Exon skipping therapy for dystrophic epidermolysis bullosa
Bremer, Jeroen

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Natural exon skipping sets the stage for antisense oligonucleotide-mediated exon skipping as a therapeutic approach for dystrophic epidermolysis bullosa

Jeroen Bremer¹, Elisabeth H. Van der Heijden², Daryll S. Eichhorn¹, Rowdy Meijer³, Hans Scheffer³, Marcel F. Jonkman¹, Anna M.G. Pasmooij¹, Peter C. Van den Akker¹²

¹University of Groningen, University Medical Center Groningen, Department of Dermatology, Center for Blistering Diseases, Groningen, The Netherlands
²University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, The Netherlands.
³Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted.
Abstract
Dystrophic epidermolysis bullosa (DEB) is a devastating blistering disease affecting skin and mucous membranes. It is caused by pathogenic variants in the *COL7A1* gene, which encodes type VII collagen, and can be inherited dominantly or recessively. We have recently shown proof-of-principle for antisense oligonucleotide (AON)-mediated exon skipping as a therapeutic approach for DEB. The precise phenotypic effect to be anticipated from exon skipping, and which patient groups could benefit, is not yet clear. Here we anticipated on the therapeutic potential of exon skipping of *COL7A1* transcripts by studying naturally occurring exon skipping in DEB patients. To gain insight into the genotype-phenotype correlation of exon skipping variants, we reviewed the literature on *COL7A1* exon skipping variants. We also incorporated new clinical and molecular data on seven patients from the Dutch EB registry. In total, we found 26 *COL7A1* variants that cause natural exon skipping, of which 15 exert a dominant effect and 11 a recessive one. Phenotypes caused by dominant exon skipping cannot be distinguished from phenotypes caused by other dominant DEB variants. Recessive exon skipping phenotypes are generally relatively mild in the spectrum of recessive DEB. For dominant DEB, AON-mediated exon skipping is unlikely to ameliorate the phenotype. The overall severity of phenotypes associated with recessive natural exon skipping lie at the milder end of the spectrum. Consequently, we anticipate AON-mediated exon skipping for recessive DEB caused by bi-allelic null variants should lead to a clinically relevant improvement of this devastating phenotype.
Dystrophic epidermolysis bullosa (DEB) is a monogenetic, heritable skin disease caused by pathogenic variants in the *COL7A1* gene, which encodes the epidermal-dermal adhesion protein type VII collagen. DEB, which can be inherited either dominantly (DDEB, OMIM# 131750) or recessively (RDEB, OMIM# 226600), is characterized by blistering of skin and mucosae upon the slightest trauma. The severity of DEB is directly proportional to the quantity and functionality of type VII collagen that is expressed. Phenotypic severity ranges from involving only nails (DDEB-na) to generalized and severe blistering and scarring (RDEB-gen sev). In general, the prognosis of DDEB is much better than that of RDEB. Patients suffering from RDEB-gen sev often die at the age of 30-40 years due to complications of blister formation and aggressive squamous cell carcinomas evoked by the complete absence of type VII collagen. Patients suffering milder DEB phenotypes, in general, have a normal life span. Currently, several therapeutic strategies are under investigation, although no curative therapy has been translated into the clinic and treatment remains primarily symptomatic.

Type VII collagen is an extracellular matrix component that secures the epidermis to the papillary dermis by forming anchoring fibrils. The 118 exons of *COL7A1* are first translated into a pro-α1-type VII collagen molecule, which comprises an ~145 kDa amino-terminal non-collagenous 1 domain (NC1), an ~145 kDa central triple helix domain (THD), and an ~30 kDa carboxyl-terminal non-collagenous 2 domain (NC2). The NC1 domain contains regions that are essential for interactions to binding partners like type IV collagen and laminin-332. The THD contains a highly repetitive Glycine-Xaa-Yaa amino acid sequence (Gly-X-Y) that is essential for proper triple helix formation. The THD, encoded by exons 29-112, is interrupted 19 times, with the flexible, so-called intrinsically disordered, central 39 amino acid ‘hinge region’ being the major interruption. Post-translational modifications subsequently lead to triple helix formation of three pro-α1 chains, followed by cleavage of the NC2 domain and antiparallel dimerization. Extracellularly, the antiparallel dimers aggregate laterally to form anchoring fibrils.

DEB-causing variants are widely spread throughout the 118 exons of the *COL7A1* gene. In total, more than 700 DEB-causing variants in the *COL7A1* gene have been reported. Variants that lead to dominant phenotypes are exclusively located in the THD, where exon 73 is a hotspot. A single glycine substitution in the THD can impair the ability to form stable triple helices and is the main cause of DDEB. RDEB-causing variants, on the other hand, are found throughout the entire gene. RDEB-gen sev is caused by bi-allelic null variants, whereas milder RDEB phenotypes are due to at least one allele capable of producing an, albeit imperfect, pro-alpha type VII collagen. The precise phenotype is determined by the combination of both alleles and the dynamic interplay with the individual’s genetic background.

Multiple *COL7A1* variants are located in splicing signals and can disturb normal
splicing. Several lead to an entire exon being spliced out from the pre-mRNA, i.e. the exon is skipped. Of the 118 COL7A1 exons, 107 are in-frame and skipping of these exons will still leave the reading-frame intact. Because the exons of the COL7A1 gene are very small (27-201 bp), with an average length of 54 bp (NM_000094.3), only short sequences will be excluded from the mRNA by exon skipping and the expressed type VII collagen is predicted to be only slightly shorter.

The small exons, in combination with the highly repetitive sequence of the THD, make COL7A1 an ideal candidate for antisense oligonucleotide (AON)-mediated exon skipping. AON-mediated exon skipping aims to bypass disease-causing COL7A1 variants by removing the mutant exon and thereby restoring the expression of a functional type VII collagen. The strong correlation between type VII collagen expression and the clinical phenotype predicts that the slightest increase in type VII collagen deposition at the basement membrane zone (BMZ) should have marked effects on the phenotype. Recently, we have shown encouraging pre-clinical results with AON-mediated exon skipping as a systemic therapeutic approach for RDEB. The precise benefit of exon skipping therapy, and which patient groups would benefit from it, is not yet clear. We hypothesized that studying patients in which COL7A1 variants induce natural exon skipping would shed light on the therapeutic potential of AON-mediated exon skipping. We therefore scrutinized the Dutch EB registry for natural exon skipping variants and reviewed the literature on this class of variants. The overview of the natural exon skipping variants presented here sets the stage for further work on AON-mediated exon skipping for DEB.

Methods

Molecular analysis of patient material
Molecular analysis at the DNA and protein level was performed as previously described. In short: DNA, isolated from peripheral blood lymphocytes was subjected to variant analysis of the COL7A1 gene (GenBank accession no. NM_000094.3) by direct Sanger sequencing. Protein expression was examined by immunofluorescence (IF) microscopy on 4 mm cryosections using monoclonal LH7.2 antibody (Abcam, Cambridge, United Kingdom) and analyzed on a Leica DMRA microscope (Wetzlar, Germany). Randomly primed two-step RT-PCR was performed on RNA isolated from 40 mm skin cryosections, using PCR primers that bind to exons at least two exons upstream or downstream of the variant, in order to be able to identify potential skipping of multiple exons.

Dutch EB registry and literature review
To gain a complete picture of COL7A1 variants that cause in-frame exon skipping, we scrutinized the Dutch EB registry, the DEB registry (www.deb-central.org), and the literature on DEB and COL7A1. “COL7A1”, “COL7A1 splicing” and “COL7A1 exon skipping” were used as queries in a search on NCBI Pubmed (www.ncbi.nlm.nih.gov/pubmed). All variants that
led to skipping of complete exons were included, regardless of the presence of additional splice variations. We excluded variants that only led to splice patterns other than in-frame exon skipping (i.e. skipping of an out-of-frame exon, in-frame or out-of-frame deletions or insertions not involving an entire exon, or partial or full intron retention). Confirmation of exon skipping at the RNA level was a prerequisite for inclusion.

Results
Exon skipping variants found in Dutch EB registry
There is a large cohort of DEB patients registered at the Center for Blistering Diseases, University Medical Center Groningen (the Netherlands). In January 2017, there were 160 DEB patients, of which 92 had a dominant- and 68 a recessive form of DEB. Of these 160 patients, 22 carried variants located in splice signals, of which seven were confirmed to result in in-frame exon skipping at the RNA level by RT-PCR. An overview of the clinical presentation and IF staining shows a varied severity of phenotype and of type VII collagen expression levels (figure 1). Seven new patients and their families from the EB register are described briefly for the first time below.

The father and son of family 1 (Pt.1) presented with generalized blistering upon slight trauma, a typical generalized DDEB phenotype (DDEB-gen); they carried the heterozygous variant c.6181-6T>G in intron 73. It was predicted that this variant would disrupt normal splicing and exon 74 skipping was indeed confirmed at the RNA level (figure S1). Since in-frame skipping of exon 74 has been described as exerting a dominant negative effect over the wild-type allele and causing DDEB, we concluded this natural skipping of exon 74 would explain their DDEB phenotype and inheritance pattern.

The father and daughter of family 2 (Pt.2) presented with a DDEB-gen phenotype due to a heterozygous deletion of the last two nucleotides of exon 87 (c.6899_6900del). This variant abolishes the exon 87-intron 87 splice site, which results in deletion of the entire exon 87 (figure S1). A dominant negative effect of in-frame exon 87 skipping has been described and also explains their DDEB phenotype.

The index patient and her father in family 3 (Pt.3) presented with a typical DDEB-acral (DDEB-ac) phenotype (figure 1): nail dystrophy and blisters predominantly on hands, knees and feet. An intronic 21 bp deletion, c.6832-23_6832-3del, was identified heterozygously in intron 86 and was predicted to result in the loss of the intron 86 splice acceptor site. RT-PCR confirmed in-frame exon 87 skipping in this family, explaining their dominant phenotype (figure S1).

The index patient in family 4 (Pt.4) presented with generalized blistering upon minor trauma (DDEB-gen). The heterozygous variant c.7894-2A>G in intron 107 was found both in the patient and his affected mother and it was predicted to lead to the loss of the intron 107 splice acceptor site. RNA-analysis confirmed skipping of exon 108 (figure S1). Because no other variant was found and skipping of exon 108 has been described to
cause a dominantly inherited phenotype, this explained their phenotype.

DNA analysis of the index patient in family 5 (Pt.5) showed compound heterozygosity for the maternal synonymous c.4011G>A variant in exon 33 and the paternal c.7769G>A (p.(Gly2590Asp)) variant in exon 104. The maternal variant in exon 33 caused skipping of the exon, shown by RT-PCR (figure S1). The index patient presented with blistering after minor trauma from birth and the RDEB-gen intermed (generalized intermediate) phenotype. No skin abnormalities were observed in the parents on clinical examination.

DNA analysis in family 6 (Pt.6) revealed the homozygous c.2710+1G>A in the index patient. The parents were unaffected but the patient presented with an RDEB-gen intermed phenotype. RNA analysis showed that the variant c.2710+1G>A, located in intron 20, resulted in three transcripts: 1) in-frame skipping of exon 20, 2) alternative splicing using a cryptic splice donor 65bp downstream in intron 20 resulting in an out-of-frame transcript, and 3) the wild-type transcript (figure S1).

The index patient in family 7 (Pt.7) has two unaffected parents but presented with a recessively inherited DEB phenotype (RDEB-gen intermed), characterized by blistering of skin and mucosa upon the slightest trauma, with scar tissue formation and loss of nails. Variant analysis identified the c.1573C>T (p.Arg525*) null variant in exon 12 on the maternal allele and the c.8227-1G>C transversion in intron 110 on the paternal allele. RT-PCR revealed that the paternal variant resulted in the in-frame skipping of exon 111 (figure S1). Since no DEB features could be detected in the father on clinical examination, the in-frame deletion of exon 111 must have acted recessively, as described previously, in contrast to the in-frame deletions in families 1-4.

**Exon skipping variants described in literature**

Our literature search revealed another 19 variants that were confirmed at RNA level, to induce in-frame exon skipping, bringing the total to 26 (summarized in table 1 and figure 2). In total, variants causing natural in-frame exon skipping represent approximately 3-4% of all DEB-causing variants. These variants are involved in either dominantly or recessively inherited phenotypes.

In contrast to recessive exon skipping variants that were found throughout the gene, and in concert with classical DDEB-causing glycine substitutions, the 15 dominant exon skipping variants were located exclusively within the THD, predominantly in its carboxyl-terminal region. More specifically, they were all located in, or in the vicinity of, interruptions in the collagenous structure: either in or directly adjacent to the hinge region (exons 71 and 74, respectively), in close proximity to interruptions in the THD or the NC-2 domain (exons 87 and 108, respectively). Exon 87 (figure 3) represents a clear hotspot for dominant natural exon skipping variants: ten different variants lead to skipping of exon 87. Exon 87 precedes a short imperfection in the collagenous structure, located two
Natural exon skipping in the COL7A1 gene

Figure 1. Clinical and molecular phenotypes of seven DEB patients associated with natural exon skipping. Left column: DEB clinical phenotypes associated with natural exon skipping depicted by representative photographs of the hands. Immuno-fluorescence (IF) staining (40x) of type VII collagen at the basement membrane zone (BMZ) is shown compared to control (middle columns). Right column: IF detail of the BMZ revealing retention of type VII collagen by basal keratinocytes (asterisks) in Pt.1, Pt.2, Pt.3, Pt.4, and Pt.7. Scale bars: 25 µm.
amino acids downstream in exon 88, and a larger imperfection of 18 amino acids further downstream in exon 89.

Recessive exon skipping variants were scattered throughout the gene and there was no apparent distribution pattern related to interruptions of the glycine-repeat of the THD (figure 2). Exons 70 and 73 are located immediately upstream and downstream of the hinge region (encoded by exons 71 and 72). Exon 111 contains a minor interruption and is located only 15 amino acids upstream of the NC-2 domain, while exons 33, 53, 95, 106, and 107 are separated by 18, 48, 15, 108, and 90 amino acids from interruptions in the glycine-repeat, respectively.

Mechanisms underlying exon skipping

The mechanism underlying exon skipping for all variants is the disruption of constitutional splice signal sequences. The lack of strong, cryptic splice signals in the vicinity of the disrupted constitutive splice signals likely explains why skipping of entire exons occurs instead of other alternative splicing patterns. 33 From the total of 26 variants, 23 were located in splice-sites. A higher number of variants was found in splice donor sites than...

Figure 2. Overview of the COL7A1 gene and natural exon skipping variants. The upper pane shows an overview of the type VII collagen protein with the noncollagenous-1 (NC1), triple-helix (THD), and noncollagenous-2 domains (NC2). Dominant and recessive variants leading to natural exon skipping are indicated above and below the pane, respectively. Interruptions of the Gly-X-Y structure are indicated in grey to scale. The NC2 cleavage site is indicated (scissors). In the middle pane, the upper bar shows all 118 exons to scale with the corresponding nucleotide (Nt) and amino acid (AA) numbers; colors correspond to the respective protein domains encoded. The second bar shows the same exon structure of COL7A1 to scale, this time indicating the exons involved in natural exon skipping and exon skipping-candidates (black), other in-frame exon skipping-candidates (white), and ‘non-skippable’, out-of-frame exons (grey). The lower pane shows the relative location of crucial domains.

SP signal peptide; CMP cartilage-matrix protein motif; FN-III fibronectin-III (FN-III) like domains 1-9; VWA Von Willebrand factor A-like domain; C/P Cytein/Proline-rich motif; hinge, the intrinsically disordered hinge region; KM Kunitz-motif like domain.
acceptor sites: 17 versus 7, respectively. The higher prevalence of donor site variants than acceptor site variants was reported in a large cohort of splice site variants. There appeared to be no difference in mechanism between dominant and recessive variants. Two small deletions and one point variant, all located in the middle of exon 87, were shown to exert their exon skipping effect through disrupting exonic splicing enhancer sequences, which is the precise class of splicing signals that AONs aim to target to induce exon skipping.

**Genotype-phenotype correlation**

**Dominant exon skipping**

The dominant phenotypes that were observed with skipping of exons 71, 74, 87 and 108 cover almost the complete DDEB phenotypic spectrum from acral, pretibial, pruriginosa, to generalized DDEB. The pruriginosa form of DDEB is a rare DEB subtype characterized by marked itching and prurigo-like nodules predominantly on the pretibial area. The phenotypic spectrum of DDEB phenotypes caused by exon-skipping could not be explained by differences in the amount of detectable type VII collagen at the BMZ, as this was reported to be normal for 13 out of 15 variants. The two other variants led to slightly reduced and strongly reduced expression, and the acral and pruriginosa forms of DDEB, respectively. Dominant skipping of exons 71, 74 and 108 resulted in the pretibial, pruriginosa, and generalized forms of DDEB, respectively. Remarkably, skipping of exon 87 was associated with a variety of phenotypes, leading to DDEB-ac, DDEB-pt, DDEB-pr, and DDEB-gen (table 1). The overall clinical heterogeneity observed in dominant exon
skipping cases is also observed for glycine substitutions. To some extent, dominant skipping leads to the retention of type VII collagen in basal keratinocytes, as shown by IF (figure 1).

**Recessive exon skipping**

In total, 11 exon skipping variants were found to cause recessive phenotypes. All recessive RDEB phenotypes were observed, i.e. acral, pretibial, generalized intermediate and even the generalized severe form of RDEB. In contrast to dominant exon skipping variants, recessive variants resulted in varying levels of type VII collagen expression, ranging from complete absence to normal (table 1). As expected, the amount of type VII collagen expression correlated well with the associated phenotypes. Normal type VII collagen expression was seen in patients with acral, pretibial, and generalized intermediate forms, whereas slightly reduced and reduced expression was observed in patients with generalized intermediate forms of RDEB. Strongly reduced expression was observed in generalized intermediate and generalized severe forms of RDEB, but the absence of type VII collagen expression was seen in generalized severe RDEB only. It was unexpected to find that, in the latter cases, the skipping of an in-frame exon was associated with a complete absence of detectable type VII collagen. Apparently, the resulting transcripts could not be translated into detectable amounts of type VII collagen.

Of particular interest, with regards to exon skipping therapy, is variant #1 (table 1). The patient carried the variant c.2005C>T (p.Arg669*) in exon 15, and a frame-shift variant c.6311_6312del (p.Ser2104Trpfs*12) in exon 76. This combination was predicted to cause a complete absence of type VII collagen and, therefore, the most severe RDEB-gen sev phenotype. However, the patient also carries the rare single nucleotide variant c.1907G>T (rs116005007, minor allele frequency 0.22%: T=0.0022/11, 1000 Genomes, https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/), in cis with the c.2005C>T variant. This variant, located in the first codon of exon 15, was subsequently shown to modify the splicing of exon 15, leading to in-frame skipping of exon 15, thus bypassing the null variant and resulting in the synthesis of some type VII collagen, which explained the substantially milder phenotype. The carrier parent had no visible DEB features.

In contrast to some milder phenotypes, six out of eleven cases were described in the literature with a severe generalized RDEB phenotype (table 1). In all six cases, type VII collagen expression was either strongly reduced or completely absent. Five of the six cases were compound heterozygous for a null variant in trans with the exon skipping variant. Only one homozygous variant, c.7929+1G>A, caused strongly reduced expression of an exon 106-skipped type VII collagen and a severe recessive phenotype. Unfortunately, detailed individual data were not available to assess and compare the expression levels and severity of the phenotypes of these severe cases.

Altogether, the phenotypic spectrum caused by recessive exon skipping variants
Natural exon skipping in the COL7A1 gene

is generally milder than RDEB caused by bi-allelic null variants, but the expression levels of the skipped protein are very important for the precise phenotypic outcome. In most cases, the expression levels of type VII collagen lacking an exon in either the NC-1 domain or the THD are directly correlated to the severity of the phenotype. In addition, individual cases show that low levels of expression of type VII collagen can already ameliorate the phenotype.

Discussion

We investigated the genotype-phenotype correlation of natural exon skipping variants causing DEB to anticipate the therapeutic effect of AON-mediated exon skipping as a treatment for DEB. We included a series of seven COL7A1 exon skipping variants from the Dutch EB registry and 19 additional exon skipping variants from the literature. For all 26 variants, exon skipping had been proven at the RNA level.

Our review shows that natural exon skipping variants can be inherited dominantly or recessively (figure 2). The reason for this is unknown. In general, and comparable to dominant glycine substitutions,34 dominant skipping exons are located closer to collagenous imperfections than recessive ones, and are found more towards the end of the THD. However, location does not seem to be the only reason, since two of the recessive exons are also located near collagenous imperfections, and five are located near the end of the THD. A possible explanation as to why some skipped exons act dominantly while others act recessively could be the ratio between expression levels of exon skipped alleles and wild-type alleles. Analogous to the finding that the wild-type versus mutant allele ratio determines the level of THD instability and consequently the phenotype,35 it is conceivable that the amount of exon skipped type VII collagen actually present is also crucial in determining its phenotypic effect. Unfortunately, it is not possible to study this hypothesis in detail as the level of exon skipping has been quantified at the RNA level for only one of the exon skipping variants (#9 in table 1). The heterozygous variant c.6215delA resulted in a 0.73:1 exon-74-skipped to wild-type type VII collagen expression ratio, and the DDEB-pruriginosa phenotype.

Intra-epidermal cytoplasmic retention of type VII collagen has been described for several variants believed to disturb proper triple helix formation. For instance, it has been reported for several glycine substitutions leading to DDEB, like the p.Gly2037Glu glycine substitution,36 bullous dermolysis of the newborn,37 and RDEB-inversa.38 The fact that we observed cytoplasmic retention in all four dominant exon skipping cases and in one of the three recessive skipping cases indicates that exon-skipping also disturbs normal triple helix formation. Hence, it makes sense that the phenotypes due to exon skipping fall in the range seen with glycine substitutions. Intra-epidermal retention in a skin biopsy of a DEB patient with unknown genetic cause could thus also point to in-frame exon skipping and should warrant RNA analysis if no glycine substitution is found.
Clearly, exon 87 is a hotspot for dominant natural exon skipping. Ten different variants that induce skipping of exon 87 were identified, of which six are located in the intron 87 splice donor site, indicating that this is a weakly defined exon. It has been shown that variants in donor sites are more likely to disrupt splicing and that the lack of cryptic splice donor signals within 50 bp downstream of splice donor sites increases the likelihood of skipping the entire exon. Using Human Splicing Finder (http://www.umd.be/HSF3/) we examined exons 86, 87, and 88 (figure S2) for such cryptic splice donor sites. Indeed, no cryptic splice donor sites were predicted downstream of the splice donor site of exon 87, whereas three and seven sites were found for exons 86 and 88, respectively. The lack of these cryptic splice donor sites in intron 87 may explain why so many variants lead to complete skipping of exon 87.

Pruritus is strongly linked to disorders of the BMZ, including EB. An association has been suggested between heterozygous skipping of exon 87 and pruritus. Indeed, six exon 87 skipping variants were reported in patients with the DDEB-pruriginosa phenotype. One other case of DDEB-pruriginosa was caused by skipping of exon 71. Four of the seven families originated from South-East Asia, a geographic region where pruritic diseases are more common in general. Whether there is a causative link between exon skipping and DDEB-pruriginosa, or that this association should be attributed to other genetic and environmental factors, needs further study.

AON-mediated exon skipping will likely not have a beneficial effect on DDEB caused by glycine substitutions. AON-mediated exon skipping will most likely skip both mutant and wild-type exons and, if this process were 100% efficient, would change a heterozygous, dominant negative glycine substitution into a homozygous exon skipping mutation. Although this would not have the problem of dominant negative interference that is encountered in heterozygous exon skipping, we could still argue that less wild-type type VII collagen will not improve the phenotype. Indeed, ten mutations that result in homogeneous skipping of exons 15, 19, 20, 33, 53, 87, 106, 107, and 111 (table 1) illustrate that it leads to a phenotype that is more severe than DDEB phenotypes at the severe end of the spectrum. Although this could partly be due to lower-than-normal type VII collagen expression, the natural exon skipping data do not provide evidence that exon skipping would benefit DDEB phenotypes. Furthermore, as AON-mediated exon skipping will be significantly less than 100% efficient and cannot be administered continuously, treatment of DDEB with exon skipping will likely introduce an exon-skipped allele in addition to the wild-type and mutant alleles, which might even worsen the phenotype. Therefore, other therapeutic strategies seem to be more appropriate for DDEB, such as allele-specific knock-down using siRNAs.

In contrast, AON-mediated exon skipping could be beneficial for RDEB-gen sev patients, where complete absence of type VII collagen is the problem. Two cases were found that illustrate what exon skipping as a therapeutic approach aims to achieve, i.e.
bypassing a null variant by excluding its harboring exon. The null variant c.2005C>T in exon 15 was rescued by the rare DNA variant c.1907G>T in the same exon, which causes skipping of exon 15 and thus bypasses the null variant.42 Although the level of expression of a type VII collagen lacking exon 15 was still rather low, the co-occurrence of both variants on the same allele led to a milder than expected RDEB-gen intermed phenotype. The second case had the c.2471dup variant in exon 19, which was predicted to cause a frame-shift with premature termination codon and the most severe phenotype. However, skipping of exon 19 led to moderate levels of exon 19 skipped type VII collagen, and a surprisingly mild RDEB-gen intermed phenotype.43 As shown by these exon 15- and exon 19 skipping cases, the induction of even small amounts of skipped type VII collagen can result in significantly less severe phenotypes.

Although RDEB-gen intermed is still a severe phenotype in itself, and improving a phenotype to this level is still far from curing RDEB, preventing the development of the RDEB-gen sev phenotype would be a major improvement for several reasons: reduced cancer risk, a longer life span, and a reduced risk and slower progression of pseudosyndactyly and esophageal strictures.

In conclusion, exon skipping therapy for DDEB patients seems unlikely to benefit patients and may even worsen their phenotype, whereas such therapy for RDEB patients has the potential to improve the RDEB-gen sev phenotypes and push the clinical outcome towards the milder RDEB-gen intermed phenotype. The focus of developing exon skipping as a therapeutic approach should therefore be on RDEB-gen sev patients with disease due to COL7A1 null variants.
<table>
<thead>
<tr>
<th>No.</th>
<th>Allele 1(1)</th>
<th>Exon / intron</th>
<th>Skipped exon</th>
<th>Dominant / recessive</th>
<th>Allele 2(1)</th>
<th>Exon / intron</th>
<th>Skipped exon</th>
<th>Dominant / recessive</th>
<th>Functional effect on COLVII</th>
<th>Associated phenotypes</th>
<th>IP</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>c.2471dup (p.Gly814_Pro863delinsAla)</td>
<td>19 19</td>
<td>recessive</td>
<td>c.2471dup (p.Gly814_Pro863delinsAla)</td>
<td>19 19</td>
<td>recessive</td>
<td>Homogeneous exon 19 skip</td>
<td>Homogeneous exon 19 skip</td>
<td>RDEB-gen intermediate</td>
<td>Slightly reduced</td>
<td>76 NA</td>
<td>Salas-Alanis et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RDEB-gen intermediate</td>
<td></td>
<td>32 NA</td>
<td>Salas-Alanis et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RDEB-gen intermediate</td>
<td></td>
<td>32 NA</td>
<td>Salas-Alanis et al. (1998)</td>
</tr>
<tr>
<td>3</td>
<td>c.2710+1G&gt;A (p.[=, Gly904delinsArg])</td>
<td>IVS20 20</td>
<td>recessive</td>
<td>c.2710+1G&gt;A (p.[=, Gly904delinsArg])</td>
<td>IVS20 20</td>
<td>recessive</td>
<td>Heterogeneous exon 20 skip</td>
<td></td>
<td>RDEB-gen intermediate</td>
<td>Slightly reduced</td>
<td>This paper (Pt6)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>c.4011G&gt;A (p.Gly1326_Pro1337del)</td>
<td>33 33</td>
<td>recessive</td>
<td>c.7769G&gt;A (p.Gly2590Asp)</td>
<td>104 NA</td>
<td>recessive</td>
<td>Homogeneous exon 33 skip</td>
<td>Homogeneous exon 53 skip</td>
<td>RDEB-gen intermediate</td>
<td>Slightly reduced</td>
<td>32 NA</td>
<td>Salas-Alanis et al. (2000)</td>
</tr>
<tr>
<td>5</td>
<td>c.4980+5G&gt;C (p.Gly1646_Arg1660del)</td>
<td>IVS53 53</td>
<td>recessive</td>
<td>Deletion spanning COL7A1 + 15 other genes</td>
<td>- NA</td>
<td>recessive</td>
<td>Homogeneous exon 53 skip</td>
<td></td>
<td>RDEB-gen intermediate</td>
<td>Unknown</td>
<td>Lee et al. (2016)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>c.58205&gt;A (p.[=, Gly1925_Pro1940del])</td>
<td>70 70</td>
<td>recessive</td>
<td>c.4036G&gt;C (p.Gly1347Arg)</td>
<td>70 70</td>
<td>recessive</td>
<td>Homogeneous exon 74 skip</td>
<td>RDEB-gen – RDEB-gen intermediate</td>
<td>Strongly reduced</td>
<td>Normal</td>
<td>Terracina et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>c.6181G&gt;T (p.Gly2061_Gln2072del)</td>
<td>IVS73 74</td>
<td>dominant</td>
<td>c.=</td>
<td>NA NA</td>
<td>NA</td>
<td>Homogeneous exon 74 skip</td>
<td>Homogeneous exon 53 skip</td>
<td>DDEB-gen</td>
<td>Normal $</td>
<td>Gardella et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>c.6211del (p.Gly2061_Gln2072del)</td>
<td>74 74</td>
<td>dominant</td>
<td>c.=</td>
<td>NA NA</td>
<td>NA</td>
<td>Homogeneous exon 74 skip</td>
<td>Homogeneous exon 53 skip</td>
<td>DDEB-pr</td>
<td>Normal</td>
<td>Kitazawa et al. (2014)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>c.6832-23_6832-3del (p.Gly2278_Gln2300del)</td>
<td>IVS86 87</td>
<td>dominant</td>
<td>c.=</td>
<td>NA NA</td>
<td>NA</td>
<td>Homogeneous exon 87 skip</td>
<td>Homogeneous exon 87 skip</td>
<td>DDEB-ac</td>
<td>Normal $</td>
<td>This paper (Pt1)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>c.6846G&gt;C (p.Gly2278_Gln2300del)</td>
<td>87 87</td>
<td>dominant</td>
<td>c.=</td>
<td>NA NA</td>
<td>NA</td>
<td>Homogeneous exon 87 skip</td>
<td>Homogeneous exon 87 skip</td>
<td>DDEB-pr</td>
<td>Normal $</td>
<td>Covaciuc et al. (2011)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>c.6855_6881del (p.Gly2278_Gln2300del)</td>
<td>87 87</td>
<td>dominant</td>
<td>c.=</td>
<td>NA NA</td>
<td>NA</td>
<td>Homogeneous exon 87 skip</td>
<td>Homogeneous exon 87 skip</td>
<td>DDEB-ac</td>
<td>Normal $</td>
<td>Sakuntabhai et al. (1998)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DDEB-gen intermediate</td>
<td>Strongly reduced</td>
<td>15 15</td>
<td>Salas-Alanis et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DDEB-gen intermediate</td>
<td>Slightly reduced</td>
<td>3 3</td>
<td>Cserhalmi-Friedman et al. (1998)</td>
</tr>
</tbody>
</table>

**Table 1.** Overview of exon skipping variants in DEB.
<table>
<thead>
<tr>
<th>Variant</th>
<th>Exon</th>
<th>Dominance</th>
<th>c.</th>
<th>Protein Effect</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.6899G&gt;A</td>
<td>6</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-pr</td>
</tr>
<tr>
<td>c.6900del</td>
<td>6</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-gen</td>
</tr>
<tr>
<td>c.6901G&gt;A</td>
<td>6</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-ac</td>
</tr>
<tr>
<td>c.6900+1G&gt;C</td>
<td>IVS87</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-pr</td>
</tr>
<tr>
<td>c.6900+1G&gt;T</td>
<td>IVS87</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-pr</td>
</tr>
<tr>
<td>c.6900+4A&gt;G</td>
<td>IVS87</td>
<td>Dominant</td>
<td>c.=</td>
<td>NA NA NA</td>
<td>Heterogeneous exon 87 skip</td>
<td>DDEB-pr</td>
</tr>
<tr>
<td>c.7929+1G&gt;A</td>
<td>IVS106</td>
<td>Recessive</td>
<td>c.7929+1G&gt;A</td>
<td>Homogeneous exon 87 skip</td>
<td>RDEB-gen</td>
<td>Unknown</td>
</tr>
<tr>
<td>(p.Gly2626_Lys2643del)</td>
<td></td>
<td></td>
<td>(p.Gly2626_Lys2643del)</td>
<td></td>
<td>RDEB-gen</td>
<td>Strongly reduced</td>
</tr>
<tr>
<td>c.7930-1G&gt;C</td>
<td>IVS106</td>
<td>Recessive</td>
<td>c.6527dup</td>
<td>Homogeneous exon 107 skip</td>
<td>RDEB-gen</td>
<td>Undetectable</td>
</tr>
<tr>
<td>(p.Gly2177Trpfs*113)</td>
<td></td>
<td></td>
<td>(p.Gly2177Trpfs*113)</td>
<td></td>
<td>RDEB-gen</td>
<td>Normal $</td>
</tr>
<tr>
<td>c.8045A&gt;G</td>
<td>IVS110</td>
<td>Recessive</td>
<td>c.1573C&gt;T</td>
<td>Homogeneous exon 111 skip</td>
<td>RDEB-gen</td>
<td>Reduced $</td>
</tr>
<tr>
<td>(p.Gly2743_Gln2768del)</td>
<td></td>
<td></td>
<td>(p.Gly2743_Gln2768del)</td>
<td></td>
<td>RDEB-gen</td>
<td>Reduced</td>
</tr>
<tr>
<td>c.8227-1G&gt;C</td>
<td>IVS111</td>
<td>Recessive</td>
<td>c.8717del</td>
<td>Homogeneous exon 111 skip</td>
<td>RDEB-ac</td>
<td>Reduced</td>
</tr>
<tr>
<td>(p.Gly2743_Gln2768del)</td>
<td></td>
<td></td>
<td>(p.Pro2906Leufs*46)</td>
<td></td>
<td>RDEB-ac</td>
<td>Reduced</td>
</tr>
<tr>
<td>c.8304+1G&gt;A</td>
<td>IVS111</td>
<td>Recessive</td>
<td>c.6127G&gt;A</td>
<td>Homogeneous exon 115 skip</td>
<td>RDEB-gen</td>
<td>Normal</td>
</tr>
<tr>
<td>(p.Gly2043Arg)</td>
<td></td>
<td></td>
<td>(p.Gly2043Arg)</td>
<td></td>
<td>RDEB-gen</td>
<td>Slightly reduced</td>
</tr>
<tr>
<td>c.8524_8527+10del</td>
<td>115</td>
<td>Recessive</td>
<td>c.6025G&gt;A</td>
<td>Homogeneous exon 115 skip</td>
<td>RDEB-pt</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*Some of the DNA variants have been shown to lead to multiple splice variants at the RNA level. For reasons of readability, only splice variants deduced to lead to protein expression are shown between brackets. For instance, p.[=, Gly1925_[Pro1940del] indicates that the DNA variant c.5820G>A in exon 70 leads to two functional protein molecules, as deduced from RNA analysis: a wild-type protein isoform (p.=) and an isoform from which exon 70 is skipped (p.Gly1925_[Pro1940del]). More information on other non-functional splice variants can be found in the references cited. 'c.=' means wild-type allele.

**Heterogeneous exon skip indicates there will be a combination of wild-type and skipped product. Mutant products (due to a missense mutation) are mentioned explicitly, where present.

1DDEB-ac (DDEB-acral), DDEB-pr (DDEB-pruriginosa), DDEB-pt (DDEB-pretibial), DDEB-gen (DDEB-generalized), RDEB-ac (RDEB-acral), RDEB-pt (RDEB-pretibial), RDEB-gen intermed (RDEB-generalized intermediate), RDEB-gen sev (RDEB-generalized severe), NA (not applicable), NF not found

$ Indicates that retention of protein in basal keratinocytes was observed.
Figure S1. RT-PCR analysis confirms natural exon skipping. For each patient, RT-PCR was performed, followed by Sanger sequencing to confirm skipping at the RNA level. The effect of the mutation (red X) on splicing is depicted in a schematic representation. Exons are depicted as boxes, introns are depicted as lines. Partial intron retention leads to a premature termination codon (PTC). Asterisks indicate heteroduplex bands.
Figure S2. Lack of cryptic splice donor sites in intron 87 may explain exon skipping hotspot. The genomic sequences of exon 86, 87 and 88 (Genbank accession no. NG_007065.1), including 100 bp up- and downstream, were analyzed by Human Splicing Finder 3.0 (http://www.umd.be/HSF3). Three cryptic splice donor sites were found at positions +26, +31, and +41 downstream of exon 86. No cryptic donor sites were found in the first 50 bp downstream of exon 87; the first cryptic splice donor sites are predicted at positions +77 and +85. Seven cryptic donor sites were found at positions +2, +4, +6, +8, +20, +30, and +38 of exon 88. Exons are indicated in grey. Potential splice donor sites are depicted by purple dots and indicated with black arrows. Y-axis: relative prediction value (0-100).
References


Natural exon skipping in the COL7A1 gene


