**K14 mRNA reprogramming for dominant epidermolysis bullosa simplex**

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The major challenge to a successful gene therapy of autosomal dominant genetic diseases is a highly efficient and specific knock-down or repair of the disease-causing allele. In epidermolysis bullosa simplex-type Dowling–Meara (EBS-DM), a single amino acid exchange in exon 1 of the keratin 14 gene (K14) triggers a severe skin phenotype, characterized by blistering of the skin and mucous membranes after minor trauma. We chose spliceosome-mediated RNA trans-splicing to specifically replace exons 1–7 of the K14 gene. In this approach, the mutated coding region is replaced by an RNA-trans-splicing molecule (RTM) that incorporates a binding domain (BD) and the wild-type sequence of K14. Since the BD is crucial for the trans-splicing functionality, we developed a fluorescence-based RTM screen consisting of an RTM library containing random BDs. Co-transfection of the library with a target molecule enabled us to identify highly functional RTMs. The best RTMs were adapted for endogenous trans-splicing in an EBS-DM patient cell line. In this cell line, we were able to detect functional, efficient and correct trans-splicing on RNA and protein levels. Scratch assays confirmed phenotypic reversion in vitro. Owing to concomitant knock-down and repair of the mutated allele, we assume that trans-splicing is a promising tool for the treatment of autosomal dominant genetic disease.

**INTRODUCTION**

Autosomal dominant genetic diseases are simple Mendelian disorders in which a single-mutant allele is able to induce an abnormal phenotype. In such heterozygotes, the mutant protein disrupts the function of the wild-type protein by a variety of mechanisms, including dominant-negative interference. One such autosomal dominant disease is epidermolysis bullosa simplex (EB) (1,2), which currently lacks a therapeutic approach. In EB simplex (EBS), heterozygosity for mutations in either the keratin 5 (K5) or keratin 14 (K14) gene, both of which are specifically expressed in the basal layer of the epidermis, leads to loss of integrity of the intermediate filament (IF) network and cytolysis of basal keratinocytes after minor trauma. Mutations in K14 are mostly dominantly inherited, causing IF fragility and characteristic alterations to cytoskeletal dynamics and solubility (3).

IF networks are important for the maintenance of cell integrity because they impart mechanical strength to cells. In keratinocytes, the IF network is made up of keratins, which are divided into group-I and group-II keratins, depending on their size, isoelectric point and sequence homologies (4). For IF formation, pairs of type-I and type-II cytokeratins heterodimerize, making up the basic modules of the IF network (5). Structurally, all keratins consist of an alpha-helical rod domain that is divided into regions 1A, 1B, 2A and 2B (6,7). The highly conserved regions 1A and 2B are of major importance for heterodimerization, and mutations in these regions lead to the most severe forms of EBS-Dowling–Meara (DM) (6,8). Mutations in other gene regions lead to other forms of EBS, which are referred to as localized EBS and generalized other EBS (1). In cell culture, EBS-DM keratinocytes are more sensitive to stress (osmotic shock, temperature, scratch wounding).

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and show faster cell migration than wild-type keratinocytes (9). In our cell model, the autosomal dominant EBS-DM hotspot mutation R125P, located in the coil 1A domain of the molecule’s rod, leads to the characteristic misfolding and aggregation of the K14 protein and alteration of cytoskeletal dynamics (10). EBS-DM cells show increased signaling of the extracellular signal-regulated kinase (ERK), rendering them even more resistant to apoptosis. These changes in the apoptotic machinery may explain the more frequent incidence of basal cell carcinomas in EBS-DM patients (11,12). However, Werner et al. (3) have shown in an in vitro model that dominance is limited and depends on the ratio of wild-type versus mutated K14 molecules. Also, for COL7A1 mutations, it was shown that overexpression of the wild-type protein can rescue the phenotype in dominant dystrophic EB (13). Furthermore, Cao et al. (14), using a mouse model, showed that dominance is dependent on a certain ratio of wild-type to mutant alleles; in their model, a neomycin-resistance gene inserted in intron 1 of K14 led to reduced expression of the mutated K14 to ≏50% of normal, resulting in phenotypically normal pups which showed no blistering. In view of this ameliorative effect, therapeutic approaches able to reduce the expression of a mutant gene product by 50% could be sufficient.

To develop a gene therapy for an autosomal dominant disease, a number of exigencies have to be fulfilled. Besides the technical issues applicable to gene therapy in general, a high level of targeting specificity and efficiency must be achieved in order to reverse a phenotype.

We chose spliceosome-mediated RNA trans-splicing (SMaRT) because this technology combines many important advantages. SMaRT reprograms mutant RNA molecules by trans-splicing a desired wild-type gene portion into an endogenous target transcript, concomitantly reducing the amount of the cis-spliced mutated allele. Thus, a double-positive effect is achieved: an increase in the amount of wild-type transcript and a decrease in the amount of mutated transcript. The wild-type gene portion to be trans-spliced is delivered by an RNA-trans-splicing molecule (RTM). The RTM consists of a number of features essential for successful trans-splicing: (a) the wild-type gene portion to be incorporated; (b) a binding domain (BD) specifically hybridizing to the target gene; and (c) essential splicing features like functional splice sites, branch point and polypyrimidine tract (15). SMaRT can be used to replace 5′, 3′ or internal exon(s), referred to as 5′, 3′ or internal exon replacement. A number of SMaRT approaches have already been used in reporter systems, including for collagen XVII and the cystic fibrosis transmembrane receptor (CFTR) (16–18). However, up to now, there are scant data pertaining to endogenous application of the technique and most data concern 3′ trans-splicing [e.g. hemophilia A, CFTR and the CD40 ligand (19–21)]. For 5′ trans-splicing, we recently showed endogenous trans-splicing of a 5′ portion of the plectin gene (22). Also a 3′ trans-splicing approach was published for the dominant MAPT gene in a double-transfection assay (23). Here we show endogenous trans-splicing of a 5′ gene portion to a K14 allele harboring a dominant-negative mutation.

**RESULTS**

A fluorescence-based RTM library facilitates identification of highly functional BDs

Our previous studies showed that RTM-BDs have significant effects on trans-splicing specificity and efficiency. Moreover, because minor sequence variations in the BD can dramatically alter the efficacy of the trans-splicing process, it is difficult to design a BD rationally. Parameters such as secondary structure formation, inhibition of splice junctions and complementarity to non-target sequences have to be considered. Therefore, we developed a screening method to identify the best BDs from a large pool of randomly cloned RTMs. This screen is based on fluorescent molecules incorporated into an RNA trans-splicing reporter molecule (RTMr), which trans-splices to an artificial target (Fig. 1). In this model, we replaced K14 by a dsRed molecule split in two. The 5′ part was cloned into the RTMr and the 3′ part into the target molecule. As endogenous trans-splicing should take place between exon 7 and exon 8 of the K14 transcript, we amplified K14 exon 7 and intron 7 from genomic DNA as a BD recognition site and cloned it upstream of the 3′ dsRed into the target molecule. BDs for the RTMr libraries were obtained by sonication of the exon/intron 7

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**Figure 1.** Schematic depiction of the trans-splicing process between the RTMr and the target molecule. The BD hybridizes with K14 exon/intron 7 from the target molecule, bringing the RTM and target into spatial proximity to allow trans-splicing to occur. The resulting mature mRNA comprises full-length GFP and a full-length dsRed sequences.
amplificate and the resulting random fragments were cloned downstream of the 5’ dsRed of the RTMr. The library contained about 10,000 clones. Analysis of a number of randomly picked clones revealed different kinds of RTMrs. (a) RTMrs with one single BD in a reverse complementary orientation, (b) RTMrs with one single BD in a parallel orientation, (c) RTMrs with two BDs in different orientations, (d) RTMrs without a BD. Most RTMs contained one BD of which ~50% were of a parallel and 50% were of a reverse complementary orientation. A full-length acGFP-coding sequence (without a termination codon) upstream of the RTMr 5’ dsRed served as an additional trans-splicing control. Hybridization of a BD with K14 exon/intron 7 of the target molecule facilitates specific trans-splicing, resulting in a full-length dsRed molecule detectable by fluorescence microscopy. When transfecting the RTMr library together with the target molecule into HEK293AD cells, we detected successful trans-splicing by the expression of red and green fluorescence. Differences in fluorescence intensity result from different trans-splicing efficiencies, different amounts of RTMr or target molecule in each cell and non-specific trans-splicing of some RTMrs.

**Isolation and characterization of a highly functional RTMr**

Reduction of the library size and continuous transfection into HEK293AD cells resulted in a small pool of highly functional RTMrs, which were then tested separately. We subjected double-transfected cells to FACS analysis, which showed double-positive cells (red/green), indicating successful trans-splicing. Fluorescence microscopy confirmed the FACS results. Because dsRed fluorescence is much weaker than GFP fluorescence, this resulted in rather low apparent trans-splicing rates (15.5% for the best RTMr, Fig. 2). However, incorporation of other fluorescent molecules resulted in much higher percentages (up to 90%, data not shown) of double-positive cells. Still, we assume that double-positive cells in the sensitive dsRed system are due to highly functional RTMrs.

**The best RTMr has a BD of 163 bp**

From our screening process, we extracted an RTMr (RTMr163) with a trans-splicing efficiency of 15.5% in the FACS analysis. Sequence analysis of the BD revealed a 163 bp sequence complementary to nucleotides 229–391 of the 561 bp K14 intron 7 (Supplementary Material, Fig. S1).

**Endogenous trans-splicing of the reporter RTMr163**

To check for correct trans-splicing with an endogenous K14 transcript, we transected RTMr163 into the K14-expressing wild-type keratinocyte cell line NEB-1 (10). Forty-eight hours after transfection, the cells were harvested and cDNA was synthesized. By PCR, using an RTMr-specific forward primer and a K14 exon-8-specific reverse primer, a product of the desired length (475 bp) was amplified and subjected to sequence analysis. The results showed an RTMr/K14 hybrid sequence for which trans-splicing had taken place in
the correct position, which is involving the 3′ splice site of intron 7 of the endogenous K14 pre-mRNA (Fig. 4).

**Adaptation of RTMr163 for therapeutic trans-splicing**

In our model of EBS-DM, the dominant missense mutation R125P is located in exon 1 of the 1A region of the K14 rod domain. Even though this is a hotspot mutation in EBS-DM, a number of other mutations underlie EBS. Our aim, therefore, was to construct an RTM covering as many mutations as possible. Thus, we replaced the 5′ dsRed sequence of RTMr163 not only by exon 1 of the K14 gene, but also by exons 2–7 (Fig. 5). **Trans**-splicing of this ‘RTM for endogenous trans-splicing’ (RTMe163) with endogenous K14 resulted in a mature wild-type mRNA with exons 1–7 of RTMe (endogenous RTM) and exon 8 from the endogenous K14 transcript. To be able to discriminate between trans-spliced (RTM-derived) and cis-spliced (endogenous) alleles, we incorporated five silent mutations in exon 6 of the RTMe163 K14-coding region. The spacer, acGFP, and, of course, the BD were all kept the same as in RTMr163. The acGFP served as an additional trans-splicing control.

**Characterization of patient EBS-DM cell line KEB-7 and detection of endogenous trans-splicing**

Before treating EBS-DM patient-derived KEB-7 cells with RTMe163, we checked for K14 expression by semi-quantitative real-time PCR (SQRT-PCR). Interestingly, we found a significant increase of K14 levels in KEB-7 cells compared with NEB-1 cells. We compared mRNA levels of pools of assimilable cell culture passages and saw an accumulation of the K14 transcript in KEB-7 cells (Fig. 6A). Also, in western blot analysis, we saw much higher K14 levels in KEB-7 cells (Fig. 6B), probably due to the resistance of the aggregates to degradation by the proteasome (24). We transiently transfected KEB-7 cells with RTMe163 and harvested the cells after 48 h of recovery. We used a silent-mutation-specific forward primer (RTMe-specific) and a reverse primer, hybridizing with K14 exon 8 to amplify trans-spliced alleles from synthesized cDNA (Fig. 7C). Subsequent sequence analysis of the 159 bp band confirmed correct trans-splicing and that the silent mutations had been amplified (Fig. 7A and B). FACS analysis of RTMe163-transfected KEB-7 cells and cells transfected with the RTM without BD (mock) showed 8.3% GFP-positive cells for the RTMe163 transfected cells and 0.8% GFP-positive cells for the mock-transfected cells (Fig. 8A). Immunofluorescence microscopy
showed the GFP distribution along the cytoskeleton and around the nucleus, indicating successful trans-splicing and expression of the GFP/K14 fusion protein (Fig. 8B). Furthermore, an 81 kDa acGFP/K14 fusion protein band was detected in western blot analysis, using an acGFP-specific antibody as well as a K14-specific antibody (Fig. 8D). A reduction of the overall amount of K14 compared with mock-transfected cells was shown (Fig. 8C).

**SQRT-PCR revealed a reduction of cis-spliced K14 alleles**

Owing to the heterozygous R125P mutation in KEB-7 cells, K14 is still expressed and is detectable by real-time PCR. In elucidating the trans-splicing efficiency in cells transfected with the RTM in reference to the cis-spliced alleles, being able to distinguish between an endogenous and a trans-spliced allele is critical. For SQRT-PCR, we used a forward primer that specifically binds the endogenous K14 allele in the position of the silent mutations of the RTM. In the case of successful trans-splicing, we expected a reduction of the endogenous allele. In fact, we found an average reduction of 55% of cis-spliced K14 alleles in RTM-transfected KEB-7 keratinocytes compared with mock-transfected cells in seven independent experiments (Fig. 9A). SQRT-PCR was normalized to the housekeeping gene GAPDH.

**K5 expression levels correlate with K14 function**

As K5 is the major binding partner of K14 in basal keratinocytes, we also analyzed K5 expression levels in KEB-7 cells (Fig. 6C). Interestingly, K5 expression also was increased in comparison with that of the wild-type control cell line NEB-1. Even though K14 expression levels were higher, there was still a significant increase in K5 transcripts. Having seen a significant reduction of the mutant K14 allele in RTM-treated cells, we wondered whether K5 expression was also correlated with these results. In fact, we saw a slight reduction of the K5 transcript compared with the mock transfections (Fig. 9B).

**Scratch assays show reduced cell migration in RTM-transfected cells**

D’Alessandro et al. (9) previously showed that R125P-harboring KEB-7 cells migrate faster than wild-type...
keratinocytes. We therefore performed scratch assays of RTMe163-transfected and mock-transfected KEB-7 cells. Cells were grown to confluency after RTM transfection for about 48 h. Pictures were taken at time $t = 0$ h and after 24 h ($t = 24$). In four independent transfections, RTMe163-transfected cells showed slower migration than mock-transfected cells.

Figure 7. (A) Schematic depiction of the mature mRNA resulting from correct trans-splicing of RTMe163 with the endogenous K14 pre-mRNA. (B) RT-PCR was performed using a silent-mutation-specific primer and a reverse primer hybridizing to exon 8. Sequence analysis of the amplified 159 bp product (C) confirmed that trans-splicing had taken place, as evidenced by the inclusion of the silent mutations (circled) in the mature mRNA. The plus sign indicates the correctly used splice junction between K14 exon 7 from the RTM and K14 exon 8 from the endogenous pre-mRNA.

Figure 8. (A) In FACS analysis, 8.3% of KEB-7 cells transfected with RTMe163 (a) and 0.8% of mock-transfected cells (b) were GFP-positive. (B) Fluorescence microscopy of RTMe163-transfected KEB-7 cells expressing full-length acGFP, which is distributed along the cytoskeleton. Three individual cells are shown from a transfected culture. Strong fluorescence around the nucleus is due to strong exposure, necessary for the visualization of the cytoskeleton. (C) Transfection of KEB-7 cells with RTMe163 resulted in a reduction of K14 in western blot analysis, approximating levels to those of NEB-1 cells. As a reference protein, annexin V was used. (D) Western blot analysis of mock- and RTMe163-transfected KEB-7 cells shows a specific band for the GFP-K14 fusion protein at 81 kDa. This band was detected with a primary antibody specific for K14 (a) as well as with one specific for acGFP (b). No band is visible in mock-transfected cells. As loading control, annexin V was used (c).
Omittance of CaCl₂ from the medium resulted in an overall slower migration, but still mock-transfected cells migrated faster (Fig. 10A and B). Furthermore, concomitantly performed invasion assays using Matrigel filter units gave similar results. Invasivness of KEB-7 cells was reduced significantly after RTMe163 transfection (Fig. 10C).

**DISCUSSION**

In our study, we showed efficient and specific endogenous correction of the K14 gene in EBS patient cells. Screening of a reporter-based library of RTMs yielded a highly functional RTMr capable of trans-splicing into exon 8 of the K14 gene, thereby replacing mutation-containing exons 1–7 by the corresponding wild-type exons. This resulted in an average reduction of 55% of cis-spliced K14 alleles, GFP colocalization along the cytoskeleton, detection of an 81 kDa GFP/K14 fusion protein band in western blot analysis and a reversion of cell migration characteristics in the patient cell line.

EBS-DM, triggered by a single amino acid exchange in exon 1 of the K14 gene, is inherited in an autosomal dominant manner and is a challenge to gene therapy. Gene therapeutic approaches like RNA interference (RNAi) are well established for some genetic diseases (25) and preliminary results are also available for K14 RNAi (3,12). However, off-target effects, activation of the interferon system and toxicity are still an issue (26). RNAi can efficiently knock-down a gene of interest. However, in the case of single-nucleotide exchanges, like in our model, allele specificity can be difficult to achieve and knock-down of both alleles of the targeted gene may result. Also, it remains to be evaluated whether allele-specific knock-down may trigger a haploinsufficient phenotype, which would have to be met by gene replacement. Considering this, we assume SMArt to be a more promising gene therapeutic approach, as it fulfills a number of exigencies for the therapy of (dominant) genetic diseases. A major advantage of SMArt is its ability to knock down the level of mutated transcripts while increasing the level of wild-type message. This double-positive effect is unique to this approach. A second advantage is that it relies on, but does not alter, endogenous control of gene expression at the promoter level. As trans-splicing can take place only in cells in which the target gene is expressed, overexpression is unlikely and targeting only the mutated allele is not necessary. Another advantage of SMArt is that it requires only distinct segments of genes to be used, thus keeping the transgene size small; this is especially relevant for large proteins such as plectin (22) or...
type-VII collagen (27). By developing a screening method for the isolation of highly functional RTMs, we elucidated the significance of the BDs in a reporter-based RTM screen, from which we learned that small sequence variations can have large effects on RTM trans-splicing efficiencies. This may be due to secondary structure formation or non-specific target binding and is of major importance for any future trans-splicing studies. Weak green fluorescence was observed when transfecting an RTM without a BD, probably resulting from low-level RTM background expression, cis-splicing to the expression vector and possibly non-specific trans-splicing. However, there was a striking difference in acGFP expression between constructs with and without the BD.

Reports of trans-splicing results are now available for a number of genes, including CFTR (18), MAPT (23), Col17A1 (16) and SMN2 (28). After having demonstrated successful 5' trans-splicing in an endogenous in vitro model for the plectin gene (22), we have now applied SmaRT endogenously to a dominant gene mutation and show correction on the protein level for the first time.

Interestingly, we saw an accumulation of K14 transcripts in non-treated KEB-7 cells in relation to NEB-1 cells. This was seen by SQR-PCR and subtractive hybridization (data not shown) and was also detected at the protein level by western blot analysis. The mechanism underlying this phenomenon is not yet known. Szeverenyi et al. (29) analyzed the K14 promoter methylation in NEB-1 cells and showed a reduction of K14 expression in NEB-1 cells compared with HaCat cells. This mechanism might be inhibited by non-functional K14 transcripts, leading to K14 accumulation in KEB-7 cells.

Another reason could be that due to keratinocyte activation in wound healing, a number of stress pathways are constitutively activated in KEB-7 cells, including the SAPK/JNK, p38 and p-ERK pathways (9,30). Lu et al. showed that IL-1 and IL-6 are significantly upregulated in keratinocytes from a K5−/− knock-out mouse model for EBS (data for K14 mutants not yet available) (31). Upregulation of the autocrine- and paracrine-acting cytokine IL-1β results in enhanced cell proliferation, higher migratory potential and expression of a specific set of genes (32). Transcription factors like NFkappaB, ATF2 and AP-1 are also activated of which, for example, the AP-1 protein family binds the K14 promoter, thereby triggering the expression of K14 (33,34). Thus, we assume that the correction of EBS-DM keratinocytes results in a downregulation of IL-1. This downregulation of segregated IL-1 also affects neighboring cells, resulting in a decrease of K14 expression also in non-transfected cells and thus to a significant by-stander effect as can be seen in real-time PCR, migration assays and western blot analysis.

In migration assays also the alteration of the expression pattern of genes involved in cell junctions after correction might be of great influence. In response to stress, mutated keratins form novel ring structures that contain non-transmembrane components of desmosomes and hemidesmosomes, which are not seen in wild-type keratinocytes (35). Liovic et al. (36) showed a significant downregulation of proteins expressed in keratin-interacting desmosomes, hemidesmosomes, adherens junctions, tight junctions and gap junctions, all involved in cell–cell and cell-substrate adhesions. The resulting lack of cell interactions and intercellular stability may explain many features of EBS-DM cells like higher migratory and invasive potential. We hypothesize that by the correction of the K14 mRNA and subsequent restoration of intracellular networks as well as intercellular adhesion complexes in a subset of patient cells, also uncorrected cells will be included in stabilizing cell associations, thus impairing their migratory potential. This might explain why 8.3% of cells showing functional trans-splicing can result in a >50% reduced invasive potential of the total of plated cells.

Finally, we conclude that trans-splicing gene therapy is a promising tool for the treatment of autosomal dominant genetic diseases. These experiments have here set the base for ex vivo gene therapy of the dominant forms of EB simplex. We envision a procedure already conducted by Mavilio et al. (37), where epidermal stem cells from an EB patient were transduced with a LAMB3-carrying retrovirus, expanded and transplanted as an epidermal sheet onto the patient.

MATERIALS AND METHODS

RTM library construction

All constructs for the RTM screen were cloned downstream of the CMV-promoter of the pCDNA3.1/D/V5-HIS vector from Invitrogen. The RTM library consists of RTM molecules mimicking an endogenous gene. The full-length 717 bp (without termination codon) reporter molecule acGFP (amplified from pacGFP vector, Clontech) was linked to the 363 bp 5' split reporter molecule dsRed (amplified from pdsRed vector, Clontech) by a 36 bp glycine–alanine–serine linker (5'-ggc-gcc-gga-gcc-tcc-ggc-gca-gga-gcc-gcc-3'). All PCR amplifications were performed using Pfu Turbo DNA polymerase (Stratagene). Amplification primers were acGFP/HindIII + Kozak forward: GATCAAGCTTCCACATGGT-GAGCAAGGCGCCGCA; acGFP/BamHI + linker reverse: TATGATATCGGATCCGGCTCCGGCGCCCTTGTACAGC TCATCCATGC; 5' dsRed/BamHI forward: TATGGAT CGCCGCGAGGAGCGCCGATGGACAAACGCG GCCGAGAC; 5' dsRed/EcoRV reverse: ATAGATATCAGACGAGG CAGATCGAGGTGCCCCACCTTGATAGTAGAAGGT. PCR products were digested with the corresponding restriction enzymes for 2 h at 37°C and cloned sequentially into the expression vector. For fragment ligation, T4 DNA ligase (NEB) was used. Ligation conditions were 1 h at room temperature.

BDs were obtained by amplification of exon/intron 7 of the K14 gene (K14Ex7fw: GATCAAGCTTACCCCTCCTCCCA GTTCTCTCTGGAT; K14int7rv: GATCGGATCCAGGAC GTTACGAGGTGAAAGCAGAC). To obtain random fragments for the library, the purified amplifyicate was sonicated three times for 5 min. Fragments were treated with a DNATerminator® End Repair Kit (Lucigen) and phosphorylated. Finally, the RTM backbone was digested with EcoRV and ligated with the random BDs. The ligation was transformed into the chemically competent Escherichia coli strain TOP10 (Invitrogen). Colony PCR revealed the random distribution of binding-domain length in RTMs.
Target construction
For an artificial target (mimicking the endogenous gene), the remaining 315 bp of the dsRed molecule were amplified (3′ dsRed/BamHI forward: GATCGGATCCCTGAGGTAA GTCAAGG GCC; 3′ dsRed/XbaI reverse: GATCTCTAGAC TACTGGAGCCGGAGTGG) and cloned into the pcDNA3.1D/V5-HIS vector (Invitrogen). The chosen K14 target site (exon/intron 7) was amplified with the primers K14Ex7fw and K14Int7rv (see above) and cloned upstream of the dsRed sequence, incorporating a functional 3′ splice site.

Sequence analysis
Sequencing was performed with an ABI Prism automated sequencer using an ABI PRISM dye terminator cycle sequencer (Applied Biosystems).

Cloning of an RTMe
The therapeutic RTMe is based on RTMr, except that the 5′ Cloning of an RTMe

RNA preparation and RT-PCR
Total RNA was isolated from cultured cells by using an RNasea Kit (Sigma). Plasmid and genomic DNA contamination was eliminated by treatment with DNaseI (Sigma). cDNA was synthesized from 100 ng total RNA by using an iScript cDNA Synthesis Kit (Bio-Rad).

Transient transfection of HEK-293AD cells
Three micrograms of the RTM library and 1 μg of the target molecule were co-transfected into HEK-293AD cells by using the lipid-based EcoTranfect delivery reagent (OZ Biosystems). Cells were grown to 80–90% confluency in Dulbecco’s modified Eagle’s medium (DMEM)/10% newborn calf serum (NCS). Before transfection, the medium was changed to DMEM without NCS and antibiotics. Transfection was performed according to the manufacturer’s protocol and cells were harvested for further experiments 48 h after transfection.

Electroporation of keratinocyte cell lines
Human keratinocyte cell lines (KEB-7, NEB-1) were grown in EpiLife™ medium (CascadeBio)/10% NCS to ~80–90% confluency. The medium was replaced with antibiotic- and serum-free medium, and then the cells were transfected with 2–4 μg of plasmid (RTMe163, RTMmock) by using an Amaxa® Nucleofector® Kit-V (Lonza). After incubation at 37°C for 48 h and 7 days, respectively, the cells were harvested for RNA and protein analysis.

FACS analysis
Cells were trypsinized and cell pellets were resuspended in 500 μl PBS. To allow living and dead cells to be distinguished, 10 μl of 7-AAD was added 10 min before analysis with a Beckman Coulter FC500 (Beckman Coulter).

Semi-quantitative real-time PCR
Primer combinations were tested by PCR of a wild-type cDNA and a silent mutation-containing RTM to determine the best conditions for high specificity. SQRT-PCR was performed with a Bio-Rad iCycler™ (Bio-Rad) and a Bio-Rad iQ™ SYBR® Green Supermix Kit (Bio-Rad). Fifteen picomoles of each primer was added to the reactions. To minimize pipetting errors, reactions were prepared as master mixes and two reactions were analyzed per sample. As a reference for semi-quantitative analysis, we used the house-keeping gene GAPDH (GAPDH: forward: aatcccatcacatctcttg; reverse: cctgcttcacctcttcttg). K14 primers were designed to hybridize with the wild-type sequence of the silent mutation region (forward) and K14 exon 8 reverse (K14 wild-type forward: CAGGAGTACAGACTCTTGCTGGACTGAG; K14 exon 8 reverse: TTTGGCAGGCTTGGAGGAGTCA). This primer combination specifically amplifies endogenous (i.e. cis-spliced) K14.

For the quantification of K5 expression, primers used were K5 forward: TCTCGCCAGTCAAGTGTGTC and K5 reverse: ATAGCCACCCACTGCCACAG.

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Cells were trypsinized and cell pellets were resuspended in 500 μl PBS. To allow living and dead cells to be distinguished, 10 μl of 7-AAD was added 10 min before analysis with a Beckman Coulter FC500 (Beckman Coulter).
Isolation of total protein from cultured cells

Cells were washed three times with PBS and scraped off with a cell scraper. After centrifugation, cells were resuspended in 200 μl cell lysis buffer (0.5 M Tris–HCl, pH 6.8, 20% glycerol, 10% SDS, 5% β-mercaptoethanol; 1× complete protease inhibitor cocktail, Roche). The lysate was passed through a 22G syringe several times to disrupt cell aggregations and incubated at 95°C for 10 min.

Western blot analysis

Total protein concentrations were determined by Bradford Assay (Bio-Rad) and equal amounts were applied on a 10% NuPAGE Bis-Tris-Gel (Invitrogen). Gels were run for 1 h at 160 v. Proteins were blotted onto Hybond ECL membrane (Amersham Pharmacia) for 1 h at 0.8 A. After blocking for 1 h with RotiBlock blocking buffer (Roti), mouse-derived monoclonal antibody LL001 (Santa Cruz Biotech) was diluted 1:500 in TBS/0.2% Tween-20, was added for K14 detection, and rabbit-derived α-GFP monoclonal antibody (Torrey Pines Biolabs, Inc.) diluted 1:1000 in blocking buffer was applied for GFP detection. Membranes were incubated overnight at 4°C. Subsequent washing with blocking buffer was followed by incubation with HRP-labeled secondary antibodies goat-anti-mouse and goat-anti-rabbit IgG2b antibody (Envision and Serotec). HRP visualization was done with Immuno-Start™ Western C Kit (Bio-Rad) according to the manufacturer’s protocol.

Scratch assays

Cells were grown to 80% confluency and transfected by electroporation with RTMe163 or mock vector. Transfected cells were seeded into 24-well plates and grown to confluency for 24 h. A scratch was done with a yellow pipet tip and the scratch area was marked with a pen at the bottom of the plate. Images were captured at 10-fold magnification and at stable zoom parameters with a digital camera immediately after making the scratch. Three images were taken per well to obtain representative data. After 24 h of growth, images were taken again using the same parameters as described above. Scratch areas were measured with AxioVision Rel. 4.7 software (Zeiss) and a mean scratch area from all wells of each transfection was calculated. Scratch area reduction was calculated relative to the scratch area at 1 = 0 and is given in % remaining area. Scratch assays were performed with and without CaCl2 supplement in the medium, as CaCl2 influences cell adhesion.

Invasion assays

We used BD BioCoat Matrigel Invasion chambers (BD Biosciences Discovery Labware) for the invasion assays. Matrigel inserts (8 μm PET membrane coated with a Matrigel basement membrane matrix) and control inserts (without Matrigel) were rehydrated with 500 μl of DMEM for 2 h at 37°C. Fifty-thousand RTMe163-transfected and mock-transfected cells in 500 μl of serum-free DMEM were seeded into each of two wells. Maintenance was performed according to the manufacturer’s protocol. Non-invading cells were removed after 24 h with a cotton swab. Cells that had invaded and were now on the bottom side of the membrane were fixed with methanol and stained with toluidine blue. For each well, stained cells in five randomly selected microscopic fields (200-fold magnification) were counted. Invasion data were expressed as percentage invasion through the Matrigel membrane relative to the migration through the control membrane.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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