Functional Correction of Type VII Collagen Expression in Dystrophic Epidermolysis Bullosa

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Functional defects in type VII collagen, caused by premature termination codons on both alleles of the *COL7A1* gene, are responsible for the severe autosomal recessive types of the skin blistering disease, recessive dystrophic epidermolysis bullosa (RDEB). The full-length *COL7A1* complementary DNA (cDNA) is about 9 kb, a size that is hardly accommodated by therapeutically used retroviral vectors. Although there have been successful attempts to produce functional type VII collagen protein in model systems of RDEB, the risk of genetic rearrangements of the large repetitive cDNA sequence may hamper the clinical application of full-length *COL7A1* cDNA in the human system. Therefore, we used *trans*-splicing to reduce the size of the *COL7A1* transcript. Retroviral transduction of RDEB keratinocytes with a 3′ pre-*trans*-splicing molecule resulted in correction of full-length type VII collagen expression. Unlike parental RDEB keratinocytes, transduced cells displayed normal morphology and reduced invasive capacity. Moreover, transduced cells showed normal localization of type VII collagen at the basement membrane zone in skin equivalents, where it assembled into anchoring fibril-like structures. Thus, using *trans*-splicing we achieved correction of an RDEB phenotype *in vitro*, which marks an important step toward its application in gene therapy *in vivo*.

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Journal of Investigative Dermatology (2011) **131,** 74–83; doi:10.1038/jid.2010.249; published online 19 August 2010

INTRODUCTION

Dystrophic epidermolysis bullosa (DEB) is a clinically severe variant of a group of inherited autosomal dominant or recessive (RDEB) mechanobullous diseases caused by mutations in the *COL7A1* gene (Pulkkinen and Uitto, 1999; Uitto and Pulkkinen, 2001). The *COL7A1* mRNA of 9.2 kb contains 118 exons and codes for the 290 kDa type VII collagen (Christiano *et al.*, 1994a). Type VII collagen is secreted into the extracellular space by both dermal fibroblasts and epidermal keratinocytes, where it self-assembles into highly organized anchoring fibrils (AFs) that extend from the lamina densa of the epidermal basement membrane into the underlying dermal connective tissue and take part in securing the epidermal–dermal adhesion (Morris *et al.*, 1986; Burgeson, 1993; Christiano *et al.*, 1994b; Bruckner-Tuderman *et al.*, 1995).

Over 400 distinct mutations spanning the entire *COL7A1* gene have been identified in DEB (Dang and Murrell, 2008). The compilation of mutation data has determined that severe generalized RDEB phenotypes are due to premature termination codons. The molecular aberrations affect synthesis, secretion, or molecular assembly of type VII collagen into AFs within the dermal–epidermal junction (DEJ), leading to separation of dermis and epidermis (Fine *et al.*, 2008). Patients with the most severe types of RDEB are prone to invasive SSC, and have lifelong severe disease with considerable morbidity and mortality (Mallipeddi, 2002).

Retroviral vectors are the most used vehicles in human gene therapy trials because of their high transduction efficiency of eukaryotic cells *ex vivo* and sustained expression of the transgene after transplantation *in vivo* (Morgan and Anderson, 1993; Khavari *et al.*, 2002). Therefore, most gene therapy efforts for DEB are focused on an *ex vivo* transfer of the wild-type *COL7A1* complementary DNA (cDNA) into affected keratinocytes or fibroblasts to correct the RDEB phenotype. For example, integration of *COL7A1* cDNA (8.9 kb) into human type VII collagen-null RDEB keratinocytes and/or fibroblasts has been achieved using PhiC31 bacteriophage integrase (Ortiz-Urda *et al.*, 2002), a lentiviral vector (Chen *et al.*, 2002), as well as a retroviral

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Abbreviations: AF, anchoring fibril; BD, binding domain; DEJ, dermal–epidermal junction; EB, epidermolysis bullosa; IRES, internal ribosome entry site; PTM, pre-trans-splicing molecule; RDEB, recessive dystrophic epidermolysis bullosa; SE, skin equivalent

Received 5 February 2010; revised 15 June 2010; accepted 8 July 2010; published online 19 August 2010

vector (Gache *et al.*, 2004). These studies resulted in reversion of many RDEB symptoms after gene transfer. However, titer and the large size of *COL7A1* cDNA are persistent problems that hamper the clinical development of this type of vector-based gene therapy.

To overcome this problem, *COL7A1* ''minigenes'' that maintain proper biochemical functions *in vitro* have been developed, and could potentially fit into retrovirus-based gene transfer vectors (Chen *et al.*, 2000). However, the efficiency of proteins of reduced size in correcting a collagen deficiency has yet to be demonstrated. An alternative strategy includes the use of *trans*-splicing technology for gene correction. *Trans*-splicing is a gene repair mechanism, using the cell's spliceosome to recombine an endogenous target pre-mRNA and an exogenously delivered RNA molecule called pre-*trans*-splicing molecule (PTM). A part of the endogenously expressed target pre-mRNA is replaced by the wild-type coding sequence from the PTM by *trans*-splicing into the 3′ or 5′ sequence of the target to generate a new reprogrammed mRNA (Puttaraju *et al.*, 1999; Wally *et al.*, 2008). Functional RNA repair through *trans*-splicing has been reported in a variety of *in vitro, ex vivo,* and *in vivo* studies (Mansfield *et al.*, 2000; Chao *et al.*, 2003; Tahara *et al.*, 2004). The feasibility of *trans*-splicing to be used for correction of a monogenic skin disease has been established in the *COL17A1* gene in an immortalized patient cell line (Dallinger *et al.*, 2003). Recently, we showed that 5′ *trans*-splicing repair of the *PLEC1* gene in patient fibroblasts increased the level of functional plectin protein by 58% (Wally *et al.*, 2008). These studies have laid the ground for further studies in the most severe forms of epidermolysis bullosa (EB). Therefore, we transduced primary human RDEB keratinocytes with a 3′ PTM to explore the correction of type VII collagen expression by 3′ *trans*-splicing. We demonstrate here that 3′ *trans*-splicing is capable of fully reverting the RDEB phenotype in cell culture, which set the basis for its application in an *ex vivo* gene therapy approach in RDEB patients.

RESULTS

Retroviral transduction of human RDEB keratinocytes with a 3′ PTM vector

To evaluate the phenotypic reversion of RDEB keratinocytes by 3′ *trans*-splicing, we used primary keratinocytes derived from an RDEB patient carrying two premature termination codons in *COL7A1* exons 14 and 106 causing type VII collagen deficiency. A plasmid was designed to express PTM-S6 to repair the 3′ part of the endogenous *COL7A1* transcript encompassing the mutation located in exon 106 (Figure 1a). PTM-S6 contains a 224-bp binding domain (BD) complementary to the last 188 nucleotides of the *COL7A1* intron 64 pre-mRNA and all 36 nucleotides of exon 65, typical 3′ *trans*-splicing elements (a 31-bp spacer, a yeast branch point, a polypyrimidine tract, and a 3′ splice acceptor site), and the 3.3-kb wild-type *COL7A1* coding sequence extending from exons 65 to 118. The BD was designed to exclude high sequence homology with other genes, which was verified by mapping the 224-bp BD sequence against the human genome using the NCBI BLASTn (somewhat similar sequences). For transduction of the RDEB keratinocytes, PTM-S6 was inserted into the retroviral vector pLZRS-IRES-Zeo (Michiels *et al.*, 2000) to generate the pLZRS-PTM-S6 vector (Figure 1b). After transduction and antibiotic selection, the RDEB cells were processed to evaluate the genetic correction and phenotypic reversion through *trans*-splicing.

Long-term serial propagation of keratinocytes in culture is sustained by the presence of epidermal cells characterized by high differentiation and clonogenic potential (Barrandon and Green, 1987). To achieve long-term propagation of the transduced primary RDEB keratinocytes for further analysis, we isolated independent colonies with a large and smooth perimeter of cell pools, and one colony (clone no. 1) that did not undergo senescence during serial cultivation was selected for all subsequent experiments to analyze the functional correction of type VII collagen expression. The selected clone no. 1 was assessed for clonogenic capacity by colony-forming assays at passages 8 and 20 (17 and 40 cell doublings). Clonal analysis indicated relatively constant colony-forming efficiency values at both passages (26–36%) (Figure 1c), suggesting that our cell cultures contain permanently transduced clonogenic cells.

PCR analysis of genomic DNA extracted from the pool of transduced cells and from the selected clone no. 1 using primers specific for the pLZRS-PTM-S6 vector showed the expected 3.6-kb amplification fragment (Figure 1d), indicating that the PTM-S6 molecule was stably integrated into the genome of infected cells. The average proviral copy number per cell evaluated by linear amplification-mediated (LAM)-PCR analysis of genomic DNA extracted from transduced cells and from the selected clone no. 1 after multiple passages (at least 40 population doublings) indicated integration of one single copy of pLZRS-PTM-S6 (Figure 1e).

Correction of type VII collagen expression in retrovirally transduced RDEB keratinocytes

Immunostaining of cultured cells using an anti-type VII collagen polyclonal antibody (pAb) was carried out to evaluate protein expression in PTM-S6-transduced RDEB keratinocytes (Figure 2a). Cytoplasmic staining of type VII collagen was observed in 100% of the transduced keratinocytes after zeocin selection and in the RDEB-PTM-S6 clone no. 1, suggesting that type VII collagen-null RDEB keratinocytes were provided with the ability to express type VII collagen after *trans*-splicing repair. Immunostaining of RDEB-PTM-S6 clone no. 1 cells after ~40 doublings also showed immunoreactivity to type VII collagen, which confirms the sustained expression of PTM-S6 (Figure 2a). No immunoreactivity was detected with parental RDEB keratinocytes.

To evaluate the expression of the *trans*-spliced collagen VII at the DEJ, skin equivalents (SEs) were obtained from wild-type, RDEB, or transduced keratinocytes seeded on a dermal equivalent composed of a fibrin matrix containing human type VII collagen-null RDEB fibroblasts. Immunostaining with pAb directed against type VII collagen showed that SEs composed of PTM-S6-transduced RDEB keratinocytes clone no. 1 at passage 6 (~13 doublings) expressed type VII collagen at the basement membrane zone, whereas parental

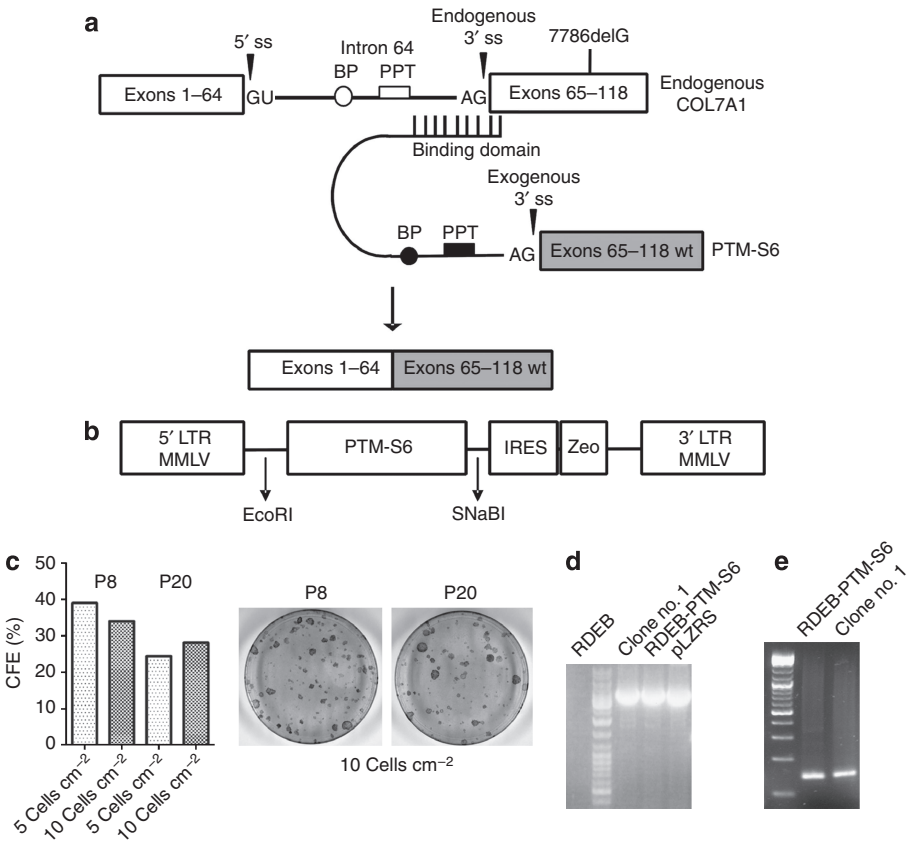

Figure 1. Retroviral transduction of recessive dystrophic epidermolysis bullosa (RDEB) keratinocytes with a 3′ pre-*trans*-splicing molecule (PTM). (**a**) *Trans*-splicing between the 5′-splice site (5′ss) of the endogenous *COL7A1* pre-mRNA and the 3′-splice site (3′ss) of the exogenously transduced PTM-S6 results in a reprogrammed *COL7A1* transcript, consisting of endogenous exons 1–64 (white) and delivered exons 65–118 (gray). (**b**) pLZRS-PTM-S6 vector comprising the Moloney mouse sarcoma virus long terminal repeat (LTR), the 3.6-kb PTM-S6, an internal ribosome entry (IRES), and a Zeocine-resistance gene. (**c**) Clonal analysis of keratinocyte cultures of clone no. 1 at passages 8 and 20 shows comparable clonogenic potential. (**d**) PCR analysis of PTM-S6 integration in the genome of RDEB- and PTM-S6-transduced keratinocytes. pLZRS, amplified product from plasmid pLZRS-PTM-S6. (**e**) Linear amplification-mediated-PCR analysis of genomic DNA of transduced RDEB cells showed one retroviral integration event. BP, branch point; CFE, colony-forming efficiency; PPT, polypyrimidine tract; wt, wild-type.

RDEB cells generated SEs that entirely lacked type VII collagen expression (Figure 2b). The intensity of the fluorescence signal was comparable to that observed in SEs obtained from wild-type keratinocytes. Localization was restricted to the basement membrane, with no expression in suprabasal cells. SEs obtained from the transduced cell clone after ~40 doublings still showed expression of type VII collagen at the DEJ (Figure 2b).

Transmission electron microscopy was performed on SEs to determine whether *trans*-splicing repair leads to restoration of AF formation at the basement membrane zone. Ultrastructural examination of the DEJ showed that the type VII collagen expressed and secreted by the corrected keratinocytes assembled into AF-like structures. Moreover, mature hemidesmosomes and a defined lamina densa indicate formation of a mature basement membrane. In contrast, SEs obtained from RDEB keratinocytes displayed a loose lamina densa, sparse hemidesmosomes, and entirely lacked AF (Figure 2c).

Reversion of the RDEB morphology and reduction of the invasive potential of RDEB keratinocyes

It has been reported that cultured RDEB keratinocytes display a different morphology from normal keratinocytes (Chen *et al.*, 2002). Moreover, compared with normal human keratinocytes, RDEB keratinocytes lacking type VII collagen show increased invasion properties *ex vivo* (Gache *et al.*, 2004), which is consistent with the proneness to develop invasive cancers in patients with RDEB (Mallipeddi, 2002; Martins *et al.*, 2009). To determine whether synthesis of type VII collagen by transduced RDEB keratinocytes exerts any functional effects on RDEB cells, we first analyzed the morphology of cells in culture before and after transduction. As shown in Figure 3a, RDEB keratinocytes in culture display an unusual size and elongated shapes with multiple cellular protrusions. These features are in contrast to the typical polygonal morphology observed in normal human keratinocytes. Compared with parental RDEB keratinocytes, PTM-S6-transduced RDEB keratinocytes demonstrated a shape and size that were similar to that of normal keratinocytes, suggesting that correction of type VII collagen expression in RDEB keratinocytes caused RDEB cells to convert to normal morphology.

To further explore the functional consequences of correction of type VII collagen expression by *trans*-splicing, cell invasion was assessed using *in vitro* invasion assays. We demonstrated that the *de novo* expression of type VII

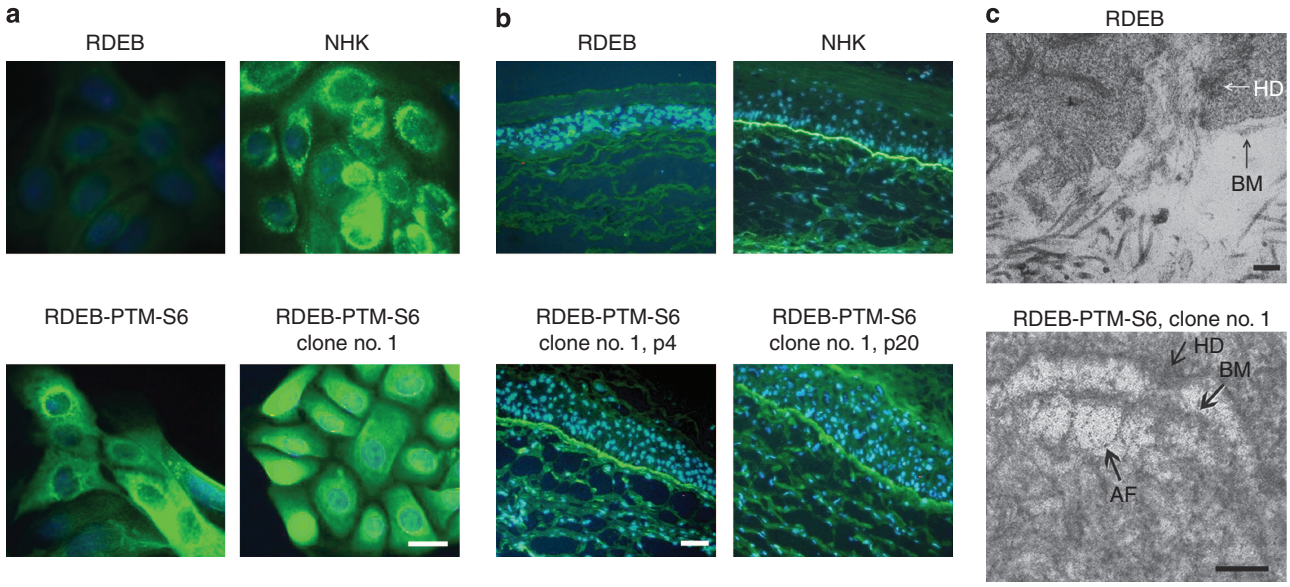

Figure 2. Reexpression of type VII collagen in 3′pre-*trans*-splicing molecule (PTM)-S6-transduced recessive dystrophic epidermolysis bullosa (RDEB) keratinocytes and at the dermal–epidermal junction of organotypic skin equivalents (SEs). (**a**) Immunofluorescence analysis of cultured keratinocytes showed no collagen VII staining in parental RDEB keratinocytes (RDEB), and positive staining in normal keratinocytes (NHK), PTM-S6-transduced cell pool (RDEB-PTM-S6), and clone no. 1 (RDEB-PTM-S6, clone no. 1). Bar = 20 μm. (**b**) Immunostaining for collagen VII is negative in SEs with RDEB keratinocytes (RDEB), and positive in SE with transduced RDEB keratinocytes at passages 4 and 20 (RDEB-PTM-S6, clone no. 1, p4/p20), comparable to the staining in normal SEs (NHK). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole dye. Bar = 100 μm. (**c**) Electron microscopy analysis shows no anchoring fibril (AF) in SEs of RDEB keratinocytes (RDEB), whereas rudimentary AFs are observed underneath the lamina densa in SEs of PTM-S6-transduced cells (RDEB-PTM-S6, clone no. 1). Bar = 100 nm. BM, basement membrane; HD, hemidesmosomes.

collagen significantly impaired the capacity of transduced RDEB keratinocytes to invade Matrigel-coated Transwell chambers (Figure 3b). These results attest the biological activity of the *trans*-spliced type VII collagen expressed by corrected RDEB keratinocytes.

Expression and secretion of full-length type VII collagen by corrected RDEB cells

Immunoblot analysis was performed on spent medium from RDEB cell cultures using anti-type VII collagen pAb (Figure 3c). The type VII collagen band was completely absent in the parental RDEB cell culture medium. Analysis of normal human keratinocyte-conditioned medium showed a band with a molecular mass of 290 kDa, corresponding to the size of full-length type VII collagen. This band was also detected in the transduced RDEB cell culture medium. No other band migrating at a different size was detectable. Expression as measured by western blot analysis was sustained for over 40 doublings *in vitro.* These results indicate that *trans*-splicing of PTM-S6 in transduced RDEB keratinocytes generates long-term expression and secretion of the full-length type VII collagen protein *in vitro*.

Increased expression levels of COL7A1 mRNA after *trans*-splicing

To assess the level of *COL7A1* transcripts, semiquantitative real-time PCR analysis was performed. RDEB patient keratinocytes that did not express type VII collagen showed much less *COL7A1* mRNA relative to *GAPDH* than did normal keratinocytes (Figure 3d). Corrected keratinocytes have a twofold increase compared with RDEB patient keratinocytes, indicating efficient *trans*-splicing. Western blot analysis showed that this level is sufficient to express full-length type VII collagen protein in almost equal amounts than expressed by normal keratinocytes *in vitro* (Figure 3c).

To be able to detect *trans*-spliced *COL7A1* mRNA, five silent mutations were included on the PTM-S6 *COL7A1* coding sequence. Reverse transcriptase-PCR (RT-PCR) analysis of RNA extracted from transduced cells using a forward primer specific for the endogenous *COL7A1* portion and a reverse primer specific for the portion of PTM-S6 carrying silent mutations showed the expected 216-bp fragment, attesting to the presence of *trans*-spliced *COL7A1* mRNA. This band was completely absent in the RT-PCR analysis of untreated RDEB cells (Figure 3e). Subsequent sequence analysis of the amplified fragment confirmed correct *trans*-splicing (not shown).

DISCUSSION

In this study, we showed that 3′ *trans*-splicing is capable of repairing functional aberrations of type VII collagen in RDEB keratinocytes. Using a designed RNA *trans*-splicing construct, we restored the synthesis of type VII collagen molecules in keratinocytes isolated from an RDEB patient. The synthesis of type VII collagen reverted the morphology and invasion capacity of RDEB cells, is restricted to the DEJ, and promotes the formation of AFs *in vitro*.

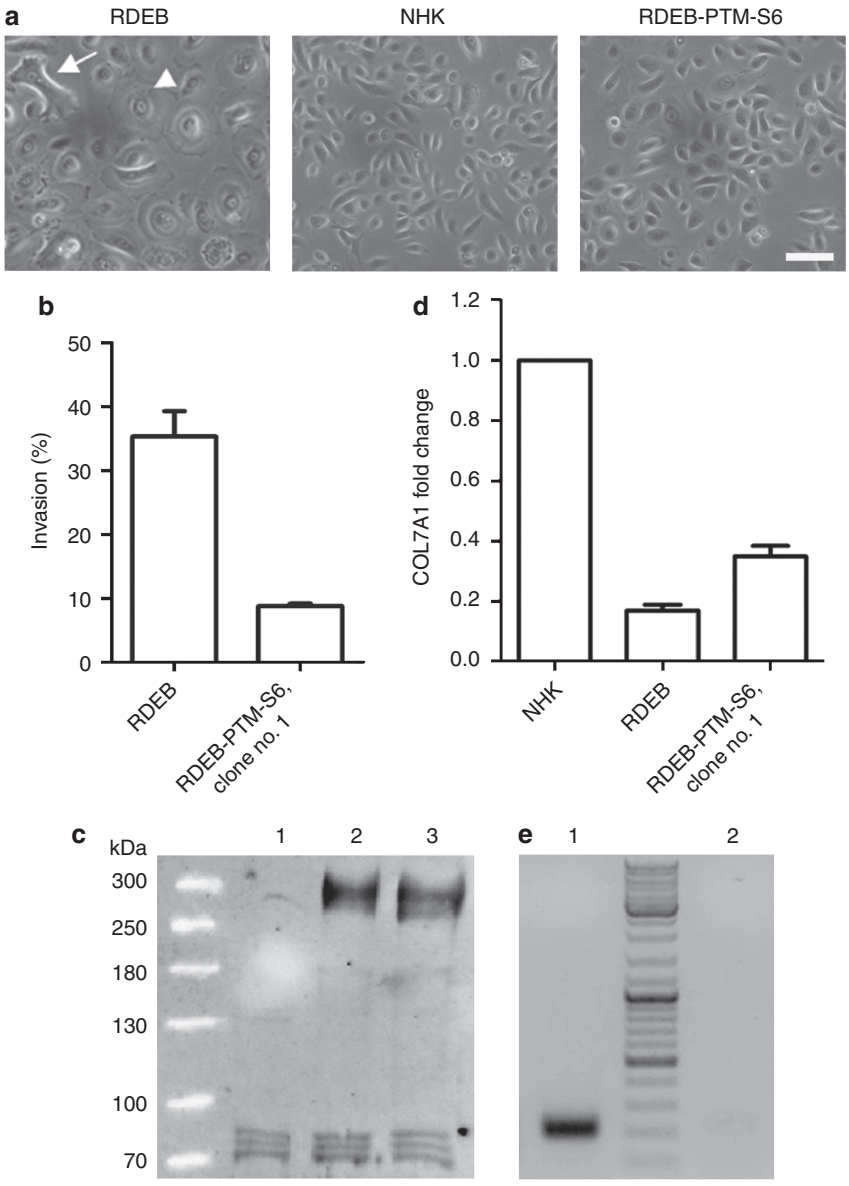

Figure 3. Functional correction of type VII collagen expression by *trans*-splicing. (**a**) Light microscopy of cell cultures. Recessive dystrophic epidermolysis bullosa (RDEB) parental keratinocytes; arrow, unusual shape; arrowhead, unusual size; normal human keratinocytes (NHK); (RDEB-3′ pre-*trans*-splicing molecule (PTM)-S6) PTM-S6-transduced RDEB keratinocytes. Bar = 10 μm. (**b**) PTM-S6-transduced RDEB keratinocytes display impaired invasive potential compared with parental RDEB keratinocytes. Shown are mean ± SD of triplicate values. (**c**) Immunoblot analysis indicates the absence of collagen VII in RDEB keratinocytes (1), whereas NHK (2) and PTM-S6-transduced RDEB keratinocytes (3) show secretion of full-length collagen VII. (**d**) Semiquantitative reverse transcription-PCR analysis of *COL7A1* mRNA expression shows low levels in RDEB keratinocytes compared with NHK, and a twofold increase in PTM-S6-transduced RDEB cells. (**e**) Reverse transcriptase-PCR analysis of *COL7A1* mRNA using *trans*-specific primers shows a band specific for *trans*-splicing in PTM-S6-transduced RDEB keratinocytes (1), undetectable in non-transduced RDEB keratinocytes (2).

In the last decade, significant research has been conducted investigating strategies to correct the RDEB phenotype caused by *COL7A1* mutations. Studies using an antisense oligoribonucleotide (Goto *et al.*, 2006), a P1-based artificial chromosome comprising a *COL7A1* gene (Mecklenbeck *et al.*, 2002), transduction of a *COL7A1* minigene construct (Chen *et al.*, 2000), or the full-length *COL7A1* cDNA with viral vectors (Chen *et al.*, 2002; Ortiz-Urda *et al.*, 2002; Baldeschi *et al.*, 2003; Gache *et al.*, 2004) have shown the possible restoration of type VII collagen expression in RDEB skin cells either *in vitro* or in grafted mice models. However, limitations of these therapy strategies include (1) the short-term correction of the phenotype (Goto *et al.*, 2006), (2) the production of a truncated type VII collagen that may induce dominant-negative interference (Chen *et al.*, 2000), (3) the unstable accommodation of the large type VII collagen transgene (9.2 kb) in viral vectors, and (4) the ectopic expression of the recombinant type VII collagen in all layers

of the reconstituted transgenic epidermis (Baldeschi *et al*., 2003; Gache *et al*., 2004). In this study, we have shown that *trans*-splicing technology allows an endogenously regulated synthesis of type VII collagen, and has the potential to circumvent the problem of truncated expression of the recombinant protein.

To correct type VII collagen defect in RDEB patient keratinocytes by 3′ *trans*-splicing, intron 64 was specifically targeted, allowing the reduction of 9.2-kb *COL7A1* cDNA to a 3.3-kb transgene. The reduction of the size of the *COL7A1* cDNA transgene may facilitate stable retroviral packaging and transduction of cells, and allows the correction of one-third of all *COL7A1* mutations described in various forms of DEB (Dang and Murrell, 2008) by a single 3′ PTM construct. Immunofluorescence analysis of PTM-S6-transduced RDEB keratinocytes isolated from a patient bearing heterozygous nonsense mutations in exons 13 and 106 showed positive staining to type VII collagen in 100% of treated cells, indicating that the delivered PTM-S6 confers successful *trans*-splicing of *COL7A1* transcripts *in vitro.* Thus, our designed PTM can correct the phenotype of RDEB cells bearing mutations downstream of exon 65. Furthermore, we have shown in SEs obtained from transduced keratinocytes that expression of the recombinant type VII collagen is restricted to the basement membrane zone, consistent with the idea that the recombinant type VII collagen is tightly regulated in the PTM-S6-corrected keratinocytes in stratified epithelia. This is different from previous studies in which RDEB keratinocytes, transduced with full-length *COL7A1* cDNA, showed ectopic expression of the recombinant protein in the suprabasal layers of the epidermis in SEs (Baldeschi *et al*., 2003; Gache *et al*., 2004).

Besides endogenous regulation, the level of expression of the recombinant protein at the DEJ is crucial for full reversion of the RDEB phenotype *in vivo*. Individuals who are heterozygous carriers of a nonsense mutation in the *COL7A1* gene do not have skin blistering, suggesting that the amount of type VII collagen necessary to prevent blistering lies somewhere between 0 and 50% (Tidman and Eady, 1985). Transgenic mice expressing type VII collagen at 10% of normal levels in the skin exhibit a milder phenotype and survive to adulthood (Fritsch *et al*., 2008), whereas type VII collagen knockout mice die early after birth (Heinonen *et al*., 1999). Therefore, a relatively low level expression of type VII collagen protein may be sufficient for restoration of the dermal–epidermal adhesion over long periods of time. Western blot analysis showed that PTM-S6-corrected RDEB keratinocytes synthesize and secrete full-length protein in an amount comparable to that of normal human keratinocytes. Using *in vitro* invasion assays, we also demonstrated that restoration of type VII collagen expression by *trans*-splicing significantly reduced the invasive migration potential of RDEB keratinocytes. These data indicate that *trans*-splicing through our delivered PTM-S6 leads to synthesis of enough type VII collagen protein to revert the invasive phenotype of RDEB cells *in vitro*.

Gene therapies using retroviral vectors may raise serious safety concerns because of the genotoxic risk associated with the insertion of viral long terminal repeat elements into the genome (Baum *et al*., 2003; Hacein-Bey-Abina *et al*., 2003; Fischer and Cavazzana-Calvo, 2005). One approach to make gene therapy safer in inherited skin diseases might be the safety preassessment of isolated genetically modified stem cells and their progeny before transplantation into RDEB patients (Del Rio *et al*., 2004; Porteus *et al*., 2006). Larcher *et al*. (2007) have recently demonstrated the long-term *in vivo* regenerative capacity of single-targeted human epidermal holoclones, using optimized organotypic culture and grafting methods onto nude mice. In this context, we have evaluated the long-term expression of the recombinant protein produced by the *trans*-splicing mechanism in transduced clonogenic cells. We showed that one clone, which underwent at least 40 population doublings, is able to synthesize full-length type VII collagen in culture and produce AFs in reconstructed SEs. The persistent expression of type VII collagen in the PTM-S6 clone indicates that, after integration site analysis, it may potentially be used for long-term *in vivo* correction of the RDEB phenotype. As an *in vitro* system cannot explore issues of long-term expression of corrective type VII collagen *in vivo,* studies are under way to test the regenerative performance of *trans*-spliced gene-corrected clones on immunodeficient mice engrafted with human skin (Del Rio *et al*., 2002; Larcher *et al*., 2007). Currently, EB mouse models to analyze 3′ *trans*-splicing are not available because of either perinatal lethality of type VII collagen knockout (Heinonen *et al*., 1999) or unsuitable location of mutations in mice with conditional inactivation of type VII collagen (Fritsch *et al*., 2008). The recently published surviving transgenic RDEB mouse model expressing human *COL7A1* cDNA with a c.7528delG mutation (Ito *et al*., 2009) is also not suitable for our approach, as *trans*-splicing repair reprograms gene expression at the pre-mRNA level and requires the presence of intronic sequences.

This is the first demonstration of an *in vitro* gene therapeutic approach for the *COL7A1* gene that exploits the endogenous RNA regulatory mechanisms of type VII collagen expression in human keratinocytes. Following the successful junctional EB gene therapy clinical assay reported by Mavilio *et al*. (2006), our long-term goal is to use the 3′ *trans*-splicing model for an *ex vivo* gene therapy approach for DEB patients. Our results may pave the way for the application of 3′ *trans*-splicing in gene therapy *in vivo* that covers mutations over 3300 nucleotides in the *COL7A1* gene. As this technology repairs mutations in genetic diseases, regardless of the mode of inheritance, it may provide a new tool for treatment of both recessive and dominant dystrophic EB.

MATERIALS AND METHODS

Cell cultures

Primary human keratinocytes and fibroblasts were obtained from skin biopsy samples from type VII collagen-null RDEB patients and healthy controls. The primary keratinocytes used for retroviral transduction were derived from an RDEB patient heterozygous for mutations R578X and 7786delG in exons 13 and 104 of the *COL7A1* gene, respectively. Keratinocytes were cultured on feeder layers of lethally irradiated mouse

J2-3T3 fibroblasts in DMEM:Ham's F-12 (3:1) (HyClone/Perbio Science, Bezons, France) (Rheinwald and Green, 1975), or without feeder layers in EpiLife medium (Cascade Biologics, Portland, OR). 3T3-J2 fibroblasts and dermal fibroblasts were grown in DMEM, supplemented with 4-mM glutamine and 10% fetal calf serum (HyClone). The Phoenix amphotropic packaging cell line was grown in DMEM, 2-mM glutamine, 2-mM sodium pyruvate, and 10% inactivated FCSII (HyClone) (Nolan and Shatzman, 1998).

Construction of PTM-S6 in retroviral expression vector

PTM-S6 consists of a 224-bp BD, complementary to 188 nucleotides of the 3′ end of intron 64 and 36 nucleotides of the 5′ end of *COL7A1* exon 65, a 31-bp spacer, which separates the 3′ splice region from the target BD, a branch point, and a polypyrimidine tract, followed by a 3′ splice acceptor site (CAG) and the 3′ wild-type coding sequence (5648–8951-bp) of human *COL7A1*. The 76-bp sequence spanning the spacer, branch point, and polypyrimidine tract was obtained by PCR amplification of PTM5 (Dallinger *et al.*, 2003) with PfuTurbo DNA-Polymerase (Stratagene, La Jolla, CA) and cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA) (Primers: F:5′-caccgaattcatcgatgttaacgagaacatt attatagcgttg-3′; R:5′-gctagcaaaaaaaagaagaggtaccag-3′). The 224-bp BD was amplified from human genomic DNA using primers F:5′-gttaaccttcctcccgtcttctccag-3′ and R:5′-gttaaccct atgcaggcacaccccta-3′. The PCR amplification product was cloned into the HpaI restriction site to yield the pcDNA3.1-S6 vector. The 3.3-kb fragment of wild-type human *COL7A1* spanning exons 65–118 (nucleotide 5648–8951; genbank accession number L02870) was obtained by a one-step RT-PCR amplification (SuperScript One-Step RT-PCR for Long Templates with Platinum Taq; Invitrogen) of total RNA purified from wild-type human keratinocytes using primers F:5′-ctaggctagcctgcagggagaacctggggaccc-3′ and R:5′-ctagg atatcgaattctcagtcctgggcagtacctg-3′. The amplified product was digested with NheI and EcoRV and cloned into the pcDNA3.1-S6 plasmid to generate plasmid termed pcDNA3.1-PTM-S6. The correct sequence was confirmed by sequencing with an ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The PTM-S6 cDNA was then subcloned into the moloney-derived pLZRS-IRES-Zeo retroviral vector (gift from GP Nolan, Stanford University, Stanford, CA) (Michiels *et al.*, 2000) between EcoRI and SNaBI restriction sites to yield the pLZRS-PTM-S6 vector. The recombinant plasmid pLZRS-PTM-S6-IRES-Zeo was amplified in the *Escherichia coli* XL10-Gold strain (Stratagene, Amsterdam, The Netherland), purified with a QIAquick kit (Qiagen, Courtaboeuf, France), and subjected to sequencing.

Retroviral transduction of keratinocytes

The recombinant plasmid pLZRS-PTM-S6 was transfected into Amphotropic Phoenix packaging cells (Phoenix-ampho) (Nolan and Shatzman, 1998). pLZRS-PTM-S6 recombinant viruses were harvested from cell culture medium 48 hours after transfection and RDEB keratinocytes (2×10^5 cells cm^{-2}) were transduced with the viral suspension in the presence of 5 μg ml^{-1} of polybrene at 32°C in humid atmosphere, 5% CO_2. Fresh culture medium was added 2 hours later and cells were incubated overnight at 32°C. Transduced cells were then fed with keratinocyte growth medium and selected in the presence of 200 μg ml^{-1} of Zeocin (Invitrogen) for 7 days.

PCR analysis of genomic DNA

For detection of transgenic PTM in transduced keratinocytes, genomic DNA was isolated from confluent cultures using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and analyzed by PCR amplification. A 3.6-kb fragment was amplified with vector-specific primers using Expand Long Range dNTPack (Roche, Mannheim, Germany). The forward primer (5′-agacggcatcgcagcttgg-3′) binds to the pLZRS vector sequence upstream of the BD, and the reverse primer (5′-tctagagaattctcagtcctgggcagtacctg-3′) is complementary to the last *COL7A1* exon on the pLZRS-PTM vector. Another PCR was performed to verify the chromosomal integration of the pLZRS-PTM and to exclude the possibility of amplification of an episomal replicated pLZRS-PTM vector using primers F:5′-atgtctgagaggggccag-3′ and R:5′-ctcctgctcctgttc cacc-3′, which bind outside the long terminal repeat regions of the retroviral LZRS vector.

LAM-PCR analysis

Proviral copy numbers were determined on 50 ng of genomic DNA extracted from PTM-S6-corrected RDEB keratinocytes. The unknown genomic DNA flanking the 5′ long terminal repeat was identified using LAM-PCR, as described (Schmidt *et al.*, 2003; Schwarzwaelder *et al.*, 2007). In brief, two 5′ biotinylated long terminal repeat-specific vector primers, 5′-tgcttaccacagatatcctg-3′ and 5′-atcctgtttggcccatattc-3′, were used for the initial linear PCR. The single-stranded genomic-proviral junction product was purified on streptavidin-coated magnetic beads, made double stranded in a randomly primed Klenow reaction, and digested with Tsp509I. The extended end was ligated to a double-stranded synthetic anchor primer (F:5′-gacccgggagatctgaattcagtggcacag-3′ and R:5′-aattctgtgcc actgaattcagatctcccgggtc-3′). The genomic-proviral junction region was then amplified by two rounds of nested PCR using primers F1:5′-gacccgggagatctgaattc-3′ and F2:5-gatctgaattca gtggcacag-3′ and R1:biotinylated 5′-gcccttgatctgaacttctc-3′ and R2:5′-ttccatgccttgcaaaatggc-3′. Products were analyzed on a 2% MetaPhor Agarose gel (Cambrex, Rockland, ME). LAM-PCR amplicons were isolated and sequenced. The sequence of the LAM-PCR product was mapped against the human genome using NCBI BLASTn (http://blast.ncbi.nlm.nih.gov/Blast). Its relation to annotated genome features was studied using the Ensembl database (http://www.ensembl.org).

Isolation of clonogenic cells and colony-forming efficiency assay

Transduced RDEB keratinocytes at passage 2 after Zeocin selection were used for the isolation of single clonal cells. Transduced RDEB keratinocytes (10^3) were seeded onto feeder layers in 100-mm Petri dishes. After 14 days in culture, cell colonies with regular perimeters and large diameters were isolated with cloning rings, trypsinized, and

individually expanded for further analysis. Colony-forming efficiency of the cultured keratinocytes was determined by plating the cells at densities of 5 and 10 cells cm^{-2} on a feeder of lethally irradiated 3T3-J2 fibroblasts. After 14 days, colonies were stained with rhodamine blue. Values of colony-forming efficiency were calculated as the percentage of inoculated cells that gives rise to colonies.

Population doublings
To calculate the population doubling level per passage, cells were removed by trypsinization at confluence and counted, and the number of doublings was calculated as log(number of cells at time of subculture/number of cells plated)/log2.

Invasion assays
For *in vitro* invasion assays, BD BioCoat Control or Matrigel Invasion chambers (BD Biosciences Discovery Labware, Bedford, MA) were rehydrated for 2 hours at 37°C by adding 500 µl of DMEM to the interior of the inserts and to the bottom of wells. Thereafter, 5×10^4 cells in 500 µl of serum-free DMEM were seeded into the upper chamber. The lower chamber was filled with DMEM containing 10% fetal calf serum and 25 ng ml^{-1} of EGF. After 24 hours at 37°C, non-invading cells were removed from the upper surface of the membrane using a cotton swab, and cells attached to the lower surface of the membrane were fixed with methanol, stained with Toluidine Blue, and counted in four randomly selected microscopic fields (× 200 magnification) of triplicate membranes. Invasion data were expressed as the percentage invasion through the Matrigel membrane relative to the migration through the control membrane. Each experiment was conducted in duplicate.

Organotypic cultures
SEs were generated using human fibrin as a scaffold (Meana *et al.*, 1998). Type VII collagen-deficient fibroblasts were embedded in the fibrin gel-matrix in six-well plates and immersed in DMEM for 24 hours at 37°C and 5% CO_2. Primary keratinocytes (2×10^5 cells per well) were seeded on the matrix, grown to confluence in DMEM:F-12 keratinocyte medium containing 50 µg ml^{-1} of ascorbic acid, and raised at the air–liquid interface for 28 days to favor stratification and differentiation into an epithelium. SEs were manually detached from the plates and processed for electron microscopy analysis or embedded in optimal cutting temperature compound (Sakura, The Netherlands), frozen in liquid nitrogen, and cut into 6-µm sections for immunofluorescence staining.

Immunofluorescence staining
For immunofluorescence analysis of monolayer keratinocyte cultures, cells (5×10^3) were seeded on glass coverslips and fed for 48 hours with medium supplemented with 50 µg ml^{-1} of ascorbic acid. Cells were permeabilized and fixed in cold methanol, and incubated for 1 hour with a pAb directed against type VII collagen (Calbiochem, San Diego, CA) diluted at 1:200 in 2% BSA. The secondary Ab used was Alexa-Fluor-488 goat antirabbit IgG (Invitrogen). Sections of frozen SEs were fixed in acetone and immunofluorescence staining of type VII collagen was performed using the same protocol as described above. Cell cultures and skin sections were analyzed using an epifluorescence Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Transmission electron microscopy
Small slices of SE specimens were fixed in phosphate-buffered formaldehyde–glutaraldehyde solution, followed by osmication, dehydration in ascending ethanol, and processing into epoxy resin (Glycidether 100, with hardeners dodecenyl succinic anhydride, methyl-5-norbornene-2, 3-dicarboxylic anhydride, and 2,4,6-tris(dimethylaminomethyl)-phenol-30; Serva, Heidelberg, Germany). Ultrathin sections (80 nm) were stained by a standardized method using tannic acid, uranyl acetate, and lead citrate (Venable and Coggeshall, 1965; Dingemans and van den Bergh Weerman, 1990). Observations and micrographic documentation were performed using an electron microscope at 80 kV (Zeiss EM 109; Carl Zeiss), using a 35-mm camera and negative film (Kodak TPF 2415 Estar base, Eastman Kodak Company, Rochester, NY). The negatives were scanned and digitized.

Western blot analysis
For immunoblot analysis, keratinocytes were grown to confluence in a conditioned medium (Epilife) and then fed for 48 hours with serum-free Epilife medium containing 50 µg ml^{-1} of ascorbic acid. Media were collected and concentrated 15- to 20-fold (Amicon Ultra-15, Millipore, Billeria, MA) in the presence of protease inhibitors (Complete Mini protease inhibitor cocktail tablets, Roche, Vienna, Austria). Equal amounts of samples were subjected to electrophoresis on a denaturing 6% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-TM-ECLTM, Amersham Biosciences, Buckinghamshire, UK) by electroblotting according to standard procedures. For type VII collagen analysis, blots were reacted with the anti-type VII collagen pAb (Calbiochem) diluted at 1:500 in blocking buffer (0.5% Western Blocking Reagent, Roche, Mannheim, Germany). A pAb to human MMP-2 (Abcam, Cambridge, UK) was used as internal control of protein quality and loading. Secondary antibodies were goat antirabbit IgG-HRP (Abcam, Cambridge, UK). Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Semiquantitative real-time PCR and RT-PCR analysis to detect *COL7A1 trans*-splicing
The BioRad CFX96™ system (Bio-Rad, Munich, Germany) was used to determine levels of *COL7A1* mRNA in normal keratinocytes, as well as in parental and transduced RDEB keratinocytes. *COL7A1* primers (F:5′-gtgaggactgcccctgag-3′ and R:5′-gactccaccttcgagaccc-3′) were designed to amplify a 210-bp fragment spanning exons 17–19. PCR reactions using 25-ng cDNA template and iQ SybrGreen Supermix (Bio-Rad) were carried out in duplicate and standard deviation was calculated from three independent experiments. Transcript levels were calculated after normalization to the

housekeeping gene *GAPDH* (F:5′-gccaacgtgtcagtggtgga-3′ and R:5′-caccaccctgttgctgtagcc-3′).

To discriminate between *trans*- and *cis*-spliced *COL7A1* mRNA, we incorporated five silent mutations in exons 65–66 on the PTM-S6 vector. A 216-bp *trans*-spliced *COL7A1* mRNA fragment was obtained by one-step RT-PCR amplification (SuperScript One-Step RT-PCR, Invitrogen) of total RNA isolated from PTM-S6-transduced keratinocytes, using a forward primer hybridizing with endogenous *COL7A1* exons 61–62 (5′-tgggccgaatggtgctgca-3′) and a reverse primer specifically binding the position of the silent mutations of PTM-S6. (5′-ctgaatctccctttcaccctacg-3′). The correct sequence was confirmed by direct DNA sequence analysis. Endogenous *cis*-splicing was detected using the same forward primer and a wild-type-specific reverse primer (5′-ctttctctcccttcctcccg-3′) to amplify a 200-bp fragment.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Lloyd Mitchell (RetroTherapy) for critically reviewing the paper. This work was supported by grants from the Oesterreichische Nationalbank (Jubiläumsfond no. 10923), Fondation René Touraine, and DEBRA Austria.

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