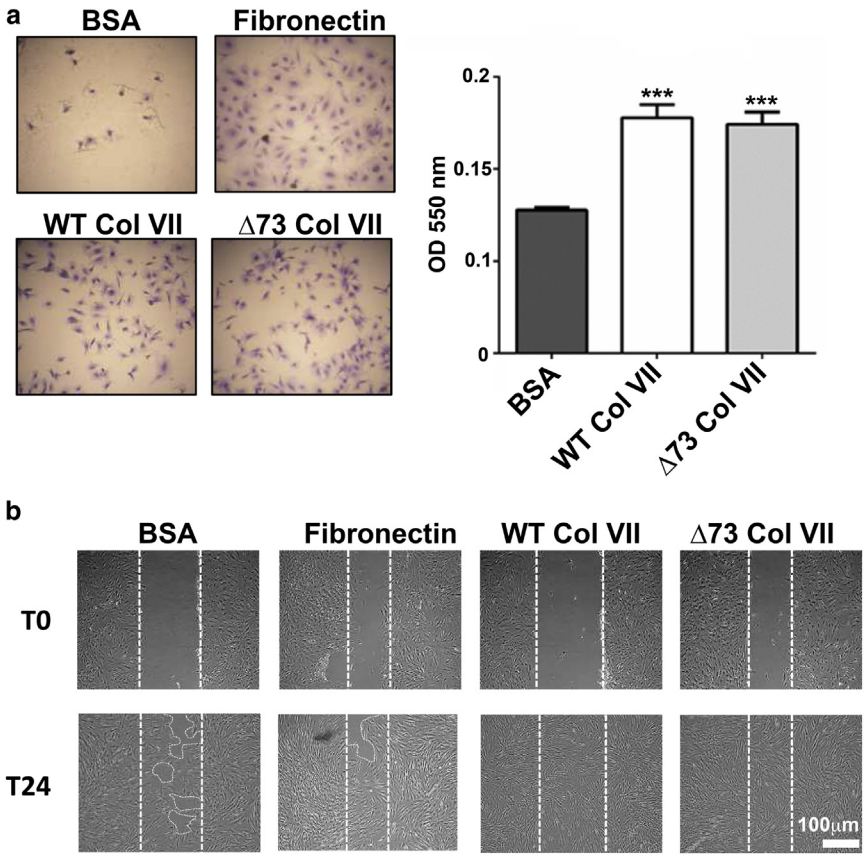
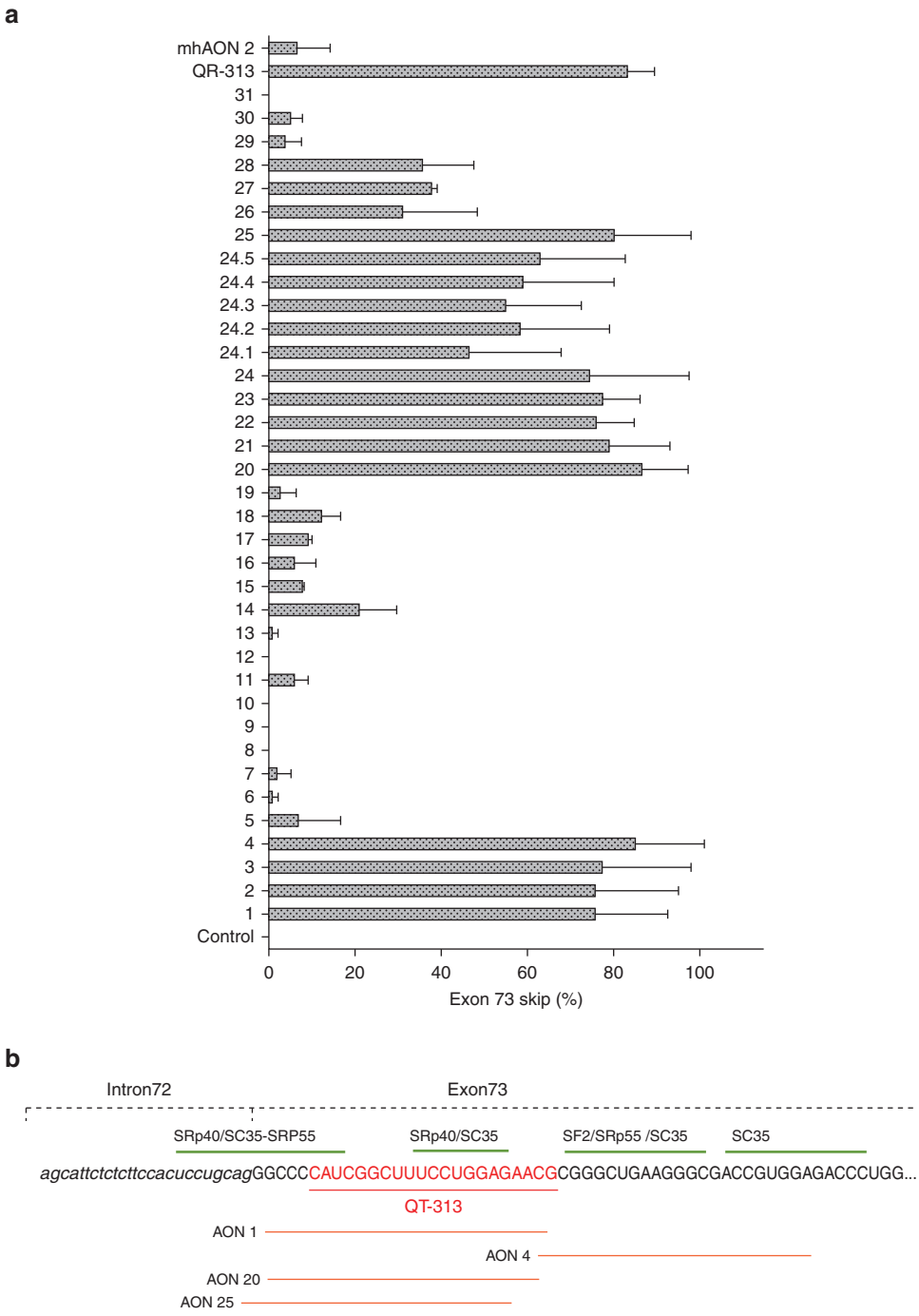
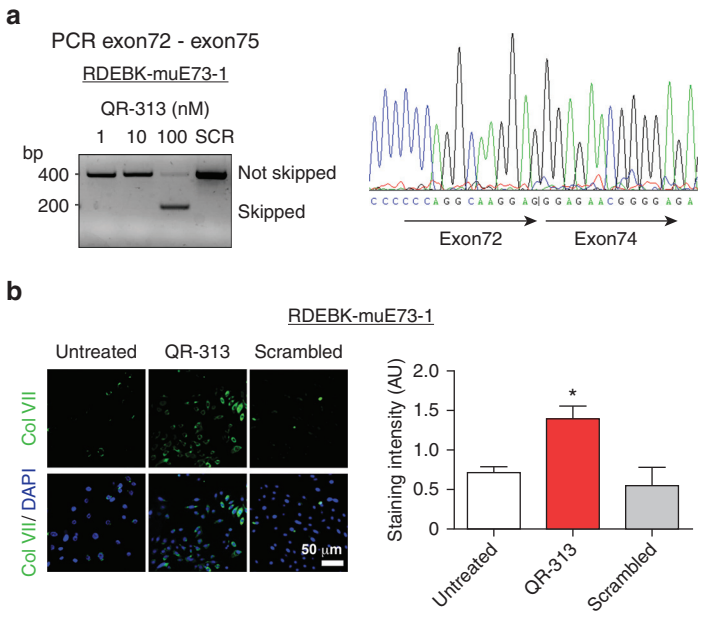


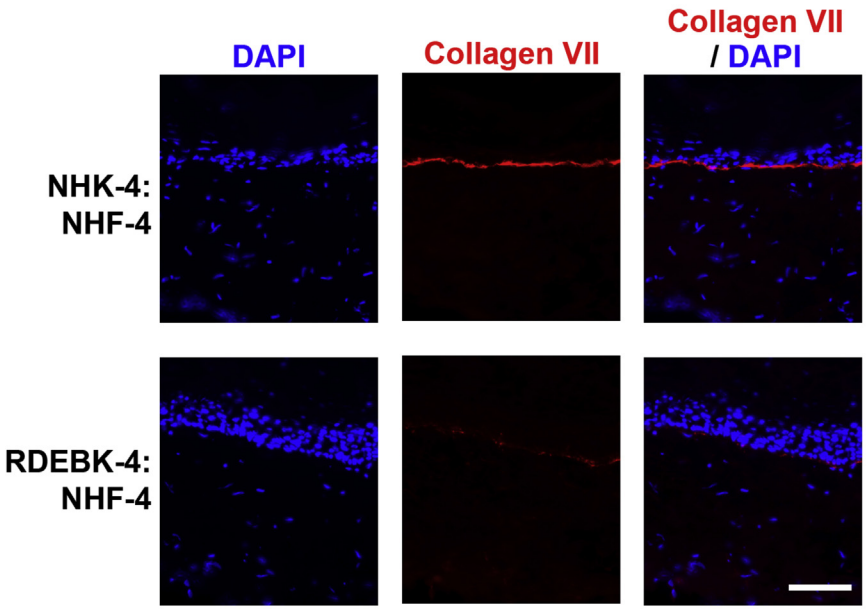
Supplementary Figure S1. Δ73 Col VII supports cell adhesion and migration. (**a**) Adhesion of RDEB fibroblasts on coated plates with BSA, fibronectin, WT Col VII, or Δ73 Col VII shows that deletion of the amino acids encoded by exon 73 in Col VII does not affect cell adhesion. Values represent mean ± SEM, $***P < 0.001$, Student's *t*-test. (**b**) In addition, migration of fibroblasts is equally supported by WT and Δ73 Col VII. Bar = 100 μm. Δ73 collagen VII, collagen VII lacking the amino acids encoded by exon 73; Col, collagen; OD, optical density; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.



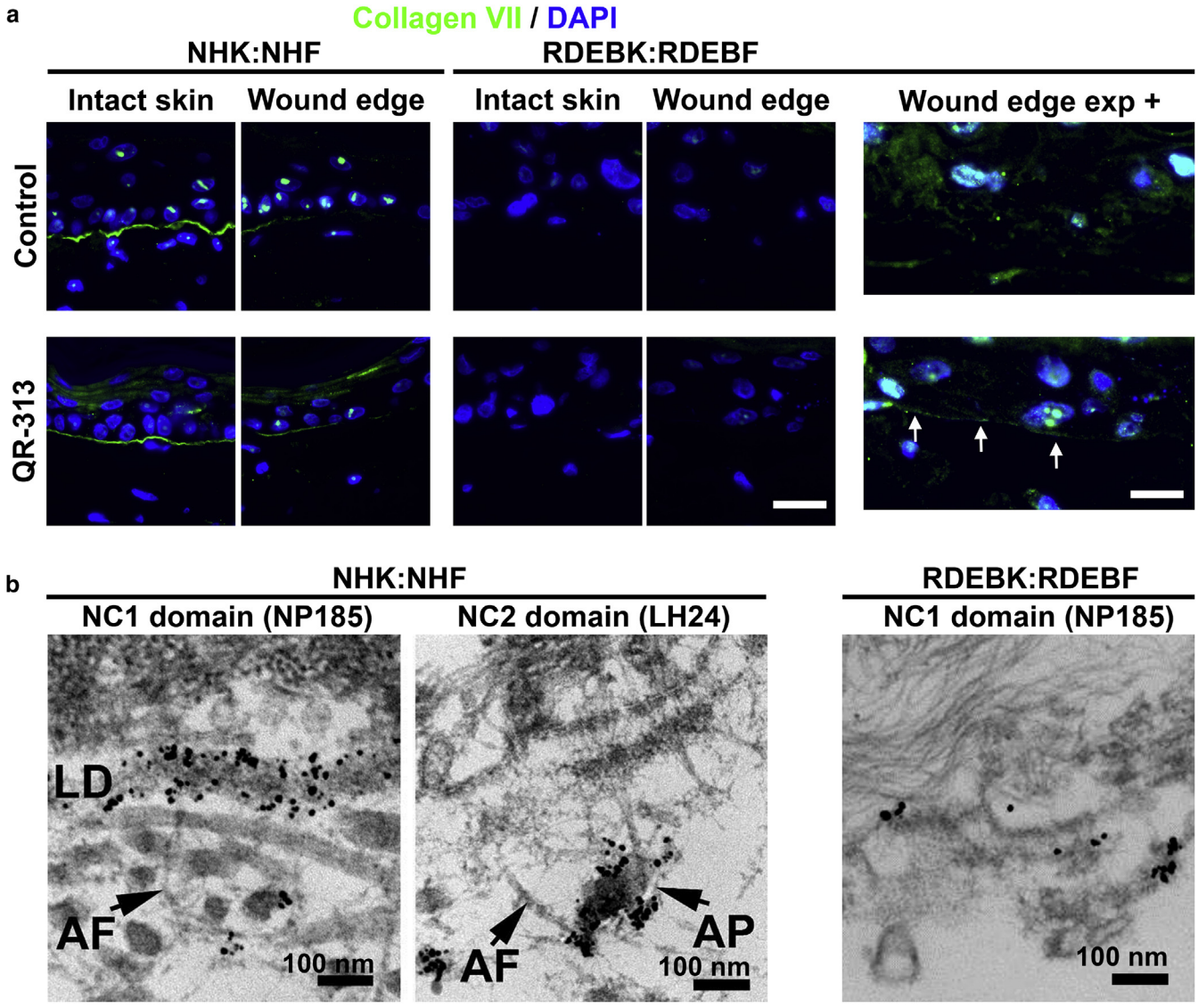
Supplementary Figure S2. Design of QR-313. (**a**) Bar graph showing exon 73 skipping efficacy in human primary pulmonary fibroblasts of screened AONs targeting exon 73 (AON1–31, AON24.1–24.5, QR-313, and a mouse-human homolog AON mhAON2). (**b**) The panel corresponds to the beginning of exon 73 with part of intron 72. In green, the predicted ESE binding sites are depicted; in orange, the binding location of the AONs with high exon-skipping efficacy. The binding location of QR-313 is indicated in red. AON, antisense oligonucleotide; ESE, exonic splicing enhancer.



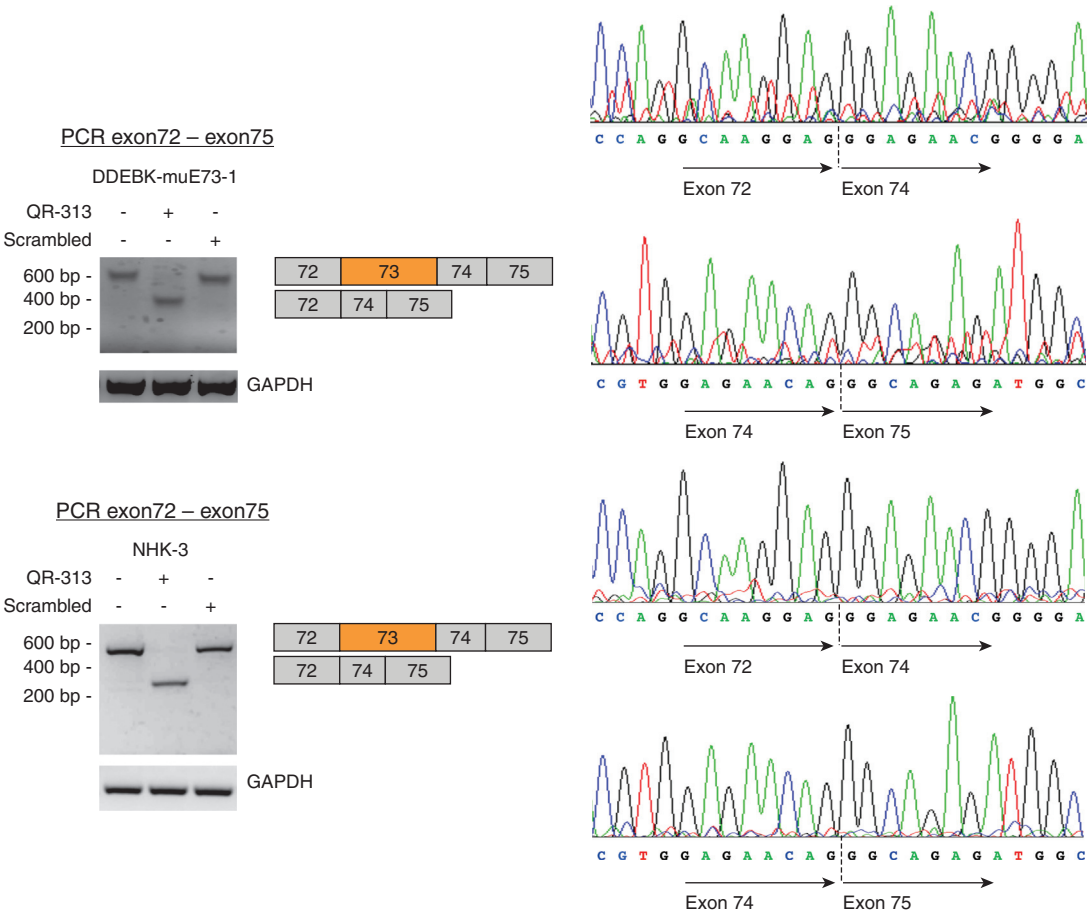
Supplementary Figure S3. QR-313 restores Col VII abundance in RDEBK-muE73-3. (**a**) KCs from control donors (NHK-1) and an RDEB donor with one heterozygous mutation in exon 73 (RDEBK-muE13-2) were treated with 1, 10, or 100 nM QR-313 or 100 nM scrambled AON. After 48 hours, mRNA was extracted and analyzed for exon skipping with primers binding in *COL7A1* exon 72 and exon 75. PCR products were sent for Sanger sequencing. (**b**) Left, cells from RDEBK-muE73-3 were left untreated or treated with 100 nM QR-313 or scrambled AON for 48 hours. After 48 hours, cells were stained for Col VII (green). Nuclei were stained with DAPI. Bar = 100 µm. Right, Quantification of three transfections as in (a). Values represent mean ± SEM, $*P < 0.05$. AON, antisense oligonucleotide; bp, base pair; Col, collagen; KC, keratinocyte; NHK, normal human keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; SCR, scrambled.



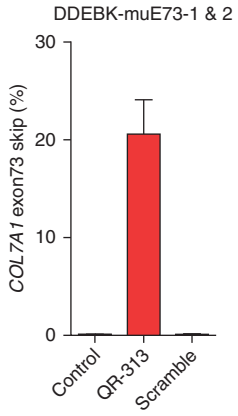
Supplementary Figure S4. Limited contribution of fibroblast-produced collagen VII to the DEJ in HSEs. HSEs cultured for 3 weeks stained for collagen VII (red). HSEs were composed of KCs from control healthy donors (NHKs) or donors with RDEB deficient in collagen VII expression (RDEBK) and fibroblasts from control donors with normal collagen VII expression (NHF). Note the low abundance of collagen VII (red) in RDEBK:NHF cocultures compared with NHK:NHF cultures despite collagen VII–producing NHFs. Nuclei counterstained with DAPI (blue). Bar = 100 µm. DEJ, dermal-epidermal junction; HSE, human skin equivalent; KC, keratinocyte; NHF, normal human fibroblast; NHK, normal human keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte.



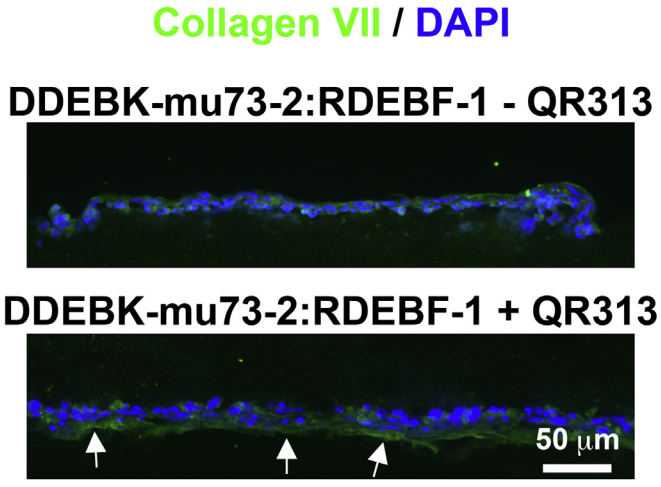
Supplementary Figure S5. QR-313 evokes some de novo deposition of collagen VII in RDEB HSEs. (**a**) Staining for collagen VII of HSEs after 2 weeks of treatment with control vehicle or QR-313. Clear signal for collagen VII at the DEJ is seen in HSEs from WT cells. Careful analysis of the wound edge of RDEB HSE treated with placebo or QR-313 shows a clear and specific signal for collagen VII only in QR-313–treated samples. Bar = 50 μm. (**b**) Transmission electron microscopy analysis of QR-313–treated HSE composed of control or RDEB donor cells stained with immunogold-labeled antibodies against collagen VII NC1 and NC2 domain indicate presence of NC1 and NC2 containing collagen VII anchoring fibrils in control HSEs. Only NC1 domain positivity was proven in QR-313–treated wounded RDEB HSEs. Bar = 100 nm. AF, anchoring fibrils; AP, anchoring plaques; DEJ, dermal-epidermal junction; exp, exposure; HSE, human skin equivalent; LD, lamina densa; RDEB, recessive dystrophic epidermolysis bullosa; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast; RDEBK, recessive dystrophic epidermolysis bullosa keratinocyte; WT, wild type.



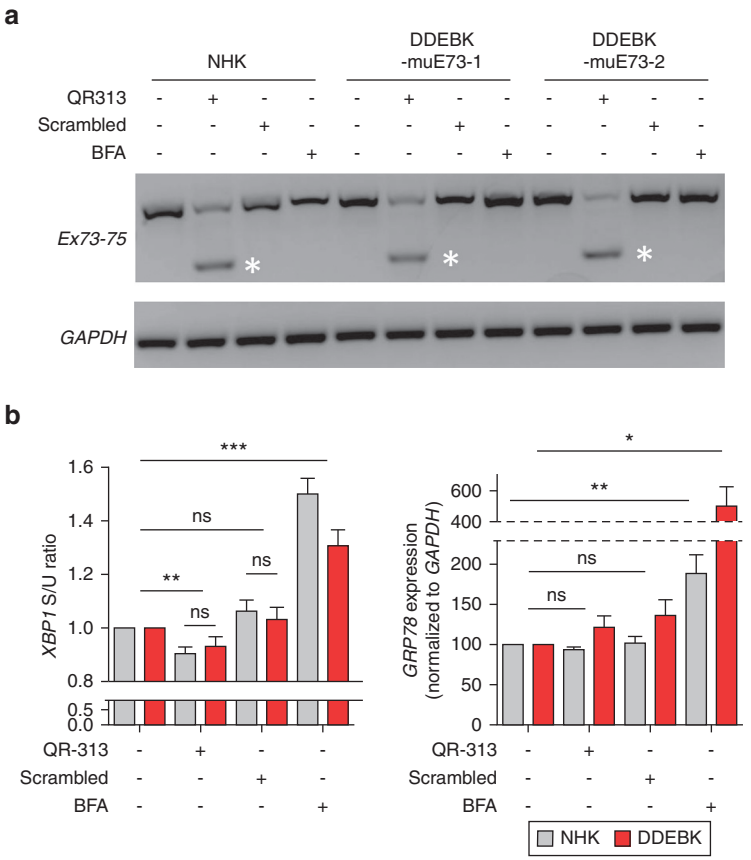
Supplementary Figure S6. QR-313 promotes correct skipping of exon 73 in exon 73–mutated DDEB KCs. Top left, agarose gel of PCR products from amplification of primers in exon 72 and exon 75 on mRNA extracted from exon 73–mutated DDEB KCs treated as indicated. Top right, sequence chromatogram from *COL7A1* PCR seen on the left side. Bottom left and right shows the same analysis for control KCs (NHK). bp, base pair; DDEB, dominant dystrophic epidermolysis bullosa; KC, keratinocyte; NHK, normal human keratinocyte.



Supplementary Figure S7. QR-313 promotes skipping of exon 73 in exon 73–mutated DDEB KCs. DDEB KCs with heterozygous mutations in exon 73 from two donors (DDEBK-muE73-1 and -2) treated with 100 nM QR-313 for 48 hours. Control cells were either left untreated or treated with 100 nM scrambled AON. After 48 hours, skipping of exon 73 was analyzed by ddPCR. Values represent mean ± SEM. AON, antisense oligonucleotide; DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; ddPCR, Droplet Digital PCR; KC, keratinocyte.



Supplementary Figure S8. QR-313 promotes collagen VII deposition in HSEs composed of exon 73–mutated DDEB KCs. HSEs composed of the indicated cells grown for 3 weeks ± addition of 100 nM QR-313 every other day, stained for collagen VII (green). Note the broader and clearer extracellular distribution of collagen VII in the HSE that had received QR-313. Bar = 50 μm. DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; HSE, human skin equivalent; KC, keratinocyte; RDEBF, recessive dystrophic epidermolysis bullosa fibroblast.



Supplementary Figure S9. QR-313 does not induce ER stress. KCs from a control donor (NHK) or two donors with exon 73–mutated DDEB (DDEBK-muE73-1 and -2) treated for 48 hours with QR-313; a scrambled AON; or BFA, a potent inducer of ER stress. (**a**) Agarose gel shows presence of unskipped or skipped exon 73 *COL7A1*; *GAPDH* was included as a control. (**b**) qPCR analyses of KCs as in (a) for ER stress markers *XBP1* S/U transcript and *GRP78*. Data confirms that BFA potently induces ER stress, whereas basal ER stress level in DDEB KCs is not increased compared with control KCs. This is likely a reflection of the low expression level of collagen VII. QR-313 does not elevate the *XBP1* S/U transcript ratio or the expression of *GRP78*, indicating no induction of ER stress. N = 2 control KCs and 2 DDEB KC donors, values represent mean SEM, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. BFA, bafilomycin A1; DDEB, dominant dystrophic epidermolysis bullosa; DDEBK, dominant dystrophic epidermolysis bullosa keratinocyte; ER, endoplasmic reticulum; KC, keratinocyte; NHK, normal human keratinocyte; ns, not significant; S/U, spliced/unspliced.