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Chapter 23

Gene Therapy for the *COL7A1* Gene

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Epidermolysis bullosa

Epidermolysis bullosa (EB) is a genetically and clinically variable disease characterized by blister formation and erosions of the skin and mucous membranes after minor trauma [1]. The inheritance of the affected genes can occur in a dominant or recessive way depending on the subform of the disease. In general, epidermolysis bullosa is caused by mutations in genes encoding structural proteins within the basal membrane zone of the skin. Absence or functional loss of one of these proteins results in a lack of stability of the microarchitecture of the connection between dermis and epidermis leading to a loss of coherence [1]. The basement membrane between the dermis and the epidermis is a complex membrane produced by basal keratinocytes and dermal fibroblasts that acts as mechanical support for the connection of both skin layers. The basal membrane also regulates the metabolic exchange between the two skin compartments [2]. Up to date, there are at least 15 genes associated with EB causing different forms of the disease. Numerous mutations in these genes that encode for structural proteins within keratinocytes or within mucocutaneous basement membranes have been identified up to now [1].

Mutations in the genes, encoding for the keratins 5 and 14 and plectin, lead to epidermolysis bullosa simplex (EBS) characterized by the cytolysis within basal keratinocytes. Junctional epidermolysis bullosa (JEB) is caused by the absence or loss of function of laminin-332, type XVII collagen or integrin-β4. JEB is a severe EB form and is characterized by the separation of the skin within the lamina lucida. Mutations in type VII collagen (encoded by *COL7A1*) lead to the dystrophic form of epidermolysis bullosa, characterized by skin separation below the lamina densa. The severity and clinical manifestation of the disease depend on the mutation type (missense mutation, nonsense mutation, splice site mutations, deletion or insertion), the mode of inheritance and the localization of the mutation within the gene. Due to this fact, diagnosis,

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course of disease and therapy vary significantly depending on the present EB subform [3]. Blister formation can be restricted to the soles of the feet or occur generalized. Severe systemic complications and extracutaneous manifestations including blistering and erosions of the cornea and mucosal tissues, stenoses or strictures of respiratory, gastrointestinal and urogenital tracts, pylorus atresia, muscular dystrophy and skin cancer are certain complications associated with different EB subtypes [1]. So far over 30 distinctive subtypes have been described and classified in a system, which was recently revised [4]. See Table 1.

A: Classification scheme for the major EB subtypes

Major EB type	Major EB subtypes	Affected proteins
EB simplex (EBS)	Suprabasal EBS	plakophilin-1, desmoplakin;
		others?
	Basal EBS	keratins 5 & 14; plectin,
		α6β4 integrin, BPAG1
Junctional EB (JEB)	JEB, Herlitz (JEB-H)	laminin-332, (laminin-5)
	JEB, other	laminin-332, type XVII collagen
		α6β4 integrin, α3 integrin
Dystrophic EB (DEB)	Dominant DEB (DDEB)	type VII collagen
	Recessive DEB (RDEB)	type VII collagen
Kindler syndrome		kindlin-1

B: Classification scheme for all known EB simplex subtypes

Major types	EBS subtypes	Affected proteins
EBS suprabasal	*lethal acantholytic EB*	desmoplakin
	plakophilin deficiency	plakophilin-1
	EBS superficialis	?
EBS basal	EBS, localized (EBS-loc)[a]	K5, K14
	EBS, Dowling Meara (EBS-DM)	K5, K14
	EBS, other generalized (EBS,gen-nonDM)[b]	K5, K14, BPAG1
	EBS with mottled pigmentation(EBS-MP)	K5
	EBS with muscular dystrophy (EBS-MD)	plectin
	EBS with pylorus atresia (EBS-PA)	plectin, α6β4 integrin
	EBS, autosomal recessive (EBS-AR)	K14
	EBS, ogna (EBS-Og)	plectin
	EBS, migratory circinate (EBS-migr)	K5

(rare variants in italics)

[a] Previously called EBS, Weber-Cockayne

[b] Includes patients previously classified as EBS-Koebner

C: Classification scheme for all known junctional subtypes

Major JEB subtype	Subtypes	Affected proteins
JEB, Herlitz (JEB-H)		laminin-332
JEB, other (JEB-O)	JEB, non-Herlitz, generalized (JEB-nH gen)[a]	laminin-332, type XVII collagen

	JEB, non-Herlitz localized (JEB-nH loc)	typeXVII collagen
	JEB with pyloric atresia (JEB-PA)	α6β4 integrin
	JEB, inversa (JEB-I)	laminin-332
	JEB, late onset (JEB-lo)[b]	
	LOC syndrome (laryngo-onycho-cutaneous syndrome)	laminin-332 α3 chain
	?	α3 integrin
(rare variants in italics)		
[a] Formerly known as generalized atrophic benign EB (GABEB)		
[b] Formerly known as EB progressive		
D: Classification scheme for all known dystrophic EB subtypes		
Major DEB subtype	Subtypes	Affected protein
DDEB	DDEB, generalized (DDEB-gen)	type VII collagen
	DDEB, acral (DDEB-ac)	
	DDEB, pretibial (DDEB-Pt)	
	DDEB, pruriginosa (DDEB-Pr)	
	DDEB, nails only (DDEB-no)	
	DDEB, bullous dermolysis of the newborn (DDEB-BDN)	
RDEB	RDEB, severe generalized (RDEB-sev gen)[a]	type VII collagen
	RDEB, generalized other (RDEB-O)	
	RDEB, inversa (RDEB-I)	
	RDEB, pretibial (RDEB-Pt)	
	RDEB pruriginosa (RDEB-Pr)	
	RDEB, centripetalis (RDEB-Ce)	
	RDEB, bullous dermolysis of the Newborn (RDEB-BDN)	
(rare variants in italics)		
[a] Previously called RDEB, Hallopeau-Siemens		

Table 1. Classification system for inherited epidermolysis bullosa. Based on Fine et al. [4].

2. Dystrophic epidermolysis bullosa (DEB)

Mutations in the gene *COL7A1*, encoding for type VII collagen, cause the dystrophic form of epidermolysis bullosa (DEB). Type VII collagen is the major constituent of the basement membrane's anchoring fibrils and belongs to the superfamily of collagens [5]. *COL7A1* comprises 118 exons and mostly short intervening introns resulting in a size of the entire *COL7A1* gene of 32kb encoding an mRNA of over 9kb [6,7]. The remarkable number of *COL7A1* mutations and the variable genotype-phenotype correlation hamper the finding of an optimal therapy for DEB patients. Nevertheless severity of clinical manifestations can often be defined by the type of the mutation and its localization within the *COL7A1* gene [3]. DEB is divided into two main subtypes according to the mode of inheritance. Dominant dystrophic EB (DDEB) is inherited in an autosomal dominant way, whereas recessive dystrophic EB (RDEB) is transmitted in an autosomal recessive mode [8]. RDEB is classified in

severe generalized RDEB (RDEB-sev gen) – formerly called RDEB, Hallopeau Siemens - and RDEB-generalized other (RDEB-O) – formerly called RDEB-non Hallopeau Siemens [3].

The DDEB phenotype is mostly generalized but mild and clinically characterized by recurrent blistering, milia, atrophic scarring, nail dystrophy and eventual loss of nails [3]. See Figure 1A,B. The fact that the defective and wildtype alleles are expressed equally explains the relative mild phenotype in comparison to RDEB [3]. Missense mutations or in frame deletions in *COL7A1* causing RDEB disturb the assembly and aggregation of type VII collagen into anchoring fibrils. As a result, the number of anchoring fibrils and their morphology is altered significantly. The resulting subforms of RDEB are classified as RDEB, generalized other [3]. RDEB-sev gen is caused by nonsense mutations in both alleles, resulting in a complete loss of type VII collagen within the basal membrane zone of the skin. Clinical manifestations of RDEB are generalized blistering, erosions, crusts, atrophic scarring, onychodystrophy, loss of nails, mutilating pseudosyndactyly of hands and feet and functionally disabling contractures in hands, feet, elbows and knees. See Figure 1C-H. Additionally, severe extracutaneous complications as gastrointestinal and urogenital tracts involvement, external eye, chronic anaemia, growth retardation and a high risk for the development of aggressive squamous cell carcinoma decrease (Figure 1 I) the life quality of the patient [3,9-14].



Figure 1. Clinical phenotype of DEB. **A,B:** DDEB with milia formation and atrophy. **C:** Atrophic scar with crusts and erosions in RDEB. **D:** Boy with severe-generalized RDEB leading to ulcerations and large non healing wounds with atrophic scarring at the back; **E:** Nail dystrophy on both feet. **F,G:** Mitten formation in hands and feet. **H:** Severe caries **I:** Squamous cell carcinoma on the foot (Photos: R. Hametner)

3. The dermal epidermal junction

The blisters characteristic for EB arise within the dermal-epidermal junction. Having a look at this compartment of the skin helps to understand the cause of blistering in EB. The dermal-epidermal junction is a complex basement membrane synthesized by dermal fibroblasts and basal keratinocytes. Adhesion of the epidermis to the underlying dermis is mechanical-

ly supported by the so called basement membrane zone (BMZ). Moreover it regulates the metabolic exchange between these two compartments. Up to now more than 20 macromolecules situated in the dermal-epidermal-junction have been detected and characterized at biochemical and genomic level [2].

Three protein-junction complexes stabilize the adherence of the basal keratinocytes to the dermis. See Figure 2. The hemidesmosomes built up by plectin, the bullous pemphigoid antigen 1 (BPAG1), $\alpha 6\beta 4$ integrin and type XVII collagen (bullous pemphigoid antigen 2 - BPAG2) link the basal keratinocytes with the basement membrane, spanning the lamina lucida and anchored in the lamina densa [2]. Different laminin isoforms are located in the lamina lucida (laminin-332, laminin 6, laminin 10) and contribute along with BPAG2 to the formation of the anchoring filaments. The lamina densa is mainly built up by type VII collagen anchoring the lamina densa to the underlying dermis by the formation of anchoring fibrils [2]. Some other antigens as uncein (19-DEJ-1 antigen), NU-T2 antigen, KF1 antigen, LDA1 antigen, nidogen, heparin-sulfate, proteoglycan, antigens AF1 and AF2, thrombospondin, type V collagen and osteonectin/BM-40 have been detected in the lamina densa but have not yet been adequately characterized [2].

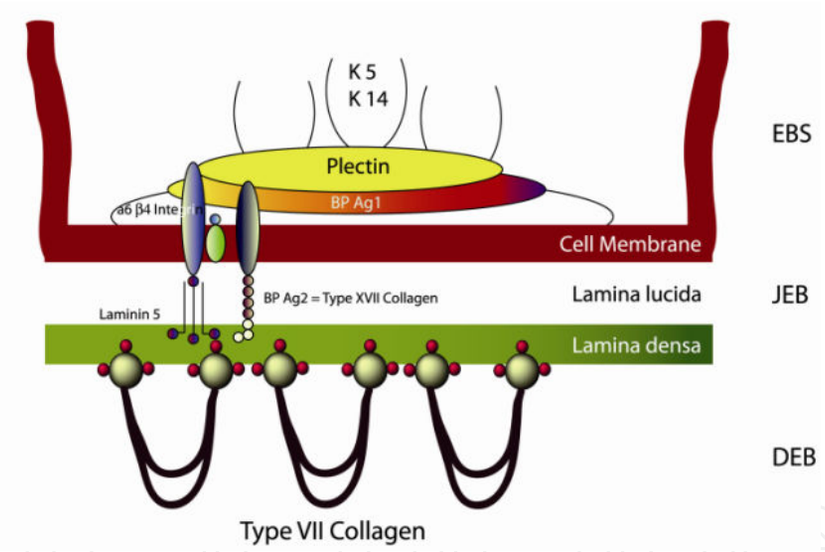


Figure 2. Schematic setup of the cutaneous dermal-epidermal junction zone and localization of structural proteins affected in inherited EB (Diagram by R. Hametner). laminin 5 = laminin 332; EBS = epidermolysis bullosa simplex; JEB = junctional epidermolysis bullosa; DEB = dystrophic epidermolysis bullosa

4. Type VII collagen

Type VII collagen is classified in the superfamily of collagens [7]. A protein domain in triple-helical conformation, which provides stability and integrity between connective tissues, is a common structural feature of all collagens [7]. Type VII collagen is a minor collagen in human

skin and demonstrates spatially restricted location but it plays a critical role in providing integral stability to the skin because it is the major component of the anchoring fibrils [6,7].

5. Biology of type VII collagen

Type VII collagen molecules are characterized by the two non-collagenous NC-1 and NC-2 domains flanking a central collagenous, triple-helical segment [7]. In contrast to other interstitial collagens the repeating Gly-X-Y collagenous sequence is interrupted by 19 imperfections due to insertions or deletions of amino acids. There is a 39 amino acid non-collagenous hinge region susceptible to proteolytic digestion with pepsin in the middle of the triple-helical domain [15]. The amino terminal NC-1 domain (approximately 145kD in size), is built up of sub-modules with homology to known adhesive proteins, including segments with homology to cartilage matrix protein (CMP), nine consecutive fibronectin type III-like (FN-III) domains, a segment with homology to the A domain of von Willebrand factor, and a short cysteine and proline-rich region [15]. The C-terminal non-collagenous NC-2 domain is with 30kD in size relatively small, and contains a segment with homology to Kuniz protease inhibitor molecule [16,17].

The 32kb gene encoding a 9,2kb mRNA has been mapped to the short-arm of chromosome 3p21.1 [18]. The encoding primary sequence and the gene structure of type VII collagen are well conserved. The mouse gene shows 90.4% identity at the protein level and 84.7% homology at the nucleotide level, indicating the importance of type VII collagen as a structural protein [19].

The expression pattern of *COL7A1* is tissue specific and restricted. Type VII collagen has been detected by immunomapping to a selected number of epithelia, including the dermal-epidermal BMZ of skin, the amniotic epithelial BMZ of the chorioamnion, the corneal epithelial basement membrane (Bowman's membrane) and the epithelial basement membrane of oral mucosa and cervix. Moreover the presence of type VII collagen correlates with the presence of ultrastructurally detected anchoring fibrils [6]. A number of cytokines modulate type VII collagen expression. Especially transforming growth factor-β is a powerful upregulator of *COL7A1* at transcription level in fibroblasts and keratinocytes [20,21].

6. Type VII collagen – A major component of the anchoring fibrils

Type VII collagen is synthesized by two cell types in the skin: keratinocytes and fibroblasts [22]. After synthesis of complete pro-α1 (VII) polypeptides, three polypeptides are associated through their carboxy-terminal ends to a trimer molecule, which is then folded in its collagenous segment into the triple-helical formation. Past to secretion into the extracellular milieu two type VII collagen molecules are aligned into an anti-parallel dimer with the amino-terminal domains present at both ends of the molecule [6]. During dimer-assembly stabilization by inter-molecular disulfide bond formation and a proteolytic removal of a part of the carboxy-terminal ends (NC-2 domain) of both type VII collagen molecules take place [23]. Large num-

bers of these anti-parallel dimers aggregate laterally to form anchoring fibrils, which then can be identified by their characteristic, centro-symmetric banding patterns in transmission electron microscopy [7].

The affinity of the NC-1 domain to bind the principal components of the cutaneous basement membrane, laminin-332, laminin-311 and type IV collagen provides stability to the dermo-epidermal adhesion on the dermal site at the lamina lucida/papillary dermis interface [6,24,25]. Arg-Gly-Asp sequences in the NC-1 domain serve as integrin mediated attachment sites for cells to adhere to extracellular matrix components such as fibronectin [26].

7. Mutations in *COL7A1*

Mutations in *COL7A1* have clinical consequences in terms of disrupted integrity of the skin, due to the complexity of the *COL7A1* gene, type VII collagen protein structures and the critical importance of its distinct domains in macromolecular interactions [7]. At least 324 pathogenic mutations have been detected within *COL7A1* in different variants of DEB up to now including 43 nonsense, 127 missense, 65 deletion, 28 insertion, 9 insertion-deletion, 51 splice-site and 1 regulatory mutations [27]. See Figure 3-5. Exon 73 constitutes a region with a high frequency of mutations, what suggests being a region in which mutations commonly affect the function of anchoring fibrils [28]. RDEB is caused by nonsense, splice-site, deletions or insertions, silent glycine substitutions within the triple helix and non-glycine missense mutations within the triple-helix or non-collagenous NC-2 domain [29]. RDEB-severe generalized originates from nonsense, frameshift or splice-site mutations on both alleles leading to premature termination codons (PTCs) [30], which result in nonsense mediated mRNA decay or truncated proteins, leading to a reduced number of collagen VII monomers, which are unable to assemble into functional anchoring fibrils [29,30]. PTC mutations do not cause a clinical phenotype if they appear in the heterozygous state, but if they are homozygous or combined with another PTC mutation they are causing severe generalized RDEB [27]. Two missense mutations or compound heterozygosity of a missense and a PTC mutation lead to severe generalized RDEB in very rare cases [31].

RDEB, generalized other, the milder phenotype, is mostly caused by PTCs, small deletions, substitutions of glycine residues in the collagenous domain, splice-site mutations within NC-2 [32-35], delayed termination codons [36], in frame exon skipping [29,36], or missense substitution mutations involving amino acids other than glycine [29,37,38], the majority involving arginine residues resulting either in the loss of an ionic charge or in the introduction of a bulky chain at an external position of the triple helix [27]. Thereby these mutations usually concern a critical amino acid and change the conformation of the protein, which then might still be able to assemble into a small number of anchoring fibrils but is likely to be unstable when they laterally aggregate. Anyhow some full length type VII collagen polypeptides can still be built up [39].

DDEB is caused by glycine substitutions within the triple helical domain of *COL7A1* or other missense mutations, deletions or splice site mutations in some cases [5,26,40-44]. Critical ami-

no acids in the structure of the triple helix are affected by these mutations and therefore the overall stability of the anchoring fibrils is disturbed. More than 100 missense mutations resulting in a Gly-Xaa substitution have been detected in the collagenous domain of *COL7A1*; half of these are situated in amino acids 1522-2791 and have a dominant negative effect [27].

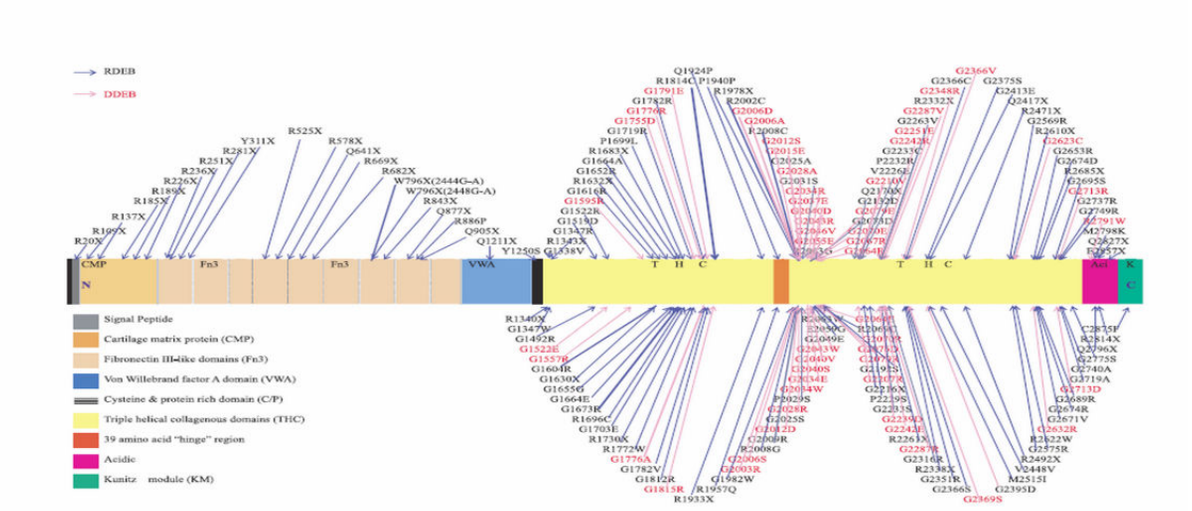


Figure 3. Missense and nonsense mutations in DEB patients. The red lettering signifies dominant and the black signifies recessive inheritance. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

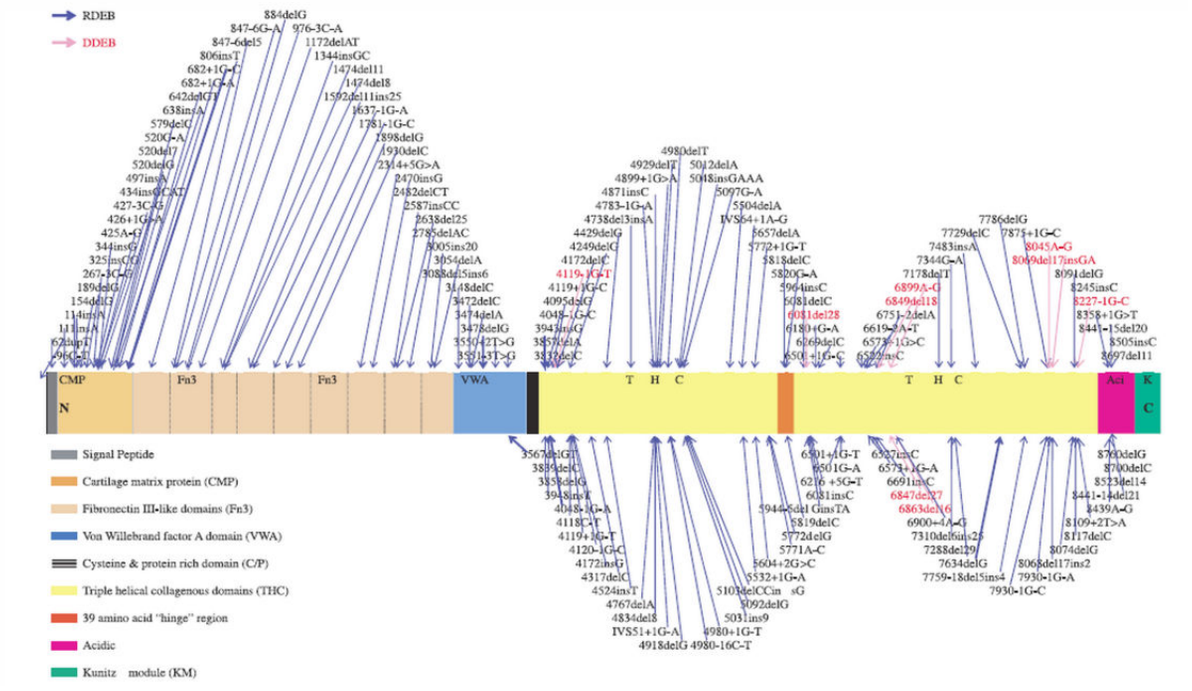


Figure 4. *COL7A1* deletions, insertions and splice site mutations in DEB patients. The red lettering signifies dominant and the black signifies recessive inheritance. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

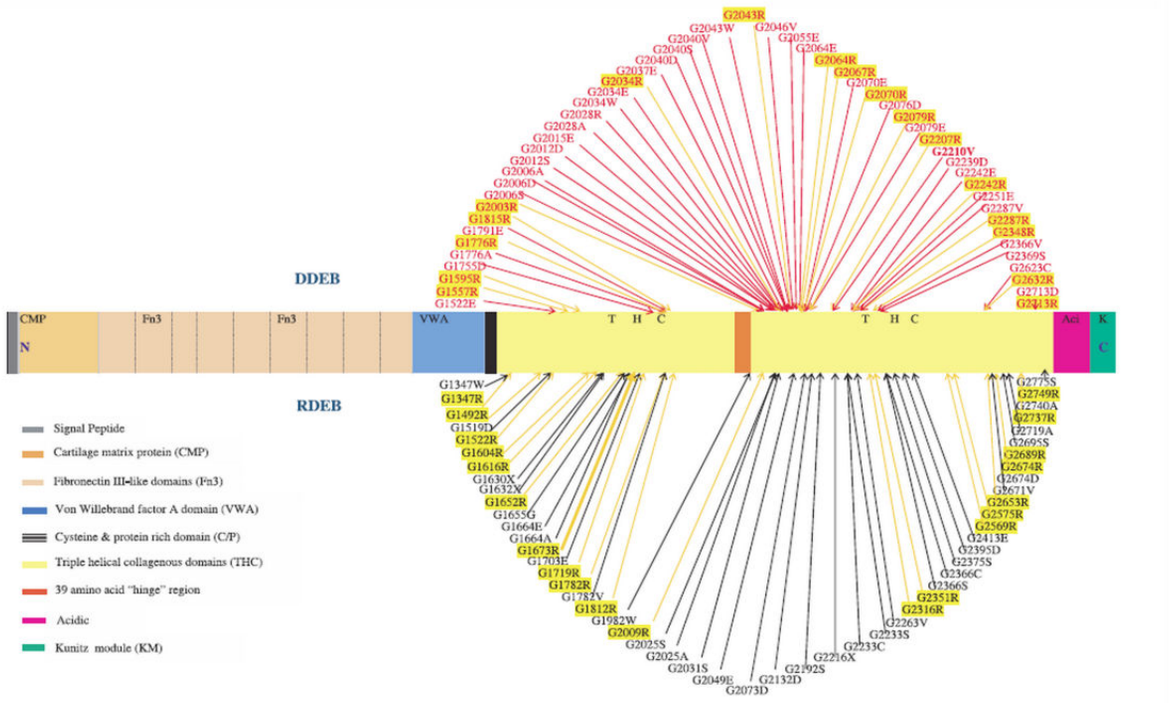


Figure 5. Glycine substitutions in DEB. These are all in the triple-helical collagenous domain; the ones above represent DDEB, the ones below RDEB. (Dang et al. [27] © 2008 Blackwell Munksgaard, Experimental Dermatology)

8. Mouse model

So far there are only two viable mouse models with defects in the *COL7A1* gene. A transgenic mouse carrying human *COL7A1* cDNA inclusive the human 7528delG mutation in exon 101, which develops the DEB phenotype gradually [45], and a collagen VII hypomorphic mouse published by Fritsch et al. 2008 [46]. In the collagen VII hypomorphic mouse reduced expression of collagen VII originates from aberrant splicing resulting from the introduction of a phosphoglycerate kinase promoter-driven neomycin phosphotransferase expression cassette (PGK-Neo cassette) in intron 2 of *COL7A1.* One out of three possible splice variants is translated into full-length type VII collagen resulting in a reduction of type VII collagen levels to about 9% of wildtype levels in *COL7A1*$^{flNeo/flNeo}$ mice. Hemorrhagic blisters on the soles of fore and hind paws, ears and mouth are developed by the collagen VII mice within the first 48 hours of life. Blisters of newborn *COL7A1*$^{flNeo/flNeo}$ mice were histopathologically classified as hemorrhagic and subepidermal. Type VII collagen immunofluorescence staining revealed weak reactivity in comparison to wildtype littermates. Ultrastructurally normal but reduced in number anchoring fibrils were detected in transmission electron microscopy of the dermal-epidermal junction of the skin. *COL7A1*$^{flNeo/flNeo}$ mice suffer from growth retardation due to malnutrition and subsequently have a reduced life expectancy. Moreover healing of the initial blistering on the paws with scarring results in the development of mitten deformities beginning at 2-3 weeks of age [46].

9. Therapy approaches

Due to the size of the *COL7A1* gene, a causal therapy for dystrophic epidermolysis bullosa is a great challenge. Symptomatic therapy is concentrated on prevention of skin trauma to minimize blister formation, prevention of secondary bacterial infection, treatment of infection, measures to improve wound healing, maintenance of good nutrition, treatment of correctable complications, and finally rehabilitation [3]. However, several gene and cell therapy strategies showed the potential to revert the disease-associated phenotype. Phenotypic correction of recessive DEB forms (RDEB) can be achieved by gene insertion therapy, in which the wildtype sequence of a mutated gene of interest is introduced into the target cells. Moreover alternative avenues including gene-, cell-, protein- and other systemic- therapy approaches have been tested to restore type VII collagen expression. See Table 2.

Author	Approach	Year
Woodley et al.	Type VII collagen minigene	2000
Sat et al.	Cosmid clone containing the entire *COL7A1* gene	2000
Mecklenbeck et al.	Microinjection of a *COL7A1*-PAC vector	2002
Urda et al.	ΦC31 bacteriophage integrase	2002
Chen et al.	Minimal lentiviral vectors	2002
Baldeschi et al	Canine type VII collagen	2003
Woodley et al	Targeting fibroblasts instead of keratinocytes (lentivirally)	2003
Gache et al.	Full-length cDNA (retrovirally)	2004
Woodley et al.	Intradermal injection of recombinant type VII collagen	2004
Woodley et al.	Intradermal injection of lentiviral vectors in vivo	2006
Goto et al.	Targeting fibroblasts instead of keratinocytes (retrovirally)	2006
Goto et al.	Targeted exon skipping using antisense	2006
Wong et al.	Intradermal injection of allogenic wildtype fibroblasts into a patient	2007
Fritsch et al.	Intradermal injection of murine wildtype fibroblasts in a DEB mouse model	2008
Remington et al.	Intradermal injection of human type VII collagen in mice	2009
Titeux et al.	Minimal self-inactivating retroviral vectors harbouring the full length human *COL7A1* gene	2010
Wagner et al.	Allogeneic bone marrow transplantation	2010
Siprashvili et al.	Full-length cDNA (retrovirally)	2010
Murauer et al.	3´ Trans-splicing of *COL7A1*	2011

Table 2. Therapy approaches to restore type VII collagen expression

Woodley et al. used a type VII collagen minigene, which contains the intact noncollagenous domains NC1 and NC2 and part of the central collagenous domain. This approach resulted after transduction into DEB keratinocytes in persistent synthesis and secretion of a 230kDa recombinant minicollagen VII [47]. However deletions in *COL7A1* have been reported to be associated with a pathologic phenotype [5,48,49]. The same group introduced recombinant human type VII collagen into mouse and human skin equivalents transplanted onto mice, by injection. As a result the injected type VII collagen was detected within the basal membrane zone leading to a reversion of the disease associated phenotype [50]. Additionally, the group expressed type VII collagen using a self-inactivating lentiviral vector, which was injected into human skin equivalents, expanded from DEB cells, placed on nude immunodeficient mice. Experiments revealed the synthesis and insertion of the protein into the basal membrane zone [51]. Using a cosmid clone, carrying the entire *COL7A1* gene, was also shown to be a promising way to direct expression of type VII collagen in skin in fetal and neonatal mice. The tested neonatal or fetal mice produced type VII collagen within the basal membrane zone of the skin showing a stable expression of the protein *in vivo* [52]. Microinjection of a P1-derived artificial chromosome (PAC) carrying the entire *COL7A1* locus resulted in production of a procollagen VII similar to the authentic one by Mecklenbeck et al. [53]. The ΦC31 bacteriophage integrase, facilitating integration only in pseudo attP sites, was used to integrate *COL7A1* stably into DEB primary epidermal progenitor cells by Urda et al. [54]. Baldeschi et al. also showed sustained and permanent expression of the transgene after transduction of canine type VII collagen into human and canine DEB keratinocytes [55]. Based on this study Gache et al. yield a full phenotypic reversion of the disease-associated phenotype of RDEB epidermal clonogenic cells after full-length human *COL7A1* cDNA introduction using a retroviral system. However, the expression of *COL7A1* was 50 times higher than the levels monitored in wildtype keratinocytes in monolayers, increasing the risk for an ectopic transgene expression and an abnormal accumulation in skin equivalents [56]. Chen et al. used a minimal lentiviral vector for *COL7A1* expression *in vitro* as an alternative to the retroviral system applied by Gache et al. [57]. Goto et al. showed that *COL7A1* treated fibroblasts of skin grafts provide higher amounts of type VII collagen for the dermal-epidermal junction than keratinocytes [58]. They have also demonstrated an antisense oligoribonucleotide therapy to maintain exon skipping of an exon comprising a premature stop codon. As a result a truncated type VII collagen variant was expressed [59]. Additionally, intradermal injection of untreated normal human or gene-corrected fibroblasts in mice can result in a stable production of human type VII collagen at the basal membrane zone of the skin [60]. Moreover, Wong et al. demonstrated an increased source of type VII collagen in the dermal-epidermal junction for at least three months after intradermal injection of allogeneic fibroblasts [61]. In a mouse model Fritsch et al. showed an accumulation of type VII collagen and restoration of a functional dermal-epidermal junction after injection of murine wildtype fibroblasts into a type VII collagen hypomorphic mouse [46]. In 2009 Remington et al. injected human type VII collagen into *COL7A1* -/- mice, also restoring type VII collagen expression and correct generation of anchoring fibrils [62]. Titeux et al. transduced *COL7A1* cDNA under the

control of a human promoter using a minimal self-inactivating retroviral vector into RDEB keratinocytes and fibroblasts leading to cell correction and long lasting expression of type VII collagen. The dermal-epidermal junction in generated skin equivalents was restored [63]. A similar strategy was shown by Siprashvili et al. using an epitope-tagged *COL7A1* cDNA, providing a long term expression of the protein in skin equivalents [64]. In a clinical trial executing a bone marrow transplantation 6 RDEB patients received allogeneic stem cells to milder the RDEB phenotype. As a result, 5 patients showed an improved wound healing, but one patient died [65].

Until now, no *ex vivo* gene therapy approach passed through a phase I/II gene therapy trial. Most of these applications are focusing on the transfer of full-length *COL7A1* cDNA into the affected patient cells. The drawbacks of the insertion of the full-length 9kb cDNA of *COL7A1* are the cloning and packaging limitations of commonly used vector systems to transduce keratinocytes or fibroblasts and the instability of the *COL7A1* gene due to possible genetic rearrangements of the large repetitive cDNA sequence [47]. Additionally, the influence of *COL7A1* over- or ectopic expression in treated cells has to be clarified for a clinical application. Using the methodology of spliceosome mediated RNA *Trans*-splicing (SMaRT) can be a promising alternative to the mentioned approaches to cope with some of the suspected issues present in full-length *COL7A1* replacement strategies. Murauer et al. demonstrated the exchange of the 3′ coding *COL7A1* cDNA region spanning from exon 65 to the last exon 118 by SMaRT [66]. Thereby the risk of genetic rearrangements of the *COL7A1* cDNA sequence should be reduced significantly. Alternatively to this, we will present in this work a 5′ exon replacement strategy using SMaRT, providing the possibility to repair also relevant mutations 5′ within the *COL7A1* gene.

10. Spliceosome mediated mRNA *Trans*-splicing

10.1. General aspects

RNA *trans*-splicing is a naturally occurring event to recombine two or more mRNA molecules to a new chimeric gene product [67]. For therapeutic purposes such products can be generated by *trans*-splicing a second RNA species from a RNA *trans*-splicing molecule (RTM) into the 3′, 5′ or internal sequence of an endogenously expressed target. See Figure 6. The main advantages of this methodology are the possibility to reduce the size of the transgene, the maintained endogenous regulation of transgene expression and the feasibility to treat dominant negative diseases [68]. Undesired gene expression due to unintended delivery or misregulation is minimized as *trans*-splicing should only occur in cells expressing the target pre-mRNA [69]. Furthermore, SMaRT offers the potential for correction of dominant negative mutations into wildtype gene products [70].

10.2. Methodology of spliceosome mediated mRNA *trans*-splicing (SMaRT)

In SMaRT constructs that are engineered to bind the introns of specific pre-mRNAs – RNA *trans*-splicing molecules (RTMs) - are the key players. These RTMs effect a *trans*-

splicing event between the target pre-mRNA and the RTM which is mediated by the spliceosome. An RTM carries three domains; i) a binding domain complementary to the target intron to localize the RTM to the target pre-mRNA; ii) a splicing domain containing splicing elements for efficient *trans*-splicing; and iii) a coding domain comprising one or more wildtype exons that are *trans*-spliced to the target. The *cis*-splicing elements and the binding domain are not retained in the modified RNA product [71]. Depending on the gene portion to replace, SMaRT can be divided into 3′, 5′ or internal exon replacement [69]. See Figure 6.

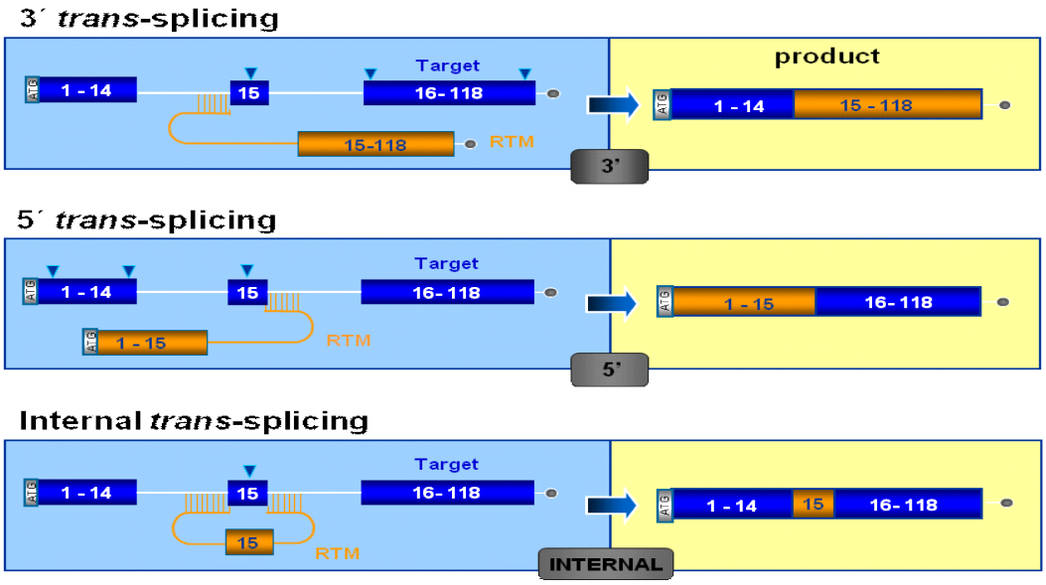


Figure 6. Schematic overview on different applications of SMaRT. **A:3`*Trans*-splicing:** If there is a mutation in the 3′ part of the target gene a wildtype mRNA can be obtained by 3′splicing. Therefore, a 3′RTM with a binding domain situated in the intron 5′ to the first exon to be exchanged is necessary. E.g. if the mutation to be corrected is in exon 15, a binding domain for intron 14 is designed. This RTM can correct mutations more 3′ as well. After binding of the RTM the two mRNAs are *trans*-spliced and combined into a wildtype mRNA. **B: 5`*Trans*-splicing:** If correction of a mutation in the 5′ part of a gene is desired a 5′ RTM with a binding domain located in the intron 3′ to the exon to be exchanged is created. If the mutation to be corrected with 5′splicing is in exon 15, a RTM with a binding domain in intron 15 is required. This RTM can repair mutations more 5′ than exon 15 as well. **C: Internal *Trans*-splicing:** There is also a method to exchange only one exon, called internal *trans*-splicing or internal exon replacement (IER). Here an RTM with two binding domains and 5′ and 3′ splice elements is applied. Arrowheads indicate mutations.

10.3. Efficiency of SMaRT

The efficiency of *trans*-splicing to correct genetic defects and acquired disorders at pre-mRNA level has already been demonstrated for 3′ as well as for 5′ *trans*-splicing in different diseases *in vitro* and *in vivo*. See Table 3.

Author	Approach	Year
3´ trans-splicing		
Puttaraju et al.	3´ repair of lacZ in a tractable system	2001
Chao et al.	3´ repair of haemophilia A mice in vivo	2003
Dallinger et al.	3´ repair in a lacZ model system in a keratinocyte specific background	2003
Liu et al.	3´ repair of CFTR mRNA (adenovirally)	2005
Rodriguez-Martin et al.	3´ reprogramming of tau alternative splicing in a model system	2005
Zayed et al.	3´ repair of DNA-PKcs in SCID (delivery via sleeping beauty)	2007
Chen et al.	3´ repair dystrophia myotonica type 1 pre-mRNA	2008
Coady et al.	3´ SMN2 trans-splicing in combination with blocking an cis-splice sit in mice in vivo	2010
Murauer et al.	Functional 3´ repair of the COL7A1 gene	2010
Wang et al.	3´ introduction of therapeutic proteins in highly abundant albumin transcripts in mice in vivo	2009
Gruber et al.	3´ reprogramming of tumor marker genes to introduce suicide genes	2011
5´trans-splicing		
Mansfield et al.	5´ repair of CFTR mRNA	2000
Kierlin-Duncan et al.	5´ repair of β-globin mRNA	2007
Wally et al.	5´ repair of the PLEC1 gene	2007
Wally et al.	5´ K14 mRNA reprogramming	2010
Rindt et al.	5´ trans-splicing repair of huntingtin at mRNA level	2012
Internal trans-splicing		
Lorain et al.	Exon exchange approach to repair Duchenne dystrophin transcripts in a minigene	2010
Koller et al.	A screening system for IER molecules	2011

Table 3. Overview on functional *trans*-splicing approaches so far.

RNA *trans*-splicing for gene correction is usually performed by 3′ RNA *trans*-splicing to exchange 3′ coding parts of a gene of interest. 3′ RNA *trans*-splicing was successfully applied to restore wildtype gene expression pattern amongst others in patient cells or in animal models of epidermolysis bullosa, cystic fibrosis, X-linked immunodeficiency and hemophilia A [66,72,73]. Primarily co-transfection experiments with RTMs and artificial targets were used to give proof of principle of functionality of the *trans*-splicing process.

So a tractable lacZ model repair system, in which user defined target introns can be *trans*-spliced into a mutated lacZ gene to test target specific 3′ RTMs by double transfection in 293T cells was developed by Puttaraju et al.. Functional lacZ correction was detected on mRNA and protein level by qRT-PCR and western blotting for one CFTR intron [74]. Chao et al. showed that the hemophilia A phenotype in factor VIII (FVIII) knockout mice can be repaired by the introduction of a 3′ RTM. After delivery of the DNA through the portal vein, the FVIII protein was detected by western blot analysis of cryoprecipitated murine plasma. Long-term correction was shown via adenoviral tail vein transduction of the specific RTM. In the classical tail-clip test all naive knockout mice died, whereas eight out of ten treated mice survived, indicating that 3′ *trans*-splicing is suitable to correct the bleeding disorder in hemophilia A [73]. Liu et al. used a recombinant adeno-associated viral vector system to target the human cystic fibrosis (CF) polarized airway epithelia from the apical membrane. The measurement of the cAMP-sensitive short circuit currents levels confirmed the CFTR correction by SMaRT [75]. Dallinger et al. showed as a proof of principle in the skin the correction of the EB-associated gene *COL17A1* by 3′ *trans*-splicing. Using a lacZ model repair system, an intron specific target molecule and a rationally designed RTM, the feasibility of SMaRT was shown by co-transfection experiments in keratinocytes [76]. Using a minigene Rodriguez-Martin et al. published functional 3′ *trans*-splicing on mRNA level after double transfection of the minigene and specific 3′RTM in COS-7 and SH-SY5Y cells for tau mRNA [77]. Zayed et al. demonstrated 3′ correction of the DNA protein kinase catalytic subunit (DNA-PKcs) gene, which is responsible for severe combined immune deficiency (SCID). Specific 3 ′RTMs were transfected into scid.adh cells using the Sleeping Beauty transposon system. After this treatment irradiated cells showed an 4.3 fold increase of surviving cells over irradiated untreated scid.adh cells. Correction of the mutation was shown via QRT-PCR and sequencing on mRNA level. Additionally, functional 3′*trans*-splicing was detected on mRNA level via sequencing and on protein level via western blotting in SCID multipotent adult progenitor cells [78]. Chen et al. corrected the dystrophia myotica protein kinase gene responsible for the most common muscular dystrophy in adults by 3′ *trans*-splicing on mRNA level [79]. Coady et al. showed *in vivo* correction of spinal muscular atrophy (SMA) by 3′*trans*-splicing in mice recently. A single injection of a repair construct *trans*-splicing *SMN2* carried by a PMU3 vector into the intracerebral-ventricular space of SMA neonates lessens the severity of the SMA phenotype in a severe mouse model and extends survival by around 70% [80]. Murauer et al. corrected mutations in *COL7A1* by 3′*trans*-splicing. RDEB keratinocytes retrovirally transduced with a 3′*trans*-splicing molecule showed an increase of *COL7A1* mRNA sqRT-PCR and recovery of full-length type VII collagen expression on protein level in western blot and

immunofluorescent staining. Moreover normal morphology and reduced invasive capacity was achieved in transduced cells. Correct localization of type VII collagen at the basement membrane zone in skin equivalents, where it assembles into anchoring fibril like structures, showed the potential of *trans*-splicing to correct an RDEB phenotype *in vitro* [66]. There are also alternative approaches in which therapeutic proteins are produced after specific 3′*trans*-splicing events into highly abundant albumin transcripts using 3 ′RTMs [81]. Another area of application of SMaRT was performed by Gruber et al. to treat malignant SCC tumors, which are life-threatening issues for RDEB patients. The transfection of RDEB SCC cells with a designed 3′ RTM lead to the fusion of the toxin streptolysin O, carried by a 3′ RTM, to MMP-9 pre-mRNA molecules, resulting in the expression of the toxin and subsequently to the cell death of transfected tumor cells [82].

5′ *trans*-splicing to correct upstream coding sequences of an mRNA of interest was first shown by a double transfection model to repair mutations in the cystic fibrosis transmembrane receptor (CFTR) pre-mRNA. Functionality tests were performed by anion efflux transport measurements. RTMs were designed capable to repair the 5′ portion of CFTR transcripts [83]. 5′ *trans*-splicing was also applied for the substitution of exon 1 of b-globin in cells co-transfected with a target molecule and an RTM in 293T cells and lead specific *trans*-splicing detected by one step RT-PCR [84]. Endogenous 5′ *trans*-splicing induced gene correction was first demonstrated by Wally et al. on the basis of the *PLEC* gene involved in the disease epidermolysis bullosa simplex (EBS). Restoration of wild-type plectin expression patterns was shown by immunofluorescence microscopy of patient fibroblasts after RTM treatment [61]. Additionally, exons 1–7 of the keratin 14 gene (*KRT14*) were replaced in an autosomal dominant model of EBS resulting in recovery of K14 on RNA and protein level, detected by SQRT-PCR, western blotting and immunofluorescence staining by transient transfection of specific 5′ RTMs chosen in a screening procedure [85]. Recently 5′*trans*-splicing correction of a disease causing huntingtin allele on mRNA level was reported by Rindt et al. [86].

Lorain et al. primarily published the methodology of internal exon replacement (IER) to correct a dystrophin minigene on mRNA level [87]. Recently, Koller et al. developed a new RTM screening system to improve double RNA *trans*-splicing for the correction of the EB associated gene *COL17A1* [88].

10.4. RTM screening systems

So far, there are no general rules for the design of highly efficient *trans*-splicing RTMs. However, recent studies revealed the influence of minor differences in length, composition and localization of the binding domain (BD) on RTM efficiency and specificity [85,88]. Due to the fact that an RTM can't be predicted rationally, we established a fluorescence-based screening system to select an efficient RTM from a pool of randomly designed RTMs. This screening system is composed of fluorescence-based RTM backbones, in which randomly created binding domains are cloned, and a gene specific target molecule. The target binding region (exon/intron sequence of a gene of interest) is PCR amplified, randomly fragmented and cloned into the RTM vector. The coding region consists

of a fluorescence reporter, divided into two (5′ or 3′ *trans*-splicing) or three parts (internal exon replacement) and distributed to both screening molecules (target molecule and RTM). The RTM library is composed of individual RTMs with various binding domains. Their efficiency can be evaluated by fluorescence microscopy and flow cytometry. For flow cytometric analysis, individual selected RTMs of the RTM library are co-transfected with the designed target molecule, including the full-length target binding region, and the missing sequence of the split fluorescence reporter into HEK293FT cells. Co-transfection of RTM and target molecule into HEK293FT cells results in the restoration of expression of the fluorescence reporter. The intensity of the fluorescence signal of the reporter molecule gives information on the functionality of the binding domain. The most efficient BDs can be tested for endogenous experiments in patient cells. After transfection of the screening-RTM, the fusion of the splitted *trans*-splicing reporter and the endogenous target is detected by RT-PCR. To develop an mRNA based gene therapy an RTM, carrying the wildtype sequence instead of the coding sequence of the fluorescence molecule, is constructed. After RTM treatment of patient cells a mutated gene part is exchanged by *trans*-splicing and wildtype transcripts are restored.

11. RTMs for the murine COL7A1 gene

We started to establish 5′ *trans*-splicing for murine *COL7A1* in order to analyze the functionality of RNA *trans*-splicing *in viv*o, due to the existence of a mouse model carrying a neo cassette in intron 2 of *COL7A1* generating aberrant splice variants, which lead to a reduction of type VII collagen expression [46]. By close similarity of this mouse model to the human RDEB phenotype and location of the defect in the 5′ part of *COL7A1*, this mouse model exhibits obviously an ideal system to test our 5′ repair molecules and investigate different application strategies.

Intron 15 was chosen as target intron because its size of about 1,5kb allows to create a large number of different binding domains. To generate a large amount of different RTMs, containing binding domains with different binding affinities to the target intron, the target exon/intron was digested out of the artificial target used in the screen with HindIII and BamHI and digested with CviJI*. The resulting fragments with a length of 50-750bp were cloned into the RTM backbone. Binding domains were identified by colony PCR using a forward primer situated in the 5′ half of the split GFP and a vector specific reverse primer. Possible binding domains with different lengths were detected on a 2% agarose gel after gel electrophoresis. To check orientation and location of the binding domain, clones with inserts were sequenced. To evaluate the created RTM library the artificial target containing the target intron (intron15) and the 3′ half of the split AcGFP instead of the 3′ part of murine *COL7A1* was cotransfected with the RTM library respectively individual RTMs into HEK293FT. The RTMs contain a transfection reporter (DsRED), the 5′ half of the split AcGFP instead of the first 15 exons of *COL7A1* and variable binding domains. The cotransfected cells were analyzed concerning their AcGFP/DsRED expression ratios by fluorescence

microscopy and flow cytometry, whereby a higher ratio indicates the presence of a more functional binding domain in the RTM. See Figures 7+8.

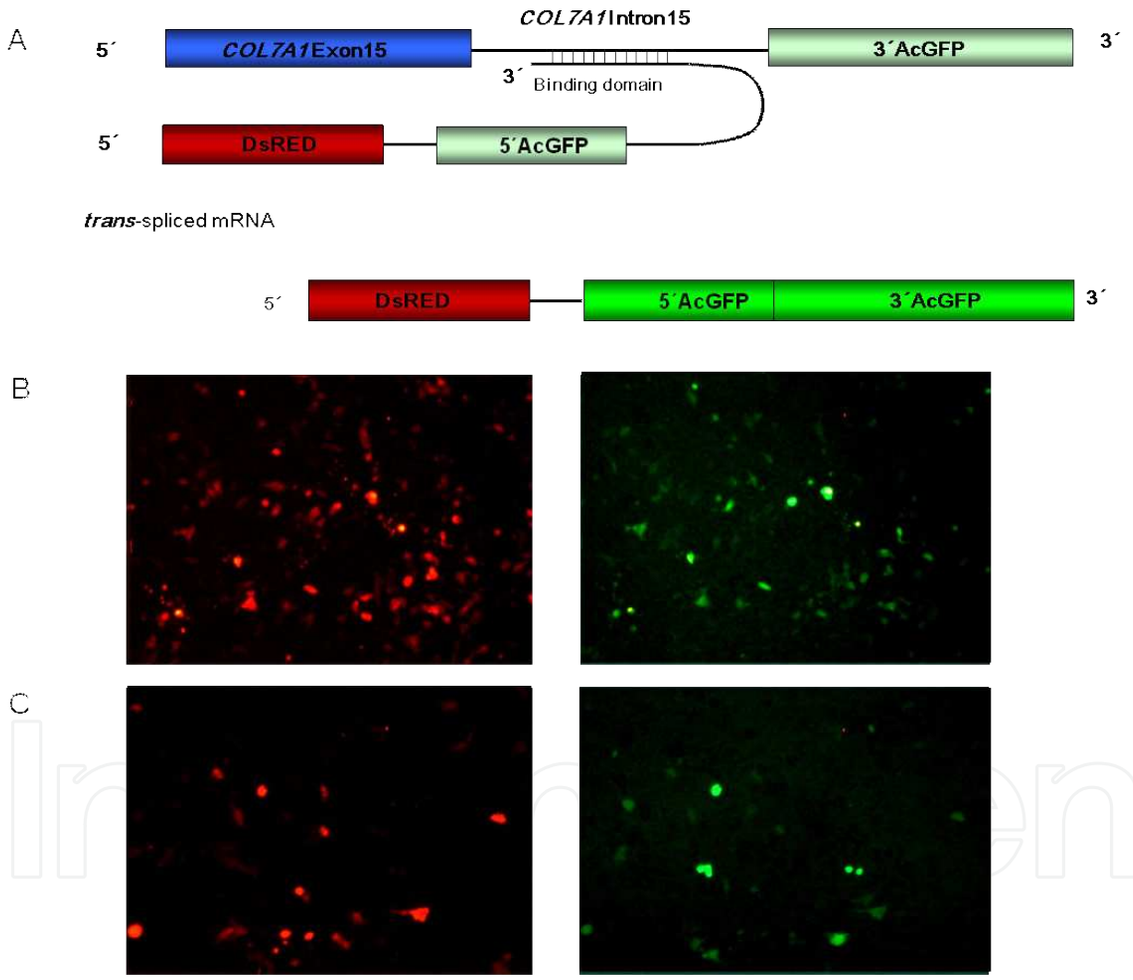


Figure 7. Fluorescence microscopy of with RTM library and target double transfected HEK293 cells. **A:** Functional binding domains lead to specific *trans*-splicing of the two pre mRNAs which are then combined into one mRNA containing DsRED and full-length AcGFP. Red fluorescence indicates the transfection of a RTM in the cells whereas red and green fluorescence indicates functional *trans*-splicing. **B:** Double transfection of an artificial target containing exon/intron 15 of murine *COL7A1* and an RTM library for this exon/intron in HEK293FT. **C:** Double transfection of an artificial target containing exon/intron 15 of murine *COL7A1* and the best RTM for this intron.

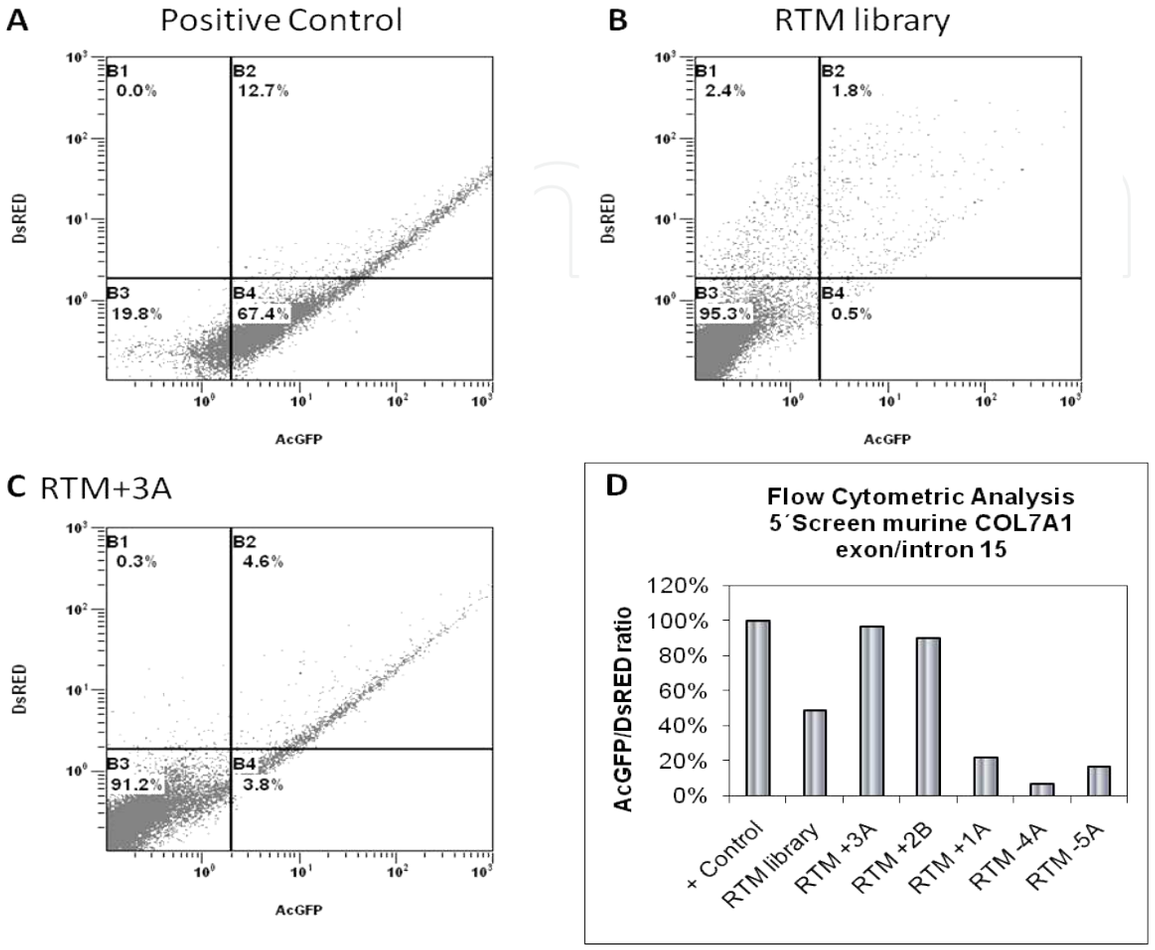


Figure 8. Flow cytometric analysis: 5´ screen for murine *COL7A1* exon/intron 15. Red fluorescence (shown on Y-axis) indicates transfection of RTMs in the cells; green fluorescence (shown on X-axis) indicates specific *trans*-splicing. **A:** The positive control is a vector containing a DsRED linker AcGFP construct. This FACS plot mimics the AcGFP and DsRED ratios expected from the product of optimal *trans*-splicing. **B:** The RTM library shows a *trans*-splicing efficiency (AcGFP/DsRED ratio) of 48,94% calculated from *trans*-splicing positive cells/all transfected cells.(B2 1,8% + B4 0,5%)/(B1 2,4% + B2 1,8% + B4 0,5%) The fact, that several cells seem to be exclusively green can be explained by the intense AcGFP fluorescence, which tends to override the weaker DsRED fluorescence. **C**: The most efficient RTM analyzed, with a *trans*-splicing efficiency of 96,55%, (B2 4,6% + B4 3,8%)/(B1 0,3% + B2 4,6% + B4 3,8%) shows a dot plot pattern similar to the positive control. Therefore the binding domain of RTM +3A was chosen to be used in further endogenous experiments. **D:** A Comparison of AcGFP/DsRED ratios of single RTMs containing different binding domains, shows a wide variability of AcGFP/DsRED ration spanning less than 10% to nearly 100%.

The RTM with the highest AcGFP/DsRED ratio (RTM+3A) was chosen for further endogenous experiments. To check endogenous functionality of the RTM was transiently transfected into an immortalized murine keratinocyte cell line [46]. The 5´ part of the split AcGFP contained by the screening RTM was specifically *trans*-spliced with its endogenous target – the murine *COL7A1* mRNA – resulting in a AcGFP-*COL7A1* fusion mRNA detected by RT-PCR analysis and subsequent sequencing. See Figure 9.

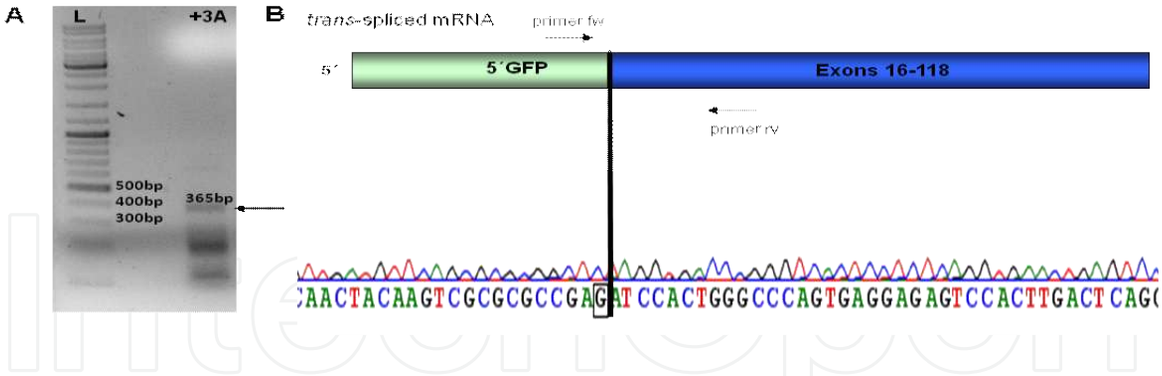


Figure 9. Endogenous *trans*-splicing into exon/intron 15 of murine *COL7A1*. **A:** RT-PCR analysis of transiently RTM+3A transfected spontaneously immortalized mouse wildtype keratinocytes [46] using primers in the 5´ part of split AcGFP and in exon 18 of *COL7A1* resulted in detection of a 365bp band after agarose gel-electrophoresis. B: The fragment was verified to be an AcGFP-*COL7A1* fusion by sequencing. **+3A** cDNA analysis of spontaneously immortalized mouse wildtype keratinocytes transiently transfected with RTM +3A **L** Ladder Mix DNA marker

12. Conclusion

RNA *trans*-splicing is a useful methodology to reprogram genes for diagnostic and therapeutic purposes. Due to a variety of advantages over traditional gene-replacement strategies, RNA *trans*-splicing is used to correct the phenotype of many genetic diseases *in vitro*, ranging from epidermolysis bullosa to neurodegenerative diseases. *In vivo* studies are in progress to accelerate the way to the medical use of this RNA-based application.

We have established all three modes of *trans*-splicing (5′, 3′ and internal exon replacement) in our laboratory on the basis of several EB-associated genes (*KRT14, PLEC, COL7A1, COL17A1*). In this work we focused on the methodology of 5′ RNA *trans*-splicing to correct mutations localized within the first 15 exons of the murine *COL7A1* gene encoding for type VII collagen. *COL7A1* is a large gene with over 9kb and is therefore suitable for this approach, in which only a short RTM has to be designed, harbouring only the first 15 exons of the gene. Using an RTM screening system, described by Wally et al 2011 [89], it should be possible to increase the *trans*-splicing efficiency of designed RTMs to a level where the phenotype of *COL7A1* deficient cells can be converted into wildtype. We analyzed the binding properties of randomly designed RTMs specific for intron 15 of murine *COL7A1* and tested the most efficient RTM in *COL7A1* deficient keratinocytes for endogenous functionality. The RTM was able to induce endogenous 5′*trans*-splicing into murine *COL7A1* pre-mRNA molecules, manifested in the fusion of the 5′ GFP part of the RTM with exon 16 of *COL7A1*. Next steps are the exchange of the 5′ GFP part by the 5′sequence of murine *COL7A1* (exons1-15) and to investigate if our RTMs are able to increase the level of full-length *COL7A1* mRNA leading to the recovery of functional type VII collagen in *COL7A1* deficient cells and in skin equivalents. In summary we demonstrated a novel RNA-based strategy to correct disease-associated mutations within *COL7A1*, thereby avoiding or minimizing many problems present in standard cDNA gene therapies including fragmentation of the large *COL7A1* gene, the size limitation of the transgene and over- and ectopic expression of the transgene.

The development of a gene therapy for type VII collagen deficiency would increase the chance to find a cure for dystrophic EB. Additionally, the improvement of the methodology of 5′ RNA *trans*-splicing will help us to move closer to the treatment of other genetic diseases caused by mutations in especially large genes.

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