**Spliceosome-Mediated RNA Trans-Splicing Facilitates Targeted Delivery of Suicide Genes to Cancer Cells**

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**Abstract**

Patients suffering from recessive dystrophic epidermolysis bullosa (RDEB), a hereditary blistering disease of epithelia, show susceptibility to develop highly aggressive squamous cell carcinoma (SCC). Tumors metastasize early and are associated with mortality in the 30th–40th years of life in this patient group. So far, no adequate therapy is available for RDEB SCC. An approach is suicide gene therapy, in which a cell death-inducing agent is introduced to cancer cells. However, lack of specificity has constrained clinical application of this modality. Therefore, we used spliceosome-mediated RNA trans-splicing technology, capable of replacing a tumor-specific transcript with one encoding a cell death-inducing peptide/toxin, to provide tumor-restricted expression. We designed 3’ pre-trans-splicing molecules (PTM) and evaluated their efficiency to trans-splice an RDEB SCC-associated target gene, the matrix metalloproteinase-9 (MMP9), in a fluorescence-based test system. A highly efficient PTM was further adapted to insert the toxin streptolysin O (SLO) of *Streptococcus pyogenes* into the MMP9 gene. Transfection of RDEB SCC cells with the SLO-PTM resulted in cell death and induction of toxin function restricted to RDEB SCC cells. Thus, RNA trans-splicing is a suicide gene therapy approach with increased specificity to treat highly malignant SCC tumors. *Mol Cancer Ther; 10(2); 233–41. ©2011 AACR.*

**Introduction**

Epidermolysis bullosa (EB) is a group of genetically and clinically heterogeneous diseases that are characterized by the formation of blisters and erosions on skin and mucous membranes following minor trauma (1). Patients suffering from recessive dystrophic EB (RDEB) harbour mutations in the COL7A1 gene that cause decay of the respective mRNA. RDEB patients have a high risk of developing aggressive squamous cell carcinoma (SCC) in early adulthood. To date, neither chemo- nor radiotherapies yield satisfactory results, and despite extensive surgical excision of the tumor and surrounding tissue, recurrence and subsequent metastases are common. This explains the high mortality rate among patients with severe RDEB, who have a 70% cumulative risk of dying from metastatic SCC by the age of 45 years (2). Therefore, it is essential to develop effective molecular-based therapies for this cancer.

RNA trans-splicing allows an endogenous RNA to be converted into a new gene product via exon replacement. This can be achieved by the introduction of a designed pre-trans-splicing molecule (PTM) that binds to an endogenous target RNA and induces a trans-splicing reaction between the target and the PTM mediated by the spliceosome. PTMs contain a binding domain (BD), which defines the target specificity, splicing elements for efficient trans-splicing, and a coding sequence that is desired to replace a part of the target pre-mRNA (3). In this manner, trans-splicing has already been successfully used to repair the mutated part of disease-causing genes, indicating its potential use in gene therapy for several genetic disorders *in vitro* and *in vivo* (4–7).

The ability of trans-splicing technology to reprogram a pre-mRNA of interest offers numerous other applications, including suicide gene therapy for cancer treatment (8). Cancer suicide gene therapy entails delivery of various cytotoxic genes such as apoptotic factors or enzyme–prodrug combinations to cancer cells, leading to cell-specific expression and cell death. Suicide gene therapy has received much attention as a potent alternative to conventional anti-cancer therapies (9–11). In this context,
Puttaraju and colleagues (12) successfully applied this method of trans-splicing to the tumor marker β-subunit of human chorionic gonadotropin gene 6 (βhCG6), which is expressed in many types of tumors but not, with a few exceptions, in normal adult cells. For this approach, intron 1 of βhCG6 was targeted using a 3′ PTM encoding diphtheria toxin subunit A (DT-A), and correct trans-splicing was observed in vitro in cultured human lung cancer cells, as well as in an animal in vivo tumor model. Moreover, specific expression of the toxin was shown in tumor cells (12).

Trans-splicing technology offers significant advantages in suicide gene therapy when compared with conventional cDNA approaches. First, cancer cells can be specifically targeted by trans-splicing of an exclusive tumor marker gene within the cell. Therefore, this system provides increased safety through the specific expression of the cytotoxic protein in tumor cells, leaving normal, healthy cells surrounding the tumor intact. Second, the expression of the endogenous tumor marker mRNA is reduced due to its reprogramming by the trans-splicing reaction. Factors that promote tumor growth or invasion can be targeted by specific PTMs that reduce the endogenous levels of target RNA, resulting in a reduction of the target molecule’s tumor-promoting effect.

Based on these facts we designed a PTM targeting the marker gene, matrix metalloproteinase-9 (MMP9), which is highly expressed in cultured RDEB SCC cells. Because little is known about the properties of BDs that promote the most effective trans-splicing reactions, we used a fluorescence-based test system to identify optimal PTMs (i.e., BDs). The selected PTM was further investigated for its ability to induce specific cell death by reprogramming MMP9 through a trans-splicing reaction to express the toxin streptolyisin O (SLO) of Streptococcus pyogenes.

Materials and Methods

Plasmids

**Target molecule.** In the fluorescence screening procedure used to identify potent PTMs for the trans-splicing reaction, we designed a target molecule containing the 5′ half of green fluorescent protein (GFP) coding sequence and intron 1/exon 2 of the MMP9 gene. The 5′ half of GFP (336 bp) was amplified from pIRES-AcGFP1 (Clontech) using Pfu Turbo DNA polymerase (Stratagene). The reverse primer inserted an EcoRV restriction site and a 5′ splice site (ss; aggt) at the GFP/intron border, the latter of which is indispensable for the trans-splicing reaction (5′GFP forward 5′ccacatgactcggccaggaggg3′, reverse 5′tgatatatctctcctccgagccactc3′). The 5′ part of GFP was then subcloned into the TOPO cloning sites of pcDNA3.1D/V5-His-TOPO (Invitrogen) according to the manufacturer’s instructions. Primers amplifying intron 1 and exon 2 of MMP9 from human genomic DNA were designed to include EcoRV and NotI restriction sites (forward 5′ggagacggagccgaataaggtgatgttcttggc3′ and the PCR product was finally cloned into pcDNA3.1D.

**PTM screening molecule.** The PTM molecule is based on pcDNA3.1D/V5-His-TOPO harboring the 3′ part of GFP as well as splicing elements [spacer, branch point, and polypyrimidine tract (PPT)] previously described by Dallinger et al. (13). The 470-bp fragment of 3′ GFP was amplified using a forward primer carrying an EcoRI site, which also served as the restriction site for ligation of variable BDs.

BDs complementary to intron 1 of MMP9 were amplified from human genomic DNA to yield 150-bp BD2 and BD4 (BD2, forward 5′ggagacgctggcggccacagctact3′ and reverse 5′tcctcgattaatctgcactaggg3′; BD4, forward 5′ggagagactctgcttgggtgc3′ and reverse 5′tcctagaatcctgctaggg3′). Oligonucleotides encoding the shorter BD1 (45 bp) were synthesized with EcoRI restriction sites (Eurofins MWG Operon), annealed, and subcloned into PTM vector.

To determine the transfection efficiency of the PTM, sequences encoding an internal ribosomal entry site (IRES) as well as the reporter gene DsRed were inserted into the vector. The wild type IRES sequence was amplified from pIRES2-AcGFP1 and ligated between EcoRV and XmaI restriction sites. The full-length DsRed gene was cut out of pDsRed Monomer (Clontech) using EcoRV and NotI restriction enzymes and cloned into the PTM vector between those two restriction sites.

The control vector for FACS analysis consisted of a full-length GFP gene, an IRES sequence, and a full-length DsRed gene.

**PTM-S1.** The MMP9 suicide PTM-S1 was designed in the vector pIRES-AcGFP1, containing 3′ splice elements identical to those of the PTM molecule used in the screening procedure. In addition, a glycine–alanine linker was inserted after the ss of the PTM to guarantee accurate expression and folding of the fusion protein after specific trans-splicing. Therefore, oligos were designed with a PstI site at the 5′ end (5′gggccgcagggagccggcaggagccggccaga3′) and an SphI site on the 3′ end (5′ctagtgaggccggctcctggctcagctggccgctc3′) of the linker. In addition, GFP was excised and the coding sequence of truncated SLO (tSLO) or thymidine kinase (tk) of herpes simplex virus was inserted into the plasmid after the linker sequence. The resulting PTM tSLO cassette was further cloned into a modified SV40 vector, which harbors fewer 3′ ss motifs, thus reducing unwanted cis-splicing events within the plasmid. A vector expressing the fusion protein of MMP9 exon 1 and tSLO served as a positive control for the suicide assays, because this fusion protein is also expressed by the cancer cells upon correct trans-splicing between the suicide PTM and the target gene MMP9. Furthermore, the resulting fusion protein should be secreted due to an endoplasmic reticulum (ER) signal present in exon 1 of MMP9. The correct recognition of this ER signal and cleavage site was predicted by using SignalP® 3.0 software (http://www.cbs.dtu.dk/services/SignalP/).
Tissue samples and cell lines

Tissue samples were taken in the course of a surgery at the Department of Children’s Surgery, University Hospital Graz, Austria, and used with informed consent. Biopsies of both SCC and nonaffected skin were taken from the same RDEB patient (COL7A1, 4048–1G>T/8440 C>T). The RDEB keratinocyte cell line (COL7A1, 3760–1G>A/unknown), E6/E7 immortalized, was kindly provided by Prof. G. Meneguzzi, Nice, France, in 2007 and the spontaneously immortalized RDEB SCC cell line (SCC–EB–GER, MN270503, COL7A1, 8244insC/8244insC) was obtained from Prof. L. Bruckner-Tuderman, Freiburg, Germany, in 2007. All cells were routinely grown in keratinocyte-serum-free medium (Gibco Invitrogen) at 37°C in 5% CO₂.

In addition, human embryonic kidney cell line, HEK293AD (Stratagene, 2007), was used for the PTM screening experiments and grown in DMEM, 10% FCS and Pen/Strep. The cell line was authenticated by the company. All experiments were done under endotoxin-free conditions.

Each initial RDEB/RDEB SCC cell line stock and random passagings were verified by detecting the specific COL7A1 mutation performing direct sequencing of genomic DNA. Mycoplasma infection was ruled out by PCR analysis, as previously reported (14), in each cell line prior to experimental use.

Semi-quantitative real-time PCR and reverse transcriptase PCR

Total RNA was isolated from RDEB SCC tissue samples using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. A Bio-Rad iCycler system (BioRad) was used to assess transcript levels of MMP genes in tissue samples as well as in cell lines. All primer pairs were designed using Primer3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The integrity of PCR products was analyzed by agarose gel and sequence analysis. All PCRs were carried out in duplicate, and the mean and standard deviation were calculated from 3 independently done experiments. Transcript levels in the various samples were calculated after normalization to the housekeeping gene for α-tubulin.

Reverse transcriptase PCR (RT PCR; SuperScript One-Step RT-PCR, Invitrogen) was used for detection of endogenous trans-splicing between the target gene MMP9 and PTM-BD1 containing tk. RNA was isolated 48 hours after PTM transfection and PCR was done using forward primer 5’gtctgtgtgtctgtctg3’ and reverse primer 5’agatgttcgcgattacttataagtagggatccgagaga3’. In addition, a nested PCR was carried out using forward primer 5’ttcctggagactctgagagatg3’ and reverse primer 5’gaagctcatcggtctgggta3’ to verify the correct PCR products.

Western blot analysis

For immunoblot analysis, cells were cultured until confluency and resuspended with lysis buffer (0.5 mol/L Tris-HCL, pH 6.8, 20% glycerine, 10% SDS, and 5% β-mercaptoethanol) in the presence of protease inhibitor (Complete Mini protease inhibitor cocktail tablets, Roche). Proteins from tissue samples were extracted using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. Samples were resolved on a denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-TM-ECLTM, Amersham Biosciences) by electroblotting according to standard techniques. The membrane was blocked with 1% blocking buffer (Roche) during 1 hour at room temperature and incubated overnight at 4°C with anti-MMP-9 pAB (Abcam). A polyclonal antibody against tubulin beta (Abcam) served as loading control. HRP labeled EnVision + anti-rabbit antibody (Dako) was used as secondary antibody. Bands were visualized using Immun-Star Western Chemiluminescent Kit (BioRad) and ChemiDoc XR System (BioRad).

Streptolysin O

Streptococcus pyogenes was obtained from patient throat swabs and routinely grown on blood agar plates (Sigma-Aldrich). PCR amplification of a truncated version of SLO from S. pyogenes was done by adapting the protocol of Yang et al (15). A smear of S. pyogenes was pretreated by incubation at 95°C for 30 minutes and the lysate was used for PCR with GoTaq hot start polymerase (Promega), 2 mmol/L dNTPs, and forward and reverse primer pairs (150 μmol/L), the latter primer harboring a BamHI restriction site for subcloning into PTM-S1 vector. SLO forward primer (5’ttcgaacaaaaacacctgct) and reverse primer (5’gtggattacttaagaggattgagaggaga) were used to amplify a 32-amino acid truncated version of SLO, named tSLO. Sequence analysis revealed that the amplified tSLO DNA differed from a previously reported sequence (Gen-Bank accession number AB050250) at 6 amino acid positions: Ser47→Asn47; Arg172→Met172; Asn182→Asp182; Asp324→Glu324; Gln470→Arg470; Trp535→Arg535. In addition, the cell-killing activity of tSLO was confirmed by lactate dehydrogenase (LDH) release assay.

PTM screen

One microgram of the target molecule and 3 μg of the PTM vector, harboring a single BD, were transiently transfected into HEK 293AD cells using EcoTransfect (OZ Biosciences). A total of 10⁸ cells were used for transfections according to the manufacturer’s protocol. Analyses using a fluorescence microscope (epifluorescence Zeiss Axioskop) and a FACScan instrument (Beckmann Coulter FC500) were done 48 hours post-transfection. For fluorescence analysis, cells were washed and resuspended in 1 mL FACScan buffer (Dulbeco’s PBS, 3% fetal calf serum) and kept on ice. Thirty thousand events were recorded and data were analyzed using CXP software (Beckmann Coulter).

Cytotoxicity studies

Recessive dystrophic epidermolysis bullosa SSC cells were transiently transfected with PTM-S1 and control
vectors using the AMAXA system (Lonza), which gives a transfection efficiency of about 40%. Cells were harvested after 24 hours under standard conditions. Permeable cells were further stained with 7-aminoactinomycin D (7-AAD) and analyzed by FACS. LDH release by membrane-permeabilized cells was monitored using a CytoTox 96 nonradioactive cytotoxicity assay (Promega) following the manufacturer’s instructions.

Cytotoxicity upon functional trans-splicing of the PTM in RDEB as well as RDEB SCC cells was assessed by an 3-(4,5 dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT) assay. Cells were transfected with the PTM-S1 or control vectors and seeded into 96-well plates. Twenty-four hours after transfection, MTT (5 g/L) was added to each well and incubated for 1 hour at 37°C. Finally, cells were lysed with glycine/dimethyl sulfoxide and absorbance of the resultant formazan product was measured at 492 nm with a photometer (Tecan).

Results

Identification of MMP9 as a target gene in an RDEB SCC cell line for suicide trans-splicing

To identify a target gene upregulated in RDEB SCC, we did semi-quantitative real-time PCR analysis using RNA isolated from RDEB SCC, as well as peritumoral tissue samples from the same EB patient. Moreover, expression levels were also analyzed in cell lines (RDEB SCC and RDEB cell lines) which were used in further in vitro studies. Due to the fact that RDEB SCC is characterized by rapid tumor growth and invasion of the extracellular matrix, we focused on the mRNA expression levels of several matrix MMPs (MMP3, MMP7, MMP9, and MMP13), which had already been described as differentially regulated proteins in EB-associated cancer (16).

Semi-quantitative real-time PCR analysis revealed increased expression of the MMP3, MMP9, and MMP13 genes in tumor tissue compared with peritumoral skin, whereas MMP7 was found to be downregulated (Fig. 1A). In contrast to tumor tissue, we could hardly detect any MMP3 and MMP7 mRNA expression in cultured RDEB SCC cells. MMP9 and MMP13 showed increased mRNA levels when normalized to the RDEB cell line (Fig. 1B). Moreover, MMP-9 protein expression was further confirmed by Western blot analysis showing an elevated level in RDEB SCC cells compared with RDEB cells (Fig. 1C). Because the amount of MMP9 mRNA was elevated compared with that of MMP13 and the difference between RDEB and RDEB SCC cells showed higher statistical significance, we chose to use the MMP9 gene to provide the proof of principle for trans-splicing technology in a suicide gene therapy approach for RDEB SCC in vitro.

PTM selection using a fluorescence-based testing system

A PTM can theoretically target any intronic region of a given RNA through its complementary BD that brings specificity to the trans-splicing reaction. However, sequence, length, and steric constraints of the BD can affect its efficiency and specificity in the reaction. To identify a PTM harboring a BD with optimized characteristics for trans-splicing into intron 1 of the target gene MMP9, we established a fluorescence-based testing system (Fig. 2) as described by Mitchell and McGarrity (17).
In this experimental setup, the fluorescent protein GFP is expressed only upon accurate trans-splicing, which fuses 2 halves of the GFP mRNA to make a full-length mRNA. We constructed a target vector harboring the 5′ half of GFP and sequence of MMP9 intron 1/exon 2. Each tested PTM comprises a BD complementary to different parts of the sequence of MMP9 intron 1, a 3′ splicing domain including a branch point, a PPT and a 3′ ss, as well as the 3′ half of GFP. Downstream of the 3′ half of GFP, we inserted an IRES and the reporter molecule DsRed to monitor transfection efficiency. Precise trans-splicing between the target molecule and the PTM in cotransfected cells leads to a complete and functional GFP, which can be examined directly by fluorescence microscopy or FACS analysis.

To select a BD that promotes trans-splicing most efficiently, we designed 3 different PTMs, each consisting of a randomly chosen antisense BD (BD1, BD2, and BD4) for MMP9 intron 1 (Supplement 1).

The target molecule and each PTM were cotransfected in HEK293 cells and the expression of full-length GFP, indicating correct trans-splicing, was monitored by fluorescence microscopy (Fig. 3A). No GFP signal was detected in cells transfected with either target molecule or PTM alone (data not shown). A plasmid simultaneously expressing DsRed and GFP served as a positive control. In this setup, we observed differences in trans-splicing reactions between the target molecule and the PTM harboring different BDs. The PTM containing BD1 produced strong GFP expression, indicating efficient trans-splicing, whereas PTM BD2 induced only weak green fluorescence signal, indicating low trans-splicing efficiency. Finally, PTM BD4 did not seem to induce trans-splicing at all, as the BD4 transfectants produced virtually no GFP expression.

To quantify the trans-splicing events we did FACS analysis using HEK293AD cells cotransfected with the target molecule and a PTM (Fig. 3B). The data are displayed as the ratio of DsRed fluorescence (PTM transfection) against the level of GFP expression and thus, the trans-splicing events (Fig. 3C). The trans-splicing performance varied between different PTMs (BD1>BD2>BD4), demonstrating the significance of both the binding site and the sequence of the BD. The results of the FACS analysis correspond with the images obtained by fluorescence microscopy. In this screening procedure, BD1 was identified as the most efficient BD and was therefore used for further in vitro studies.

Trans-splicing in RDEB cancer cells

Next, we sought to show trans-splicing between the PTM and the endogenous target mRNA in vitro at the mRNA level. Therefore, the RDEB SCC cell line was transfected with a vector expressing PTM-BD1 and a cDNA of tk from herpes simplex virus. In the presence of the antiviral drug ganciclovir, tk produces toxic metabolites that disrupt DNA synthesis and result in the death of dividing cells. Forty-eight hours after transfection and without addition of ganciclovir, correct trans-splicing was confirmed by RT PCR using primers with specific binding sites within exon 1 of MMP9 and tk (Fig. 4A). The PCR product was further verified by direct sequencing, confirming successful PTM-driven trans-splicing of the target gene MMP9 (Fig. 4B and C). With this experimental approach, we were able to show trans-splicing between endogenous MMP9 and PTM-BD1 at the mRNA level, proving the functionality of our designed PTM.

PTM containing the toxin SLO is functional in a cell culture model

For endogenous suicide studies, we designed a new PTM, designated as PTM-S1, containing the binding domain BD1 (Supplement 2) and a truncated version (del32aa) of the cell death-inducing peptide SLO (15,18), designated as tSLO. tSLO was inserted into PTM-S1 with the goal to induce specific cell death in MMP9-overexpressing tumor cells. In our experimental approach, the toxin is expressed as a fusion protein with MMP9 exon 1 upon correct trans-splicing. Because both the MMP9 gene and SLO contains an ER-targeting signal,
we used a truncated version of SLO lacking the ER-targeting signal to guarantee correct processing and avoid secretion of SLO without trans-splicing with MMP9. In addition the del32aa version has been used successfully in a suicide gene therapy approach in vivo and in vitro in a study by Yang et al (15). The resulting fusion protein upon correct trans-splicing was predicted to be secreted by the transfected tumor cells and this enables the toxin to attack the tumor cell also from the outside, suggesting higher efficiency in RDEB SCC cells.

To monitor specific cell death upon PTM-S1 treatment, we did 2 independent cell culture assays. First, the amount of dead cells after transfection of RDEB SCC cells with PTM-S1 containing tSLO was monitored in a 7-AAD uptake experiment (Fig. 5A). Cells transfected with the suicide PTM-S1 showed high levels of 7-AAD cell staining. Twenty-four hours after transfection, 22.9% of the PTM-transfected SCC cell population was dead, as shown by 7-AAD positive staining. This indicated expression of the MMP-9 exon 1/tSLO fusion protein upon trans-splicing (Fig. 5A). We also detected cytotoxicity (18%) in cells transfected with a control plasmid (mock, backbone of PTM), which resulted from the transfection procedure itself. Similar levels of cytotoxicity were observed between PTM-S1 and the positive control (24%) encoding the fusion protein MMP-9/tSLO. In a second approach, we wanted to investigate whether the cytotoxicity of PTM-S1 was caused by cell membrane permeabilization resulting from SLO expression. We measured the release of cytosolic LDH, an assay specifically used for the measurement of cell lysis by perforins or other pore-forming toxins. As shown in Figure 5B, PTM-S1 treatment of RDEB cancer cells resulted in increased LDH concentration detected in the supernatant, indicating that correct trans-splicing led to expression of MMP-9/tSLO fusion protein.
Selective cytotoxicity by specific PTM-S1 in MMP9-expressing cancer cells

We further determined whether the PTM-induced toxin expression is selective for MMP9-expressing cancer cells. Therefore, we transiently transfected the vector encoding PTM-S1 into RDEB SCC cells expressing high MMP9 levels, as well as RDEB cells that express low levels of MMP9 mRNA to assess the target specificity of PTM-S1. The MTT assay showed significant cytotoxicity in both cell lines when transfected with the positive control construct that encodes the fusion protein MMP9/tk fusion mRNA. Primer locations are marked by arrowheads. B, specific trans-splicing was shown in PTM-transfected RDEB SCC cells by the presence of a 210-bp PCR product, which is shown on the agarose gel (I) in contrast to untransfected cells (II). C, further sequence analysis revealed that tk was successfully trans-spliced to the target gene MMP9. The PCR band shown in lane II was also analyzed by direct sequencing and proven to be a nonspecific PCR product.

In the first series of experiments, we identified MMP9 as being upregulated in both SCC tissues from an RDEB patient and an RDEB SCC cell line compared with non-SCC RDEB skin tissue and an RDEB cell line. MMP-9 is a zinc metalloendopeptidase that degrades and removes extracellular matrix molecules from tissues and is involved in tissue remodeling, wound healing, and cancer invasiveness (19). Although previous immunohistochemical studies of several MMPs in RDEB SCC tissue reported that MMP-9 expression is restricted to inflammatory cells (16), we showed here that RDEB SCC cells in culture also express MMP9 at the mRNA level as well as the protein level. Based on these data, we evaluated a PTM designed to replace the target gene MMP9 with the toxin SLO through a trans-splicing reaction. We have shown that PTM-S1 induces correct trans-splicing to the endogenous MMP9 mRNA, leading to expression of functional SLO and thus cancer cell death. Notably, the PTM caused cytotoxicity in MMP9-positive cancer cells, indicating target-specific RNA replacement.

The BD of a PTM molecule is the most critical element for accurate trans-splicing. It ensures specificity for target–gene binding, which in turn defines the specificity of tumor cell targeting. This approach thus offers an important advantage compared with other conventional therapies. Most trans-splicing studies have been done with PTMs containing BDs in the range of 70–250 bases (6,20,21). It has been shown that enlargement of BD size improves trans-splicing efficiency (22). In previous studies, we observed that localization of the binding site within the target intron also influences the trans-splicing efficiency. Therefore, we analyzed the trans-splicing activity of 3 different BDs complementary to MMP9 intron 1 using a fluorescence-based model system. Puttaraju et al. (12) showed improvement of PTMs by extension of BD length. Our data here show that not only the size but also the position of the binding site in the intron may contribute to stringent binding because the shorter BD1 (45 bp) showed superior trans-splicing activity. It is also likely that BD properties are difficult to generalize because the PTMs are designed for different intronic regions of various genes. Therefore, these data emphasize that screening experiments for the best BDs should be done with every new target gene that is selected to be used in trans-splicing suicide gene therapy.

The efficiency of RNA trans-splicing in vitro is an important question because the low transfection rates of tumor cells still constitute a limiting factor (23). Detection of endogenous trans-splicing efficiencies was done in gene correction studies (e.g., of factor VIII in hemophilia A), showing trans-splicing levels of 3%–7%, depending on the PTM construct (24,25). Therefore, trans-splicing seems to be a low-efficiency event; however, it is expected that in suicide gene therapy, this low trans-splicing efficiency can be at least partly offset by the high efficacy of most toxins, as only a few molecules are usually required for cell death induction, for example, a single molecule of DT-A can kill a target cell (26). Moreover, in conjunction...
with suicide gene therapy \textit{in vivo}, adjunct therapies to stimulate the immune system could also be employed to combat tumor formation in cancers such as SCC, which take measures to evade immune recognition (27, 28).

Activation of the immune system can be achieved by boosting immune recognition and response using strong danger signals such as products of cell injury or necrotic cell death, as well as signals generated by inflammation (29). Therefore, SLO might be a suitable suicide gene for cancer gene therapy because it induces cell death by necrosis (15) and thus releases heat shock proteins (HSP). Because endogenous HSP70 is a natural ligand of mammalian Toll-like receptor 4, HSP70 may serve as an immune-activating danger signal in the tumor environment (30). This fact could further increase the efficacy of suicide gene therapy and may thus partly compensate for the lower transfection rates obtained with standard techniques.

Attention has also been drawn to the specificity of the PTM because nonspecific \textit{trans}-splicing events have already been observed. For this reason, we tested the specificity of PTM-S1 toxicity toward MMP9-expressing cells. In cells that expressed low levels of MMP9, we observed some toxicity, although the levels were not significantly different from those of the nontransfected control cells. As the MMP9 levels are low but not zero in the RDEB cell line, we expect that the observed low-level toxicity resulted from MMP9-specific \textit{trans}-splicing rather than from nonspecific \textit{trans}-splicing events.

**Figure 5.** \textit{Trans}-splicing of target gene induces tSLO expression and subsequent cell death. A, the uptake of 7-AAD by transfected RDEB SCC cells was monitored by FACS analysis 24 hour after transfection. The percentage of dead cells (+ 7-AAD) was calculated using CXP software (Beckman Coulter). The positive control encodes the fusion protein of MMP-9 exon 1 and tSLO, which will also be present after correct \textit{trans}-splicing events. The cytotoxicity of the transfection method itself was investigated by transfection of the PTM backbone plasmid (mock). The experiment was repeated 4 times (mean ± SEM) and 1 representative histogram of 7-AAD stainings is shown. B, LDH release assay upon cell-membrane permeabilization caused by SLO. The release of cytosolic LDH into the culture medium was measured using a CytoTox 96 nonradioactive cytotoxicity assay kit. RDEB SCC cells were transfected with 1 μg of each construct and culture media was harvested for 24 hours. Data were generated by measurement of absorbance at 492 nm. One representative experiment in triplicate ± SD out of 3 is shown.

**Figure 6.** Specific induction of suicide gene activity by MMP9-specific \textit{trans}-splicing PTM-S1 in RDEB SCC. Cell lines were transfected with PTM-S1 or the positive control and analyzed for cell-specific cytotoxicity after 24 hours using MTT assay. Cell viability is represented as a percentage relative to RDEB SCC or RDEB cells transfected with water. Data are shown as the mean of 3 independent experiments ± SD. Significance \( P < 0.05 \) (*), \( P < 0.005 \) (**).
In summary, the results presented here indicate that 3' trans-splicing constitutes an alternative tool for suicide gene therapy. We showed that spliceosome-mediated RNA trans-splicing is able to target tumor cells by using specifically designed molecules (PTMs) that induce binding and trans-splicing of an endogenous tumor-associated marker gene. We also showed that targeted cell death in RDEB tumor cells can be achieved by a specifically designed PTM delivering the toxin SLO. Therefore, this work shows a promising application of trans-splicing technology to be used in further in vivo suicide gene therapy studies.

Disclosure of Potential Conflicts of Interest

J.W. Bauer has a patent pending on trans-splicing.

References


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