Citation: Molecular Therapy—Nucleic Acids (2014) 3, e157; doi:10.1038/mtna.2014.10

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LETTER TO THE EDITOR

Considerations for a Successful RNA *Trans*-splicing Repair of Genetic Disorders

Molecular Therapy—Nucleic Acids (2014) **3**, e157; doi:10.1038/mtna.2014.10; published online 8 April 2014

To the Editor: Spliceosome-mediated RNA *trans*-splicing (SMaRT) has emerged as a novel technology for the repair of mutations in monogenic disorders. SMaRT can be used to replace 5′, 3′, or internal sequences in an exon-wise manner. For this purpose, RNA *trans*-splicing molecules (RTMs) are engineered, which comprise (i) the wild-type coding region to be replaced, (ii) a splicing domain that includes essential splicing elements such as branch point and polypyrimidine tract, (iii) a binding domain (BD) hybridizing to a selected target region to bring RTM and target transcript into close proximity, and (iv) a spacer sequence for steric reasons. The replacement of exonic portions provides several advantages relative to other gene therapeutic approaches, such as complementary DNA (cDNA) therapy. First, for very large genes, the sequence to be replaced can be reduced to a size that facilitates viral packaging and transduction. Second, the increase of wild-type alleles and the concomitant decrease of mutated alleles leads to an enhanced shift in their quantitative proportions, making this technology interesting for the correction of dominant mutations. Third, as *trans*-splicing (TS) takes place at the messenger RNA (mRNA) level, target gene expression remains under the control of the respective endogenous promoter, and problems arising from transgene overexpression can be excluded.

The first publications on SMaRT date back to the 1990s, when minigene systems were used in cotransfection experiments. Accordingly, a selected target region for RTM binding is cloned into an expression vector and transfected, together with a rationally designed RTM, into cell lines that do not express the gene of interest.[1,2] This approach facilitates the monitoring of correct TS but does not reflect a real disease setting, wherein the amount of the target molecule is often significantly reduced due to missense or nonsense mutations. Successful TS in an authentic, endogenous environment requires a highly functional and specific RTM to provide a therapeutically relevant level of correction and to exclude unspecific splicing events due to the relative underrepresentation of the target molecule.

Currently, RNA TS has evolved into an elegant tool to reprogram endogenous pre-mRNAs. By specifically replacing a portion from the gene of interest, a reprogrammed, mature wild-type mRNA is generated, encoding a functional protein.[3] With increasing experience, it turned out that the composition and characteristics of the BD sequence play a crucial role in the TS process. Therefore, during the past years, the main focus has been on the identification and design of highly functional BDs. Due to the diversity of primary and secondary structure characteristics of target regions (mostly introns), no reproducible principles for BD design applicable for a majority of target regions have emerged. Therefore, we developed a fluorescence-based RTM screening system, which we have successfully applied for several genes underlying the heterogeneous skin blistering disease epidermolysis bullosa.[4–7] In this letter, we want to share our expertise and provide insight into a method that facilitates the identification of highly functional BDs for any gene of interest.

The choice of using 3′ TS, 5′ TS, or internal exon replacement (IER) depends mainly on the type and number of mutations known in a gene of interest. Whereas 5′ and 3′ TS are used to replace large upstream or downstream regions of a pre-mRNA, thereby covering all potential mutations in this region, IER is used to replace one or more central exons.[8] The latter is especially suitable for genes with mutational hotspot mutations because the size of the respective RTM can be minimized, facilitating cloning and transfections. Another method of TS, without targeting endogenous transcripts, was recently shown by Koo *et al.*[9] In their study, the cDNA (>11 kb) of the Duchenne muscular dystrophy gene was transported into skeletal muscles of mdx mice using three independent adeno-associated virus vectors carrying sequential parts of the coding sequence of the Duchenne muscular dystrophy gene . TS between the three vectors led to expression of the full-length protein. Although this method is an elegant way of full-length cDNA replacement, it does not provide the advantages of corrective TS. However, it shows the wide applicability of TS, and general aspects of RTM design will also be valid for this approach.

For the design of RTMs that target endogenous pre-mRNAs, there is not much scope regarding the composition of the sequence to be replaced, the spacer, and the splicing domain. However, the BD is very variable, and even slight variations can change the efficiency of the TS process significantly. Therefore, the characteristics of the targeted intron also have to be considered because longer introns provide the possibility of obtaining a library with high BD diversity. Finally, the inclusion of potentially existing isoforms in the TS process, or the exclusion of isoforms, has to be borne in mind. To select optimal BDs, applicable for the replacement of any gene and gene portion of interest, we developed a fluorescence-based screening system. For each targeted pre-mRNA, an artificial target and an RTM library is needed. RTM backbones are cloned, comprising a spacer, splicing domains, and respective parts (5′, 3′, or a central part) of a fluorescent reporter molecule (*e.g.*, green fluorescent protein), which mimics the endogenous

target. Adjacent to the spacer, a blunt-end restriction site facilitates the inclusion of randomly generated BDs. High diversity of BDs is obtained by the amplification of the target region and the random fragmentation (using either enzymatic or ultrasound processes) of the respective polymerase chain reaction product. Library backbones can be generated for any SMaRT approach, be it 5′ TS, 3′ TS, or IER. For a successful screen, a corresponding target molecule is also needed. The target molecule comprises the remaining part of the fluorescent molecule and the region with which the BD is supposed to hybridize. This region is usually composed of the last (for 5′ TS) or the first (for 3′ TS) exon incorporated in the coding region to be replaced and the following or preceding intron and exon (**Figure 1**).[10] On cotransfection, a full-length fluorescent molecule is generated. The intensity of the fluorescence, measured by fluorescence-activated cell sorting analysis, sheds light on the functionality of the RTM, which is directly related to the composition and efficiency of the BD. Regarding IER, we primarily recommend the evaluation of BDs for 5′ and 3′ TS separately before cloning of the most efficient BDs into a double-RNA RTM.

By using this fluorescence-based screening system, we were able to exemplify some common features of highly functional BDs. These are as follows: (i) The length and the GC content of the BD influences the stringency of the target binding. (ii) The binding position plays a crucial role in RTM– target interaction. Especially the binding close to the competitive donor (5′ TS) or acceptor (3′ TS) splice site, or that covering them, increases the TS efficiency.[6,7] As splicing enhancers and silencers can be found in introns and in exons, including an exon in the target region and covering the complete target exon—thereby blocking potential *cis*-splice regulators—can make TS have the edge over *cis*-splicing. These considerations proved true in cotransfection experiments and in an endogenous context.[7,8] For example, for the *PLEC* gene, most RTMs that were highly efficient in the screen partly or fully covered the targeted exon 9.[6] However, Lorain *et al.*[8] found that the most functional BD in their study hybridizes to a central intron target region.[8] Such exceptions from the rules underline the usefulness of performing RTM screens with randomized BDs, for each targeted gene.

On successful screening for an appropriate BD, this BD can be included in an RTM for the correction of the respective gene, *i.e.,* an endogenous RTM. Thereafter, the green fluorescent protein part is exchanged by the coding region to be replaced and can then be used for experiments on

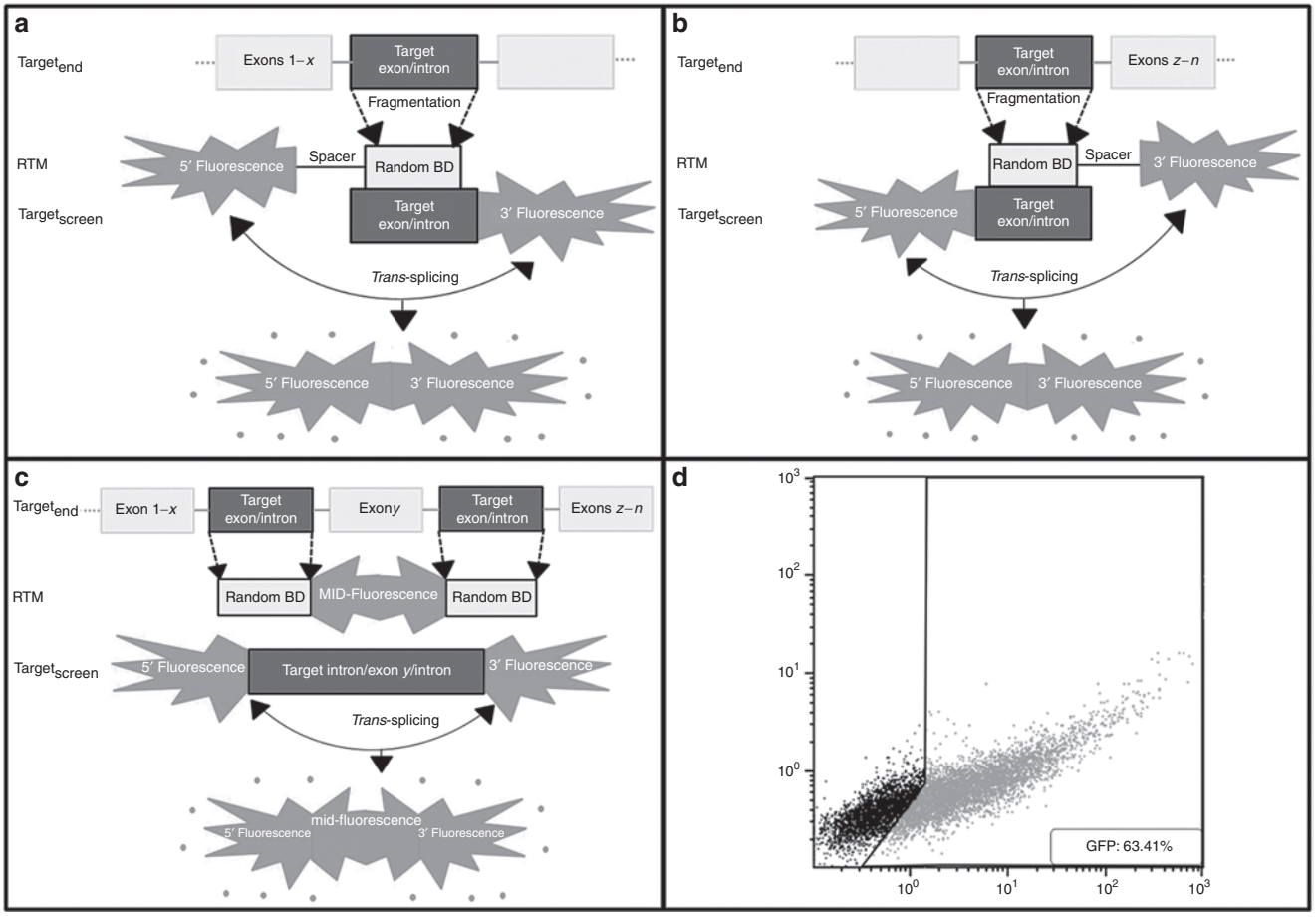

Figure 1 Schematic depiction of fluorescence-based RTM screening constructs. Schematic representation of (**a**) 3′ *trans*-splicing, (**b**) 5′ *trans*-splicing, and (**c**) internal exon replacement between GFP target and GFP–RTM constructs. (**d**) FACS analysis of cotransfected test cells expressing GFP after successful *trans*-splicing. BD, binding domain; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IER, internal exon replacement; RTM, RNA *trans*-splicing molecules.

patient cells. Moreover, we were able to confirm the advantage of blocking the competitive target pre-mRNA splice sites to enhance the RTM's functionality.[7] On viral transduction of an RTM carrying a BD selected from the fluorescence-based screen, which covers the intron 64/exon 65 junction of the *COL7A1* pre-mRNA, we saw a highly efficient correction of *COL7A1* in recessive dystrophic epidermolysis bullosa keratinocytes.[7,11] *COL7A1* correction was detectable on the mRNA and protein levels and, most importantly, within the basement membrane zone of three-dimensional skin equivalents generated from corrected recessive dystrophic epidermolysis bullosa cells.

Finally, the TS efficiency can also be enhanced with the help of antisense oligonucleotides capable of reducing competitive *cis*-splicing.[12] Moreover, the addition of a downstream intronic splice enhancer in the RTM can drastically enhance its repair efficiency.[8] A remaining issue is the risk of direct RTM expression due to in-frame termination codons (5′ TS) or start codons (3′ TS) within the BD or the vector backbone. A negative interference of a truncated, nonfunctional protein might create a dominant negative effect. Recent studies showed that a 3′ acceptor splice site within the RTM's splicing domain leads to alternative splicing events during the TS process. The result is an out-of-frame fusion of the RTM's coding sequence with the endogenous mRNA.[7] Although it is likely that a nonfunctional mRNA will not be translated, there is a potential risk of side effects remaining. This is of particularly high interest in the context of TS for suicide tumor therapy, where a toxin ("suicide" gene) is *trans*-spliced to a tumor marker transcript. The resulting fusion consists of the first exon of the marker gene, followed by an in-frame sequence of the toxin. This method provides the preferable expression of the toxin in tumor cells, and the reduction of tumor mass with minimal impact on other tissues. However, a significant decrease of background toxin expression and a concomitant maintenance of the RTM functionality is essential and can be achieved by the deletion of start codons within and upstream of the BD.[13] Furthermore, for 5′ RTMs, the deletion of the poly-A signal leads to a retention of the RTM in the nucleus and thus reduces its direct translation in the cytoplasm.[14] This is especially interesting because preliminary experiments on murine *CoL7a1* showed that this not only minimizes background expression but also leads to increased TS levels (data not shown). Finally, considerations regarding codon optimization of the gene portion to be replaced to provide highly efficient translation of the *trans*-spliced mature mRNA or the inclusion of splicing enhancers can contribute to the therapeutic success.[14,15]

Table 1 summarizes recommended steps and considerations for the development of highly functional RTMs.

To sum up, using RTM libraries, we were able to select highly efficient for the repair of mutations in numerous epidermolysis bullosa–associated pre-mRNAs, most of them encoding structural proteins within the basement membrane zone of the skin.

Table 1 Recommended steps for the design of a highly functional RTM

Steps to RTM design	Criteria
Selection of the *trans*-splicing strategy (5′, 3′, and IER)	• Distribution of mutations within the gene
	• Presence of hot-spot mutations
	• Presence of transcript isoforms
	• Size of mRNA transcript
Planning and preparation of the screen	• Preparation of library backbones
	• Choice of method for BD library cloning (enzymatic and ultrasound fragmentation of target region)
	• Selection of an appropriate cell line, which does not express the target mRNA
	• Cloning of appropriate target molecules
Selection of BDs	• Cotransfection of target and RTM library
	• Flow cytometer analysis and flow cytometer sorting of highly fluorescing cells
	• Isolation of RTMs from the sorted cells
	• Sequence analysis of the isolated BDs
	• Choice of BDs for endogenous *trans*-splicing after consideration of beneficial BD characteristics (*e.g.,* presence of cryptic splice sites and start/termination codons)
Design of endogenous RTM	• Exchange of reporter gene with coding sequence of targeted gene
	• Prediction and deletion of cryptic splice sites within RTM backbone and proximal vector sequences
	• Inclusion of silent mutations within RTM coding sequence to distinguish between *cis*- and *trans*-spliced transcripts
	• Use of strong splice elements
	• Use of strong promoter (if tissue-specific promoter is not necessary)
	• Codon optimization of RTM coding sequence
	• Inclusion of splicing enhancers within the RTM
Choice of vector for delivery	• Viral vectors: consider packaging capacities and safety. Retroviral and lentiviral vectors facilitate the packaging of large constructs (up to 10 kb). To avoid *cis*-splicing events during transduction, we recommend reverse complement cloning of the RTM. Adeno-associated virus vectors provide the advantage of the possibility of using tissue-specific serotypes, thereby increasing specificity. However, a packaging capacity of about 4 kb has to be considered.
	• Transient expression: consider the feasibility of nonintegrating expression vectors for your experiments.
	• Transposon systems

BD, binding domain; IER, internal exon replacement; mRNA, messenger RNA; RTM, RNA *trans*-splicing molecules.

Scientists who would like to use the fluorescence-based screening constructs are invited to e-mail Eva Murauer at e.murauer@salk.at or Ulrich Koller at u.koller@salk.at.

Acknowledgment. This work was supported by DEBRA Austria. The authors declared no conflict of interest.

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