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The design and optimization of RNA *trans*-splicing molecules for skin cancer therapy

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ABSTRACT

Targeting tumor marker genes by RNA *trans*-splicing is a promising means to induce tumor cell-specific death. Using a screening system we designed RNA *trans*-splicing molecules (RTM) specifically binding the pre-mRNA of *SLCO1B3*, a marker gene in epidermolysis bullosa associated squamous cell carcinoma (EB-SCC). Specific *trans*-splicing, results in the fusion of the endogenous target mRNA of *SLCO1B3* and the coding sequence of the suicide gene, provided by the RTM. *SLCO1B3*-specific RTMs containing *HSV-tk* were analyzed regarding their *trans*-splicing potential in a heterologous context using a *SLCO1B3* expressing minigene (*SLCO1B3*-MG). Expression of the chimeric *SLCO1B3-tk* was detected by semi-quantitative RT-PCR and Western blot analysis. Cell viability and apoptosis assays confirmed that the RTMs induced suicide gene-mediated apoptosis in *SLCO1B3*-MG expressing cells. The lead RTM also showed its potential to facilitate a *trans*-splicing reaction into the endogenous *SLCO1B3* pre-mRNA in EB-SCC cells resulting in *tk*-mediated apoptosis. We assume that the pre-selection of RTMs by our inducible cell-death system accelerates the design of optimal RTMs capable to induce tumor specific cell death in skin cancer cells.

Abbreviations: BD, binding domain; BP, branch point; DT-A, diphtheria toxin subunit A; EB, epidermolysis bullosa; EB-SCC, epidermolysis bullosa-associated squamous cell carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCV, ganciclovir; GCVTP, ganciclovir triphosphate; HSV, herpes simplex virus; IRES, internal ribosomal entry site; MG, minigene; MMP9, matrix metalloproteinase 9; MTT, 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PPT, poly pyrimidine tract; RDEB, recessive dystrophic epidermolysis bullosa; RTM, RNA *trans*-splicing molecule; RTMm1, RTMm2, RTM mutated 1, RTM mutated 2; RTMmut, splicing deficient RTM; RTMorg, RTM original; SCC, squamous cell carcinoma; SLCO1B3, solute carrier organic anion transporter family member 1B3; SMaRT, spliceosome-mediated RNA *trans*-splicing; SqRT-PCR, semi-quantitative real time PCR; SS, splice site; βhCG6, β-subunit of human gonadotropin gene 6; STS, segmental *trans*-splicing; *tk*, thymidine kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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1. Introduction

Most gene therapy strategies for tumor elimination focus on producing toxin-mediated death of the tumor cells. A transgene encoding a toxin is delivered into the tumor cells, resulting in death of the cells. Inefficient expression of the therapeutic gene due to suboptimal delivery methodology and low targeting specificity toward tumor cells remain the major obstacle to this technology (Altaner, 2008). An established suicide gene is the thymidine kinase (*tk*) gene from herpes simplex virus (HSV). This enzyme exhibits high substrate affinity for the nucleoside analog ganciclovir (GCV) (Elion et al., 1977). The nontoxic prodrug GCV is converted into the metabolite GCV triphosphate (GCVTP), which produces an intracellular toxic effect (Fillat et al., 2003): upon phosphorylation, GCV is incorporated into replicating DNA where it blocks DNA polymerization, resulting in cell death by apoptosis (Altaner, 2008; Hamel et al., 1996).

RNA *trans*-splicing is a technology to combine exogenous genetic information into a target mRNA by exploiting the cell's endogenous spliceosome. The *trans*-splicing reaction is facilitated by RNA *trans*-splicing molecules (RTMs) consisting of a binding domain (BD) for gene targeting, a splicing domain for efficient *trans*-splicing, and a coding domain comprising the sequence to be introduced. RTM binding to the target pre-mRNA induces specific *trans*-splicing between both molecules, resulting in a hybrid gene product consisting of the endogenous mRNA and the coding sequence provided by the RTM. This system, known as spliceosome-mediated RNA *trans*-splicing (SMaRT), has already been applied *in vitro* and *in vivo* to correct mutated genes in several genetic diseases, including epidermolysis bullosa (EB) (Wally et al., 2012; Koller et al., 2011; Murauer et al., 2011; Wally et al., 2010), cystic fibrosis (Liu et al., 2005; Song et al., 2009), hemophilia A (Chao et al., 2003), and spinal muscular atrophy (Coady and Lorson, 2010). In addition, SMaRT can be utilized to produce high levels of therapeutic proteins and antibodies *in vivo* by generating chimeric molecules (Wang et al., 2009).

Because RTMs provide cell-specific expression by binding to a defined target gene, RNA *trans*-splicing has also been considered for cancer gene therapy. For example, Puttaraju et al. (1999) demonstrated accurate *trans*-splicing between the β-subunit of human chorionic gonadotropin gene 6 and an RTM *in vitro* and *in vivo* (Puttaraju et al., 1999). More recently, we provided proof of principle for SMaRT in a suicide gene-therapy approach in recessive dystrophic EB-associated squamous cell carcinoma (RDEB-SCC). In that study we described the construction of an RTM targeting the *MMP-9* gene, which is over-expressed in cultured EB cancer cells, and delivery of the exotoxin streptolysin O. *Trans*-splicing between the RTM and the target gene led to toxin-mediated death preferentially of EB cancer cells compared to a non-cancerous EB cell line (Gruber et al., 2011).

Since non-specific *trans*- (Kikumori et al., 2001) and *cis*-splicing events (Murauer et al., 2013; Wang et al., 2009) within the expression vector have already been observed further attention has to be turned on specificity aspects when using *trans*-splicing in a suicide gene study *in vivo*. Therefore, for clinical application of suicide RTMs in EB cancer, the efficiency and specificity have to be improved so as to provide increased cell-killing ability with as few side effects as possible. Therefore, we used RTMs containing the clinically evaluated HSV-*tk*-GCV system (Immonen et al., 2004; Nasu et al., 2007; Schwarzenberger et al., 2011; Xu et al., 2009). In a double-transfection system, we analyzed RTMs targeting the solute carrier organic anion transporter family member 1B3 (*SLCO1B3*), a marker gene associated with various human cancers, including colorectal adenocarcinomas (Lee et al., 2008), breast (Muto et al., 2007) and lung cancer (Nagai et al., 2012) and, most importantly for our purposes, RDEB-SCC (Cole et al., 2009). Here we describe a suicide-RTM screening system which provides information on the ability of individual RTMs to induce tumor-specific cell death. RTM sequence optimization improved the specificity of the *trans*-splicing reaction, thereby identifying a potent RTM for subsequent *in vivo* applications in RDEB-SCC.

2. Materials and methods

2.1. RTM screening constructs

2.1.1. SLCO1B3 target molecule

The target molecule harbors the 5′ coding region of AcGFP (nt1–nt336), a functional 5′ splice site (ag/gtaag) and nucleotides 4–1200 (first 3 nucleotides of intron 3 were removed to avoid the creation of a possible competitive 5′ splice site) of intron 3 of *SLCO1B3*. The intron 3 portion was amplified by PCR using Gotaq DNA polymerase (Promega), genomic DNA of a healthy donor, and an intron-specific primer pair (fw: 5′-gatc**gatatc**gaatgggttttatattttcaaactaaaataagttaatggaaaattttt-3′, rv: 5′-gaga**gcggccgcg**atttgaatatacatttctcaaaagaagacatacaaatagc-3′) incorporating restriction sites for cloning. The PCR product was ligated into the expression vector pcDNA 3.1D/V5-His-TOPO downstream of the 5′ AcGFP sequence using the restriction sites for EcoRV and NotI. Gel-extractions of amplified PCR products were performed using a GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Plasmid preparations were performed using a Plasmid Mini Prep Kit (Sigma–Aldrich), according to the manufacturer's protocol. Sequence analysis of all plasmids and PCR products was carried out using a 3130 ABI Prism automated sequencer and ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems).

2.1.2. RNA trans-splicing molecule

The RTM backbone consists of a splicing domain carrying a short spacer region and 3′ splicing elements (branch point, polypyrimidine tract, 3′ acceptor splice site) for efficient splicing, and a coding domain incorporating the missing 3′ coding sequence of AcGFP (nt337–nt720) and the full-length DsRed gene expressed under the translational control of an internal ribosomal entry site (IRES) (Dallinger et al., 2003; Gruber et al., 2011). A highly diverse RTM library specific for intron 3 of *SLCO1B3* was created according to Bauer et al. (2013). The cloning procedure of the binding domain (BD) library includes PCR amplification of the intron 3 portion of *SLCO1B3*,

fragmentation of the PCR products by sonication (~10 min on ice) and finally, cloning of the resulting end-repaired (DNATerminator® End Repair Kit, Lucigen Corporation) fragments into the RTM vector, upstream of the splicing domain using the restriction site for HpaI.

2.2. Constructs for the HSV-tk-based inducible cell death system

2.2.1. SLCO1B3 *minigene*

To simulate the endogenous *trans*-splicing scenario as closely as possible, a *SLCO1B3* minigene (*SLCO1B3*-MG) was constructed which consists of the first coding exon of *SLCO1B3* (exon 3: 84 nucleotides) and the first 1200 bases of intron 3 (due to the huge size of the entire intron; 39,205 nucleotides). The exon/intron 3 region of *SLCO1B3* was PCR-amplified from genomic DNA of a healthy donor using Gotaq DNA polymerase (Promega) and a specific primer pair (fw: 5′-gatc**aagctt**atggaccaacatcaacatttgaataaaacagc-3′, rv: 5′-gaga**gcggccgcg**atttgaatatacatttctcaaaagaagacatacaaatagc-3′). The resulting PCR product was cloned into the pcDNA 3.1D/V5-His-TOPO vector (Invitrogen) using the restriction sites for HindIII and NotI.

2.2.2. RTMs

First, one of the most efficient BDs, evaluated in our fluorescence-based screening system, was cloned into the pIRES2-AcGFP1 vector (Clontech) together with the 3′ splicing elements using the restriction sites for EcoRI and PstI. The coding sequence (CDS) of the thymidine kinase gene from herpes simplex virus (*HSV-tk*) without the start codon was amplified from the *HAX1*-targeting vector, kindly provided by Dr. Peckl-Schmid (Peckl-Schmid et al., 2010). Prior to PCR amplification the PstI restriction site within the CDS of *HSV-tk* was mutated using the QuikChange Lightning site-directed mutagenesis kit (Stratagene), according to the manufacturer's protocol. We used a forward primer including a PstI restriction site (5′-ctag**ctgcag**cccacgctactgcgggttta-3′), a reverse primer including a BamHI restriction site (5′-gagaga**ggatcc**tcagttagcctcccccatctc-3′) and Pfu turbo polymerase (Stratagene) for PCR amplification. The resulting PCR product was further subcloned into the RTM vector.

For RTM optimization, start codons and potential cryptic splice sites upstream of the splicing domain were modified with the QuikChange Lightning site-directed mutagenesis kit (Stratagene) resulting in three different RTMs: RTMorg, RTMm1, RTMm2. The sequence of each construct is provided in Supplementary Figure 1.

2.2.3. *Positive control*

As a positive control we used a plasmid expressing a fusion protein of *SLCO1B3* and HSV-*tk* (*SLCO1B3*-*tk* fusion) representing the accurate *trans*-splicing product derived from RTM and *SLCO1B3*-MG. To create this construct, exon 3 of *SLCO1B3* was amplified from *SLCO1B3*-MG using the Pfu turbo polymerase and the following primers (fw: 5′-gaga**gaattc**atggaccaacatcaacattt-3′, rv: 5′-ctag**ctgcag**cttgaatccattgcagcgtc-3′). The PCR product was cloned into the RTM-vector using EcoRI and PstI restriction sites, thereby removing the binding and splicing domains of RTM. Finally, the remaining PstI restriction site was removed from the CDS by using a QuikChange Lightning site-directed mutagenesis kit (Stratagene).

2.3. Constructs for retroviral delivery

2.3.1. *RTM*

The RTM sequence (including BD, spacer and *tk* coding region) was PCR amplified using a pIRES2-AcGFP1 vector specific forward primer (5′-gatc**ggatcc**cgctagcgctaccggactcagatctcg-3′), a *tk* specific reverse primer (5′-gatc**gcggccgc**tcagttagcctcccccatctcc-3′), the Pfu turbo polymerase (Stratagene) and RTMm2 as template for PCR amplification. The resulting PCR product was cloned into the retroviral vector pMXs-IRES-Blasticidin (Cell Biolabs) using the restriction sites for BamHI and NotI.

2.3.2. RTM*mut*

An RTM with an inactive splice site served as negative control. A point mutation at the 3′ splice site was inserted using the QuikChange Lightning site-directed mutagenesis kit (Stratagene) to convert the sequence from c**a**g/c to c**g**g/c.

2.3.3. *Positive control*

The *SLCO1B3*-*tk* fusion was PCR amplified from the pIRES2-AcGFP1 vector (screening procedure) carrying the *SLCO1B3*-*tk* fusion using a *SLCO1B3* exon 3 specific forward and a *tk* specific reverse primer (fw: 5′-ctag**gaattc**atggaccaacatcaacatt-3′, rv: 5′-gatc**gcggccgc**tcagttagcctcccccatctcc-3′) and the Pfu turbo polymerase (Stratagene). The resulting PCR product was cloned into the pMXs-IRES-Blasticidin vector (Cell Biolabs) using the restriction sites for EcoRI and NotI.

2.3.4. *FLAG constructs*

A FLAG-tag for detection of the *trans*-splicing product on protein level was cloned into all constructs (RTMm2, RTMmut and positive control) by PCR amplification using the forward primers mentioned above and a *tk* specific reverse primer (5′-gatcGCGGCCGCTCAtttatcatcatcatctttataatcGTTAGCCTCC CCCATCTCC-3′) including the FLAG epitope and finally cloning of the resulting PCR products into the pMXs-IRES-Blasticidin (Cell Biolabs) vector as described above.

2.4. Cell culture and transfections

For all screening experiments the human embryonic kidney cell line HEK293AD (Stratagene) was used. HEK293AD cells were grown in DMEM supplemented with 10% FCS and 100U/ml penicillin/streptomycin (Biochrom) at 37 °C and 5% CO_2 in a humidified incubator. The cells were passaged every 4 days by Trypsin-EDTA (Biochrom) treatment following centrifugation at 250 g for 5 min. SCCRDEB2 cells (previously described by Watt et al. (2011)), were routinely grown in DMEM/Ham's F-12 (2:1) (Hyclone) containing 10% serum and growth factors according to the protocol of Rheinwald and Green (1975). For better understanding, SCCRDEB2 cells are termed EB-SCC cells throughout this paper. Plasmid transfection in HEK293AD was performed in a 60 mm tissue culture dish at a 60% cell density using the jetPEI reagent (Polyplus-transfection SA) according to the manufacturer's protocol.

2.5. Retroviral RTM delivery into EB-SCCs

For production of retroviral particles, the Phoenix™ Retrovirus Expression System (Orbigen) was used. As previously described, Phoenix cells were transfected at a confluence of approximately 60% with 10 μg of viral plasmid using a standard calciumphosphate transfection and cultured over night at 37 °C and 5% CO_2 (Murauer et al., 2011). After 12–16 h the cells were washed once with PBS and further cultivated in fresh medium at 32 °C and 5% CO_2. After 48 h cell culture supernatant containing infectious viral particles was harvested every 8 h. Medium containing viral particles was filtered through a 0.22 μm filter to remove cell debris and added to EB-SCCs twice within 48 h. To increase transduction efficiency 5 μg/ml Polybrene were added to the virus and spin-oculation was performed for 1.5 h at 600 g and 32 °C. The whole transduction procedure was performed at 32 °C and 5% CO_2. 24 h after the last transduction step, cells were washed 4 times with PBS and cultivated under standard cell culture conditions. For the selection of transduced cells, cell culture medium was supplemented with 10 μg/ml Blasticidin.

2.6. RNA isolation and cDNA synthesis

RNA was isolated from treated HEK293AD cells two days post-transfection using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Purified RNA (2 μg) was digested with DNase I for 30 min at room temperature (RT) and then used as a template for cDNA synthesis using the iScript™cDNA Synthesis Kit (Bio-Rad). For cDNA synthesis, a mixture of oligo(dT) and random hexamer primers was provided.

2.7. Semi-quantitative RT-PCR (SqRT-PCR)

SqRT-PCR was performed to detect *SLCO1B3-tk* fusion transcripts in EB-SCC or HEK293AD cells co-transfected with *SLCO1B3*-MG and RTM. For PCR analysis, an *SLCO1B3*-specific forward primer (5′-ggaccaacatcaacatttgaataaaacagcagag-3′), an HSV-tk-specific reverse primer (5′-gtaagtcatcggctcgggta-3′ or 5′-agatgttcgcgattgtctcggaa-3′), cDNA of treated cells and GoTaq®qPCR Master Mix (Promega) were included. For amplification of *SLCO1B3* and *SLCO1B3*-MG transcripts the following primer combinations were used i.) *SLCO1B3*: fw: 5′-gggtgaatgcccaagagata-3′, rv: 5′-attgactggaaacccattgc-3′, ii.) *SLCO1B3*-MG: fw: 5′-ggaccaacatcaacatttgaataaaacagcagag-3′, rv: 5′-gattaaaaactgatttaaatcaatataggg-3′. The PCR was performed under the following conditions with a Bio-Rad CFX96™ system: 95 °C for 2min, and 40 cycles of 20sec at 95 °C, 20sec at 64 °C, and 20sec at 72 °C. The experiment was carried out in duplicates and repeated two times. Correct PCR products were verified by direct sequencing.

2.8. Western blot analysis

For immunostaining of the *SLCO1B3-tk* fusion protein, HEK293AD cells were resuspended in RIPA lysis buffer (Santa Cruz) and the extracted proteins were separated on a NuPAGE 4–12% BisTris gel (1.0 mm × 12 well, Invitrogen) under denaturating conditions for 2 h at 120 V. After equilibration of the SDS-gel in standard blotting buffer for up to 30 min at RT, the proteins were electro-blotted onto a nitrocellulose membrane (Amersham Hybon-ECL, GE Healthcare) for 75 min at 0.25 A. Subsequently, the membrane was soaked in blocking buffer (5% milk powder in Tris buffered saline + Tween (TBS-T)) for 1 h at RT and incubated overnight at 4 °C with first antibodies: goat anti-HSV-*tk*1 IgG (diluted 1:1000 in TBS-T) (Santa Cruz Biotechnology) and rabbit anti α-actinin IgG (diluted 1:1000–1:5000 in TBS-T) (Santa Cruz Biotechnology). After three washings steps with TBS-T, the membrane was incubated with the secondary antibodies, polyclonal rabbit anti-goat HRP conjugate (diluted 1:200–1:1000 in TBS-T, Dako) and HRP-labeled Envision$^+$ anti-rabbit antibody (diluted 1:500–1:1000 in TBS-T, Dako), respectively. The blot was washed three times and visualization of specific protein bands was performed using an Immun-Star WesternC Kit (Bio-Rad) and a ChemiDoc XRS Imager (Bio-Rad).

2.9. Immunoprecipitation

For endogenous detection of the *trans*-splicing protein, *SLCO1B3-tk*, immunoprecipitation was performed. Briefly, transduced EB-SCC cells (3×10^6) were resuspended in lysis buffer (25 mM Tris, 140 mM NaCl, 1 mM EDTA, 0.5% IPGAL) and incubated for 45 min at 4 °C rotating. 1 μg of anti-FLAG antibody (Sigma–Aldrich) was added to the cleared lysate and incubated over night at 4 °C rotating. Then, the cell lysates were added to 30 μl protein G sepharose (GE Healthcare) and incubated for additional 2 h at 4 °C rotating. After three washing steps of sepharose G (once with lysis buffer and twice with PBS), the bound protein was denatured from the polymer and transferred to SDS-Page, followed by Western blot analysis described in detail above. Additionally, FLAG fusion proteins were isolated from RTMm2 transduced RDEB-SCC cells (2×10^6) by adding 40 μl of anti-FLAG M2 Affinity Gel (Sigma–Aldrich) and a subsequent incubation over night at 4 °C rotating. After three washing steps the detection of *SLCO1B3-tk* fusion proteins was performed as described in the Western blot analysis section.

2.10. Cell viability assay

Cell viability after RTM treatment was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma). Forty-eight hours after RTM transfection, HEK296AD cells were seeded into a 96-well plate (10,000cells/well) and treated with 100 μM GCV (Cymevene®, Roche) the next day and incubated for 72 h. 20 μl of MTT (5 g/L in PBS) were added to each well (200 μl) and incubated for approximately 2 h at 37 °C. Then, all media was removed and the cells were lysed with 100 μl DMSO/glycine (6 vol DMSO + 1 vol 0.1 M glycine/NaOH, pH 10.2). After 10 min incubation on a plate shaker (500 rpm) the absorbance of the resultant formazan product was measured at 492 nm/620 nm with a plate photometer (Tecan). The percentage of cell viability was calculated by the equation: (OD GCV-treated/OD GCV-untreated) × 100/negative control (OD GCV-treated/OD GCV-untreated). HEK293AD cells transfected with *SLCO1B3*-MG alone or EB-SCC cells served as a negative control in this assay. Statistical analysis was performed with GraphPad Prism 5.03 software.

2.11. *Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay*

Detection of apoptosis was performed using an *In Situ* Cell Death Detection Kit, TMR red (Roche) according to the manufacturer's protocol. Briefly, cells were washed with 1× phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde for 60 min at RT and permeabilized with 0.1% BSA/0.2% Triton X-100 in PBS for 5–10 min on ice. After 3 washing steps, the cells were incubated with TUNEL reaction solution for 1 h at 37 °C in the dark and finally analyzed by fluorescence microscopy (Axiophot, Carl Zeiss) and flow cytometry (FC500, Beckman Coulter).

3. Results

3.1. *Screening for an efficient RTM binding domain*

A binding domain (BD) screen accelerates the construction of a highly efficient RTM for endogenous applications. Exon 3 of *SLCO1B3* gene constitutes the first coding exon, therefore intron 3 was examined for potent RTM binding sites. Moreover the huge size of intron 3 of 39,205 nt did not allow a screening procedure covering the whole intronic sequence, so we used the first 1200 nt of intron 3 for cloning into the target vector. Using our fluorescence-based RTM screening procedure (Bauer et al., 2013; Murauer et al., 2013), we were able to select a promising BD specific for intron 3 of *SLCO1B3* out of a pool of randomly created BDs with various binding localizations within the target intron (Supplementary Figure 2).

Binding of an RTM to intron 3 of the target molecule induces fusion of the split segments of AcGFP by 3′ RNA *trans*-splicing, manifested in the expression of the full-length reporter in co-transfected cells (Figure 1A). In total, 22 individual 3′ RTMs, binding all over the first 1200 nt of intron 3 of *SLCO1B3*, were tested for their *trans*-splicing efficiency by flow cytometric analysis after co-transfection with the designed target molecule into HEK293AD cells. The intensity of the fluorescence signal and the amount of AcGFP-expressing cells correlate with the *trans*-splicing efficiency of

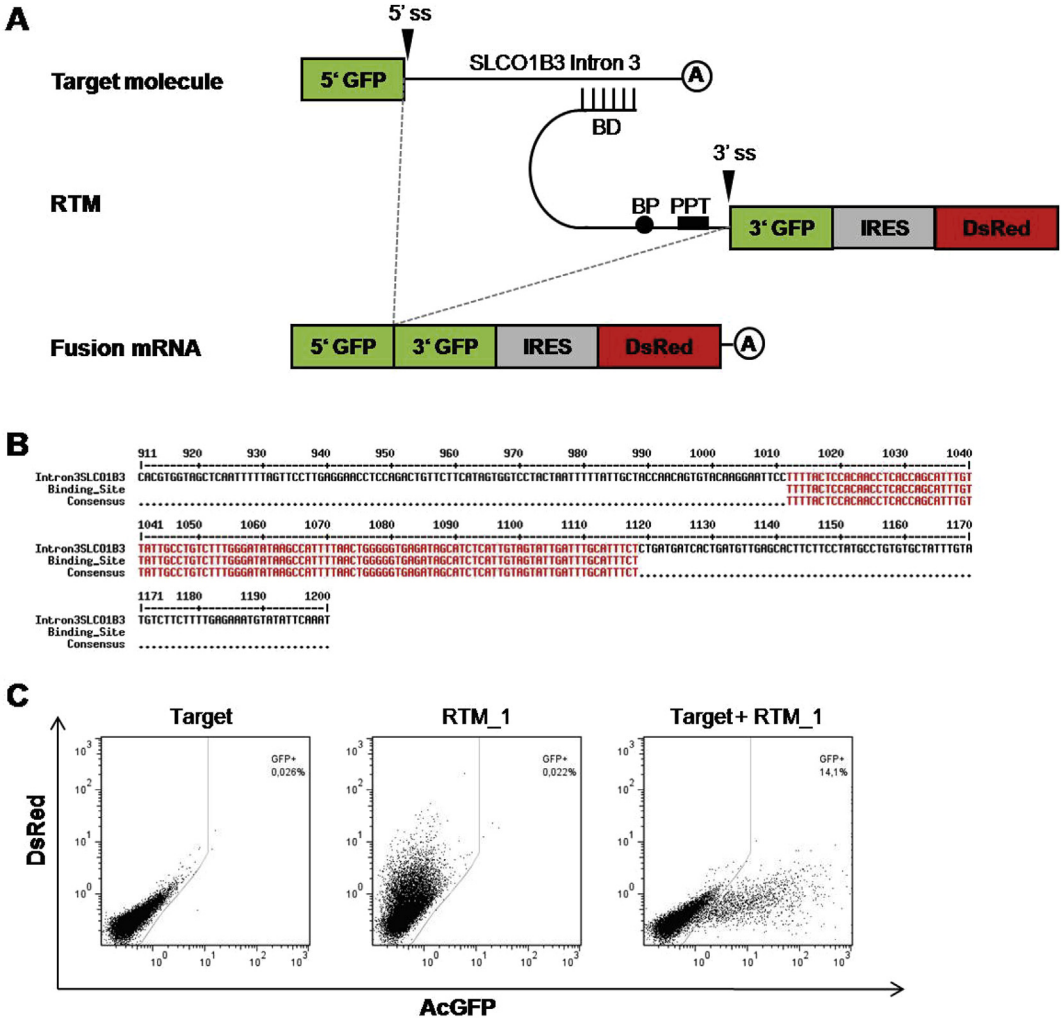

Figure 1 – Evaluation of a specific binding domain (BD) for efficient *trans*-splicing. (A) Schematic diagram of the screening procedure used for evaluation of BDs. (B) Binding position of RTM_1 within the target intron 3 of *SLCO1B3* (nt1012-nt1118). (C) Analysis of AcGFP expression by flow cytometry in HEK293AD cells transfected with the target molecule, RTM_1, or both the target molecule + RTM_1. Only cells co-transfected with the target molecule and RTM_1 showed green fluorescence signal, resulting from fusion of both parts of the split AcGFP gene by RNA *trans*-splicing. BD: Binding Domain, BP: Branch Point, PPT: Poly Pyrimidine Tract, 3′ ss: 3′ splice site.

the transfected RTM (The geometric means of GFP expression, as a measure of *trans*-splicing efficiency of each RTM is given in Supplementary Table 1). The analysis revealed RTM_1 (binding position in intron 3: nt1012–nt1118) as one of the most efficient RTMs, and thus it was selected for the initial tests in the *tk*-based inducible cell-death system (Figure 1B+C). The short BD of RTM_1 probably reduces unwanted direct HSV-*tk* expression from the RTM backbone as the amount of in frame ATGs and cryptic 5′ splice sites, leading to new potential start sites, are decreased significantly.

3.2. *Trans-splicing between* SLCO1B3-*MG and RTM is accurate*

To further evaluate the specificity of RTMs in a suicide gene therapy approach we cloned the BD from RTM_1 into the suicide RTM plasmid containing the *tk* sequence from HSV. The sequence upstream of the splicing domain was mutated to reduce potential cryptic splice sites and start codons, resulting in 3 different RTMs, termed RTMorg, RTMm1 and RTMm2. Detailed information on sequence modifications of the RTMs is provided in Supplementary Figure 1.

To mimic the endogenous RDEB-SCC marker expression in heterologous HEK293AD cells, we designed a minigene (MG) vector carrying the first coding exon (exon 3) of the tumor marker gene *SLCO1B3* as well as the first 1200 bases of intron 3. Intron 3 was selected as RTM binding site due to the fact that the fusion of the first coding exon of the gene and HSV-*tk* by accurate *trans*-splicing minimizes the size of the resulting fusion product and probably maintains its functionality. *HSV-tk*- and *SLCO1B3-tk*-expressing vectors served as positive controls (Figure 2A). Upon correct *trans*-splicing of the RTM with the MG pre-mRNA, a fusion mRNA of *SLCO1B3* exon 3 and *HSV-tk* is generated, encoding a fusion protein that is approximately 3 kDa larger than the original HSV-*tk* (Figure 2B).

3.3. *The fusion protein of* SLCO1B3-tk *exhibits suicide gene activity*

Next we determined whether the fusion protein that results from the *trans*-splicing reaction between *SLCO1B3*-MG and the RTM is expressed correctly and still possesses its functionality as a suicide gene. To this end, we created the chimeric cDNA of *SLCO1B3* exon 3 and *HSV-tk* and confirmed its expression in whole-cell lysates of HEK293AD cells by Western blot analysis (Figure 3B, lanes 9, 10). The *SLCO1B3-tk* fusion protein was detected at the predicted molecular weight of 40 kDa, 3 kDa larger than the original HSV-*tk* (37 kDa). Furthermore, the biologic activity of *SLCO1B3-tk* fusion protein was assessed in an MTT cell viability assay (Figure 4A). HEK293AD cells were transfected with the plasmid HSV-*tk* or the fusion construct and treated with 100 μM GCV. The *SLCO1B3-tk* fusion protein

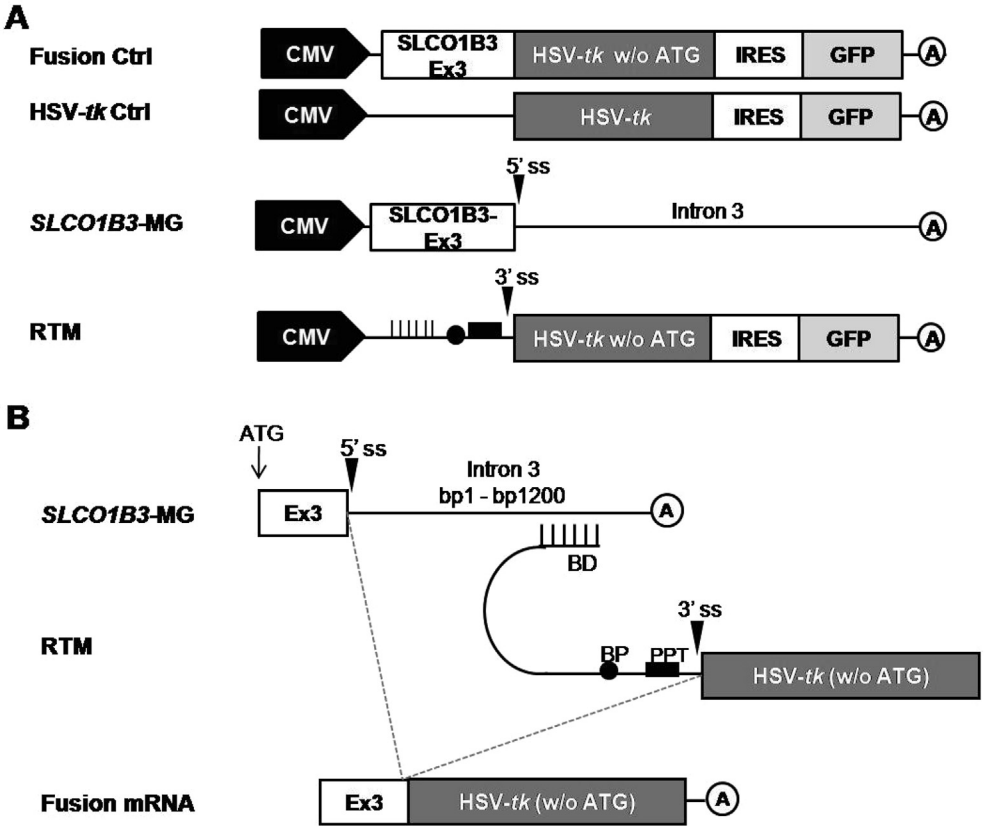

Figure 2 – (A) Constructs used in the experimental procedure. (B) Schematic diagram of the double-transfection system used for RTM evaluation. Correct *trans*-splicing of the RTM to *SLCO1B3*-MG results in a fusion mRNA consisting of exon 3 of *SLCO1B3* and *tk*. BD: Binding Domain, BP: Branch Point, PPT: Poly Pyrimidine Tract, 3′ ss: 3′ splice site, A: polyadenylation signal.

was able to convert the prodrug GCV into the toxic metabolite GCV triphosphate (GCVTP) which induces cell death, producing a reduction of cell viability similar that caused by the original HSV-*tk*. Therefore, these data confirmed the functionality of the fusion construct that is expected to result from the *trans*-splicing procedure in our experimental setup.

3.4. *Detection of* SLCO1B3-tk *fusion at the mRNA and protein level*

To investigate accurate *trans*-splicing between *SLCO1B3*-MG and each individual RTM (RTMorg, RTMm1, RTMm2) we first performed sqRT-PCR. We detected the correct fusion mRNA in all three co-transfected cell populations, represented by a 205 bp PCR product on an agarose gel (Figure 3A). In addition we performed Western blot analysis to confirm synthesis of the *trans*-spliced protein product. As shown in Figure 3B, high background expression of HSV-*tk* (37 kDa) was observed in cells transfected with RTMorg alone, whereas very low background expression was seen in cells transfected with the improved RTMs, RTMm1 and RTMm2. Furthermore, double transfection of cells with RTMorg plus the MG resulted in generation of both the *trans*-spliced fusion protein (40 kDa) and the background HSV-*tk* protein (37 kDa). In cells double-transfected with RTMm1 or RTMm2 and the MG, sufficient expression of the *trans*-spliced fusion protein was observed, while hardly any background expression of HSV-*tk* was detected. These data indicate that improvement of RTMs by sequence optimization is an important step for reducing unwanted expression of HSV-*tk*.

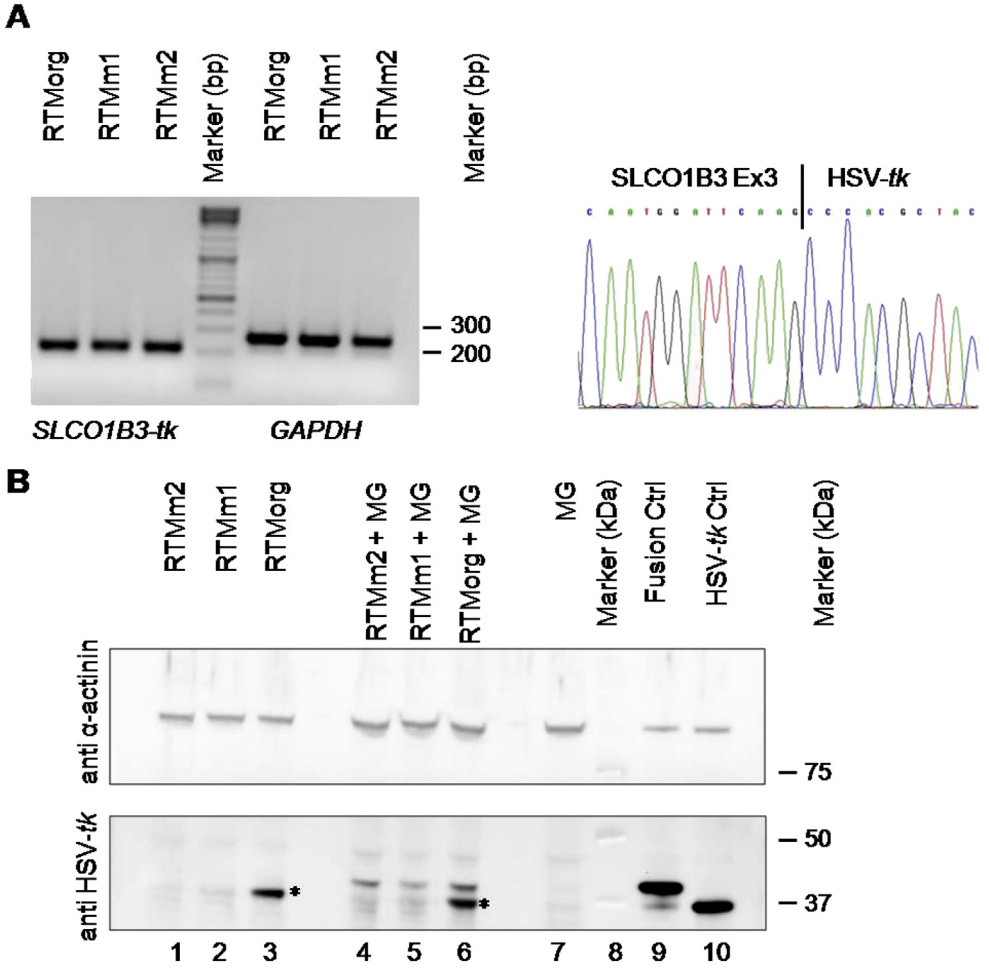

Figure 3 – Detection of accurate *trans*-splicing on the mRNA and protein level. (A) SqRT-PCR analysis of HEK293AD cells double-transfected with *SLCO1B3-MG* and either the original RTM (RTMorg), or the mutated RTMs (RTMm1 and RTMm2). The correct PCR product of the fusion mRNA of *SLCO1B3* and HSV-*tk* (205 bp) is shown. *GAPDH* was used as housekeeping gene. All PCR products were verified by direct sequencing, demonstrating correct *trans*-splicing between *SLCO1B3-MG* and the RTMs on the mRNA level. A representative figure shows the sequence of the *SLCO1B3*-HSV-*tk* junction (right panel). (B) Sequence optimization leads to reduced background expression of RTMs as shown by Western blot analysis: Upper panel: α-actinin staining (100 kDa) was used as a loading control. Lower panel: Western blot analysis demonstrated the expression of the *SLCO1B3*-fusion protein (40 kDa) in all cell populations double-transfected with the *SLCO1B3*-MG (MG) and each RTM [lane 4, 5, 6]. Sequence optimized RTMm1 and RTMm2 showed greatly reduced background expression of HSV-*tk* (37 kDa) in both single [lane 1, 2] and double [lane 4, 5] transfected HEK293 cells as compared to the RTMorg [lane 3, 6] (marked by asterisk). Cells transfected with the MG alone [lane 7], either the fusion [lane 9] or HSV-*tk* [lane 10] construct served as negative and positive controls, respectively. Lane 8: Precision Plus Protein WesternC Standards (Biorad).

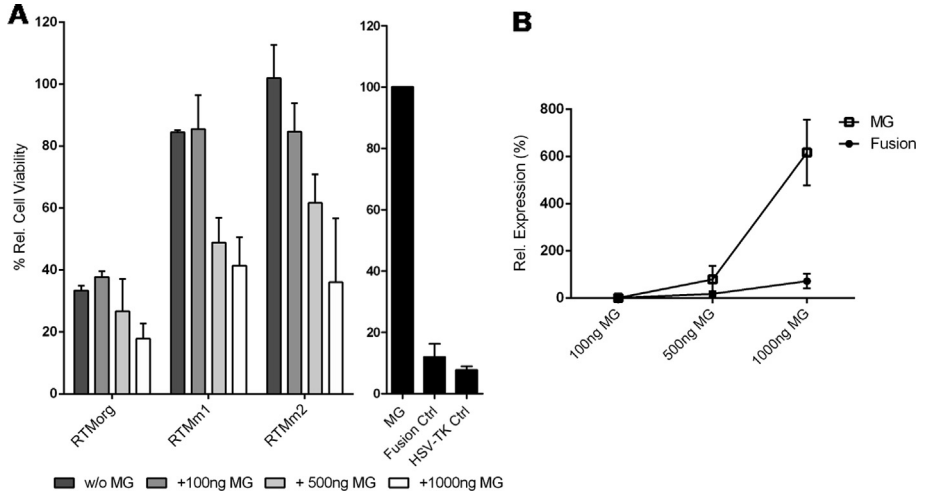

Figure 4 – Biological activity of the fusion protein *SLCO1B3* and HSV-*tk* resulting from accurate *trans*-splicing. (A) MTT assay was performed to measure reduction in cell viability. 100 ng of each RTM as well as different concentrations of *SLCO1B3*-MG (100 ng, 500 ng, 1000 ng) were transfected into HEK293AD cells and the cells were treated with 100 μM GCV for 72 h. HEK293AD cells transfected with *SLCO1B3*-MG alone, the *SLCO1B3-tk* fusion or HSV-*tk* served as negative and positive controls, respectively. The mean ± SD of two independent experiments is shown. Six replicates of each sample were measured in every experiment. The percentage of cell viability was calculated by the equation: (OD GCV-treated/OD GCV-untreated) × 100/negative control (OD GCV-treated/OD GCV-untreated). (B) SqRT-PCR analysis of HEK293AD cells co-transfected with RTMm2 (100 ng) and different amounts of *SLCO1B3*-MG (100 ng, 500 ng, 1000 ng). Both, the transcripts of *SLCO1B3*-MG and the fusion mRNA of *SLCO1B3* and HSV-*tk* were amplified. *GAPDH* was used as housekeeping gene. Data were calculated relative to the transcript level in HEK293AD cells transfected with 100 ng MG and 100 ng RTMm2.

3.5. *Suicide gene induction by RNA trans-splicing*

Trans-splicing provides gene- and cell-specificity, thereby reducing unwanted side effects that accompany other toxin-mediated cell-death approaches. In the present system, cell death is mainly induced in cells expressing the up-regulated tumor marker gene *SLCO1B3*. Expression of the fusion constructs at the protein level (Figure 3B) correlates with the reduction of cell viability measured in MTT assays (Figure 4A). Single transfection of RTMorg induced apoptosis in HEK293AD cells, which can be explained by the presence of alternative start codons (ATGs) and splice sites upstream of and within the BD. These would allow alternative in-frame *tk* proteins to be expressed from the RTM vector alone, leading to a toxic effect in GCV-treated cells. However, by deleting the superfluous ATGs and potential cryptic splice sites within and upstream of the BD sequence of the RTM by site-directed mutagenesis, we were able to reduce this unwanted expression significantly. RTMm1 and RTMm2 alone showed only a minimal ability to induce apoptosis using 100 ng plasmid per transfection. Furthermore, addition of different amounts of *SLCO1B3*-MG (100 ng, 500 ng, 1000 ng) led to fusion of *SLCO1B3* exon 3 with the *tk* sequence of the RTM by *trans*-splicing, resulting in expression of a functional *SLCO1B3-tk* fusion protein in transfected cells. Both RTMm1 and RTMm2 induced a clear dose-dependent reduction in cell viability after MG addition (Figure 4A). Additionally, data achieved by sqRT-PCR confirmed that the increased levels of *SLCO1B3*-MG expression go along with an enhanced *trans*-splicing efficiency of RTMm2, manifested in the expression of *SLCO1B3-tk* fusion transcripts (Figure 4B). The TUNEL assay performed on transfected HEK293AD cells underlines the reliability and functionality of the inducible cell-death system by *trans*-splicing constructs (Figure 5A). Co-transfection of *SLCO1B3*-MG (1 μg) and RTMm2 (100 ng) into HEK293AD cells induced apoptosis in up to 63.8% of cells, as revealed by flow cytometry. This level of killing is comparable with the cell lethality rate reached by using the *SLCO1B3-tk* fusion control vector. Light microscopy performed on HEK293AD cells co-transfected with *SLCO1B3*-MG and RTMm2 also showed reduced numbers of healthy cells in comparison to cells transfected with RTMm2 or the *SLCO1B3*-MG alone (Figure 5B).

3.6. *Pre-evaluated RTMm2 induced trans-splicing reaction with endogenous* SLCO1B3 *in EB-SCC cells*

In order to demonstrate the functionality of the pre-evaluated RTMm2 also on the endogenous level, we tested its *trans*-splicing ability in EB-SCC cells, over-expressing *SLCO1B3* (South A.P.; Manuscript in preparation). To simplify the monitoring of the *trans*-splicing efficiency manifested in the expression of the *SLCO1B3-tk* fusion protein over a longer period of time, RTMm2 was cloned into a retroviral vector and transduced into EB-SCC cells. A retroviral vector carrying the *SLCO1B3-tk* fusion gene and a splicing deficient RTMmut (3′ splice site was removed from RTMm2 by site directed mutagenesis) were included as positive and negative controls in this experiment, respectively. RTMm2 transduction of the tumor cells resulted in a long lasting expression of the RTM facilitating the detection of the *SLCO1B3-tk* fusion molecule on RNA and protein level. The *SLCO1B3-tk* fusion was detected in RTMm2 transduced EB-SCC cells by sqRT-PCR, demonstrating

the endogenous functionality of our designed RTM with a relative *trans*-splicing efficiency of 4.9% in comparison to total amplified *SLCO1B3* transcripts. RTMmut did not induce a *trans*-splicing reaction (Figure 6A + B). We were able to confirm the translation of the *SLCO1B3-tk* fusion by immunoprecipitation using two different purification methods, via protein G sepharose or FLAG affinity gel (Figure 6C). Finally, we analyzed the functionality of *SLCO1B3-tk* fusion protein in transduced EB-SCC cells upon GCV treatment (Figure 6D). MTT assays demonstrated a reduction of cell viability in fusion Ctrl (to 19.5%) and RTMm2 (to 45%) transduced cells in comparison to untreated EB-SCC cells, attesting to the functionality of the fusion protein also on the endogenous level. RTMm2 showed a significant reduction in cell viability compared to RTMmut ($p = 0.006$), indicating induction of apoptosis in EB-SCC cells via *trans*-splicing specific suicide gene transfer.

4. Discussion

In this work we present a screening system accelerating the design of "suicide" RTMs capable to induce apoptosis in tumor cells by accurate *trans*-splicing into the tumor marker *SLCO1B3*.

Targeted elimination of tumor cells is challenging for many reasons. One significant hurdle is finding a means to discriminate between normal and malignant cells. We expedite spliceosome mediated RNA *trans*-splicing (SMaRT) as a promising approach for cancer cell targeting. RNA *trans*-splicing is a technology that exhibits its potential in different applications: such as therapeutic gene correction, production of therapeutic proteins, molecular imaging and suicide gene therapy approaches (as reviewed by Wally et al., 2012). A *trans*-splicing-mediated approach for tumor cell targeting was previously demonstrated by Nakayama et al. applying a "segmental *trans*-splicing" (STS) strategy. In this work the 5′ and 3′ fragments of the Shigatoxin gene were separately introduced into tumors, combined in the target cells by accurate *trans*-splicing leading to the subsequent expression of a Shigatoxin protein and a marked suppression of tumor size over time (Nakayama et al., 2005). However, the delivery of two vectors may hamper the chance of a therapeutic success. Therefore we utilize the increased endogenous expression of a tumor marker gene to maintain the *trans*-splicing-mediated expression of a fusion protein exhibiting cell killing potential predominantly in tumor cells. This should increase the tumor-specific expression of the toxin as preferable those cells are targeted by the delivered RNA *trans*-splicing molecule (RTM), in which the marker gene is expressed at high levels.

The feasibility of 3′ *trans*-splicing technology in a suicide gene therapy approach was initially demonstrated by Puttaraju et al. (1999). They have designed an RTM capable to *trans*-splice the coding sequence of diphtheria toxin (DT-A) to the tumor marker gene β-subunit of human chorionic gonadotropin gene 6 (βhCG6). After transfecting tumor bearing athymic mice (H1299 human lung cancer cells) with the RTM a correct splicing product consisting of βhCG6 and DT-A was detected on the mRNA level. More recently we also provided proof of principle to this technology in cancer cells (SCC) from patients suffering from recessive dystrophic epidermolysis bullosa (RDEB) (Gruber et al., 2011). Utilizing a fluorescence based screening system we defined an RTM targeting the matrix metalloproteinase 9 (*MMP-9*), which was shown to be an upregulated mRNA transcript in RDEB-SCC *in vitro*. The RTM accomplished accurate *trans*-splicing on the endogenous mRNA level in cell culture thereby generating a new chimeric fusion mRNA consisting of exon 1 of *MMP-9* and the suicide gene HSV-*tk* encoded by the RTM. Furthermore, cell death was induced in *MMP-9* positive cancer cells by the RTM, delivering the coding sequence of exotoxin streptolysin O, indicating target-specific RNA replacement.

However, since non-specific *trans*- and *cis*-splicing events induced by a designed RTM can reduce the pool of specific and functional RTMs significantly, our present work concentrates on the construction and optimization of RTMs in order to reduce these unwanted side effects which have to be eradicated prior to *in vivo* investigations.

First, we constructed an RTM targeting the gene *SLCO1B3*, coding for the organic anion transporter polypeptide OATP1B3 (Kalliokoski and Niemi, 2009) which was shown to be an upregulated mRNA population in EB-SCC cells. OATP1B3 is mainly expressed at the membrane of human hepatocytes and act as influx transporters for its substrates from blood (Niemi, 2007). An increased expression of *SLCO1B3* mRNA was previously observed in colorectal cancer, where it provides apoptotic resistance to colon cancer cells (Lee et al., 2008) and more recently, also in head and neck SCC tumor tissue (Zolk et al., 2013). Although, *SLCO1B3* demonstrated to be a potent target for the evaluation and improvement of RTMs in our suicide gene therapy approach, some aspects have to be considered when used in a more translational perspective. For systemic application, the targeting and killing of *SLCO1B3*-expressing hepatocytes by the RTM can be prevented by, e.g. the usage of a skin specific K14 promoter (Staggers et al., 1995; Hosseini et al., 2010). An alternative route for a possible application might also constitute the local injection of the RTM into the epidermal tumor tissue. However, the most promising RTM introduction has to be evaluated in future *in vivo* studies which can be viral- or non-viral-based. Additionally, these experiments will also answer the question of possible side effects in non-cancerogenous cells/tissue with a lack of *SLCO1B3* expression.

Due to the huge size of *SLCO1B3* intron 3 (39,205 nt) we only screened the first 1,200 nt for potent RTM binding sites. To date, few rational parameters are known for the design of a BD, and future screening experiments may also identify even more potent BDs in the 3′ part of intron 3. Recently, Murauer et al. suggested that by blocking the endogenous 3′ intron/exon junction the splicing machinery may favor the use of 3′ ss provided by the RTM leading to increased *trans*-splicing efficiency (Murauer et al., 2013). However, due to the huge size of our target intron we preferred to select the 5′ region of intron 3 near the first 5′ coding exon for RTM binding.

The two critical points concerning development of a suicide RTM are: (i) the occurrence of nonspecific *trans*-splicing events between the RTM and pre-mRNAs of other genes; and (ii) direct expression of the suicide gene from the RTM vector or functional suicide gene expression through *cis*-splicing within the RTM backbone sequence. In this work we increased

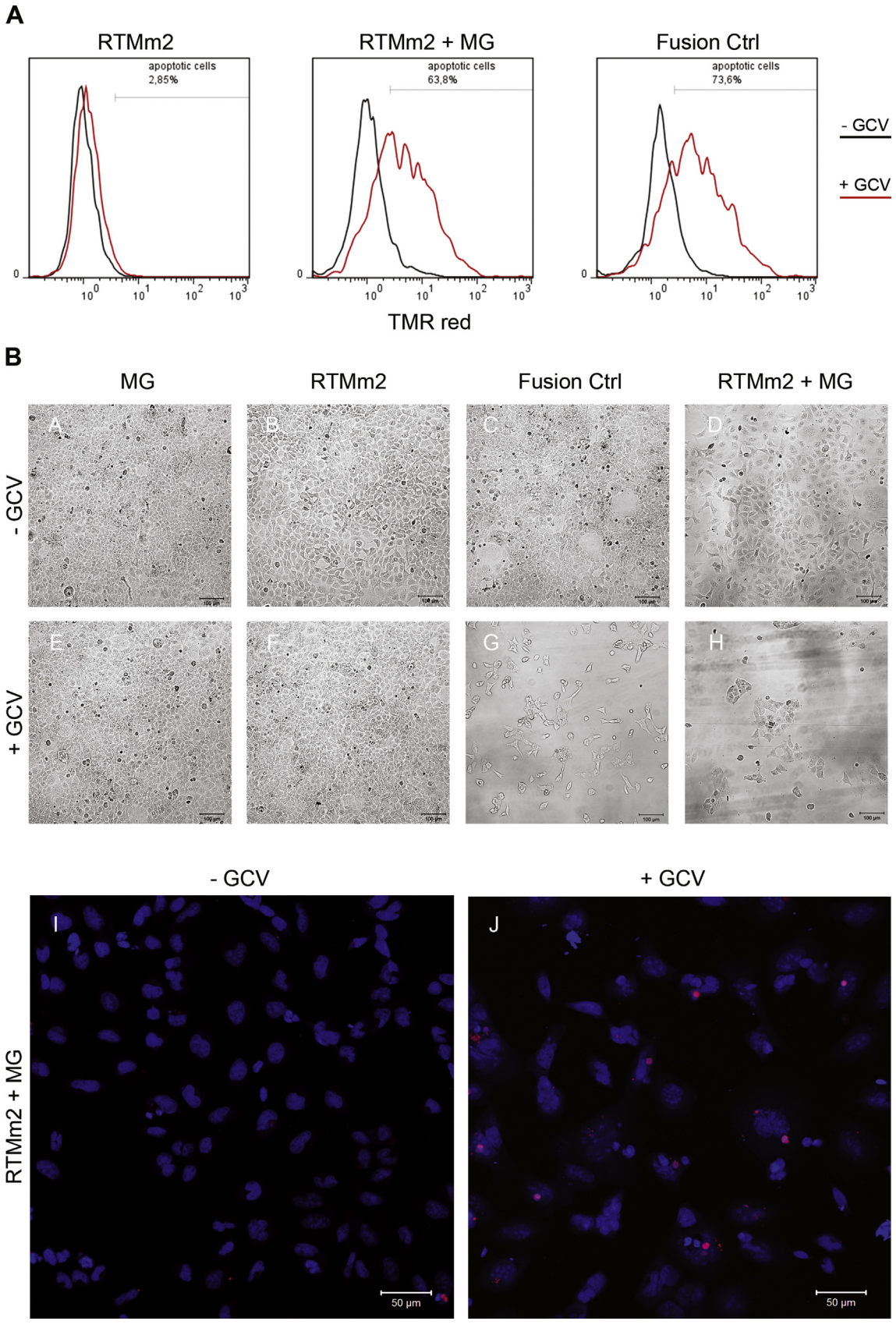

Figure 5 – Detection of cell death after RNA *trans*-splicing. (A) *In situ* detection of cell death by TUNEL assay of HEK293AD cells treated with RTMm2 and RTMm2+MG was assessed by flow cytometric analysis. The *SLCO1B3-tk* fusion served as positive control. Overlay of TMR red positive cells is shown. The black line represents the GCV-untreated and the red line the GCV-treated cell populations. (B) The reduction in cell number of transfected and GCV-treated (100 µM) HEK293 cells (E–H) in comparison to GCV-untreated cells (A–D) was visualized by light microscopy analysis. A decrease in cell viability of *SLCO1B3-tk* and RTMm2+*SLCO1B3*-MG transfected cells 72 h after addition of GCV was detected. TMR red staining (TUNEL assay) and nuclear counterstaining with DAPI of RTMm2 and *SLCO1B3*-MG co-transfected HEK293 cells revealed a significant higher rate of apoptotic cells after GCV treatment (J) in comparison to the level seen in the GCV-untreated cell population (I).

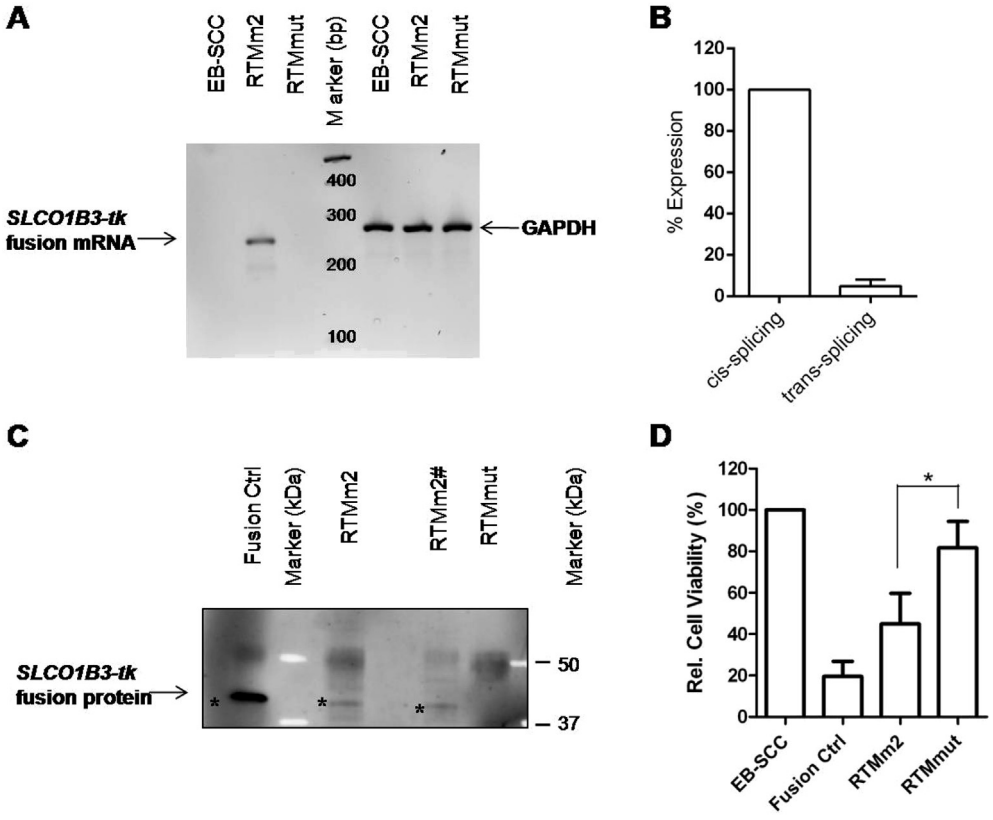

Figure 6 – T*rans*-splicing into endogenous *SLCO1B3* pre-mRNA. (A) SqRT-PCR confirmed a correct *trans*-splicing product (245 bp) only in RTMm2 transduced EB-SCC cells. The housekeeping gene GAPDH served as control for quality and quantity. PCR products were verified by direct sequencing (data not shown). (B) mRNA level of endogenous *trans*-splicing compared to *cis*-splicing within the *SLCO1B3* gene in RTMm2 transduced EB-SCC cells. Specific PCR products were amplified by sqRT-PCR and *trans*-splicing efficiency (total *SLCO1B3-tk* transcripts) was calculated relative to the *cis*-splicing level (total *SLCO1B3* transcripts = 100%). *GAPDH* was used as housekeeping gene (C) Detection of *SLCO1B3-tk* fusion protein (marked by asterisk) by immunoprecipitation (using anti-FLAG antibody) in combination with Western blot analysis (using anti-HSV-*tk* antibody) in cells containing transduced RTMm2 or the fusion ctrl. In contrast, no specific protein was isolated from RTMmut transduced cells. Fusion proteins were purified using protein G sepharose (Fusion Ctrl, RTMm2 and RTMmut) or FLAG affinity gel (RTMm2#) and visualized with anti-HSV-*tk* antibody by Western blot analysis. (D) Expression of *SLCO1B3-tk* fusion protein resulted in reduced cell survival as detected by MTT assay. The mean of at least 4 different experiments ± SD is shown. *$P < 0.05$ Student's *t* test, unpaired, two-tailed.

the *trans*-splicing specificity by modifying the RTM's BD and deleting ATGs and *cis*-splice sites upstream of the suicide gene (HSV-*tk*) sequence within the RTM. The HSV-*tk* system provides an easy monitoring of the *trans*-splicing efficiency and specificity by the RTM as the toxicity of the *tk* gene is only activated after ganciclovir treatment of the cells. A significant reduction of background *tk* expression for both RTMm1 and RTMm2 was detected by Western blot analysis and confirmed by MTT assays. Additionally, GCV treatment of HEK293AD cells co-transfected with RTM and *SLCO1B3*-MG led to a significant reduction of cell viability in comparison to cells transfected with *SLCO1B3*-MG or an RTM alone. Although the HSV-*tk* system is known to show a "bystander effect" (Freeman et al., 1993) which may influence our experimental system, we clearly observed a reduction of cell viability after the *SLCO1B3*-MG addition in a dose dependent manner. The cell viability was found to decrease as the amount of *SLCO1B3*-MG-expressing plasmid increased, owing to higher *trans*-splicing rates in the nucleus. However, the current inducible cell-death system has to be further optimized due to persistent, albeit significantly reduced background *tk* expression caused by the high amounts of RTM transfected in the heterologous system. In addition, our results support the construction of RTMs for subsequent tests in endogenous model systems since optimized RTMm2 demonstrated its functionality also in EB-SCC cells. Here we used a retroviral vector system for delivery and stable expression of the RTM to ease the monitoring and detection of the *SLCO1B3-tk* fusion protein resulting from accurate *trans*-splicing in EB-SCC cells. Although retro- or lentiviral vectors are previously described as transport systems for RTMs in gene correction studies, where a long term restoration of mutated genes is favored (Murauer et al., 2011; Wally et al., 2010; Avale et al., 2013), we assume that for a suicide gene therapy approach a viral integration system might not be necessary because cell killing by a strong toxin do not require long lasting expression of the RTM.

We detected a relative *trans*-splicing efficiency (*SLCO1B3-tk* fusion transcripts versus total *SLCO1B3* transcripts) of about 5%. Still we observed a significant reduction of cell viability

of RTM transduced tumor cells after ganciclovir treatment. These data indicate that in this cancer suicide gene approach the *trans*-splicing efficiency plays a minor role, as compared to the effects of the toxin. In future experiments variations in RTM administration, RTM delivery systems or the selected toxin for apoptosis induction will provide further information concerning the efficiency, specificity and safety of the RNA *trans*-splicing-induced cell death system in comparison to conventional cancer therapy approaches already applied. Taken together, these results confirm the potential of SMaRT to induce the death of target cells expressing *SLCO1B3* and we suggest SMaRT as a promising tool to target and eliminate specific tumor cells in a suicide gene therapy approach to treat patients suffering from EB-SCC.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2013.08.005.

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