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High-Throughput Screening for Highly Functional RNA-*Trans*-Splicing Molecules: Correction of Plectin in Epidermolysis Bullosa Simplex

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1. Introduction

Epidermolysis bullosa (EB) is a very heterogeneous hereditary disease of the skin and mucous membranes, characterized by erosions and blistering after minor traumatization. Up to now at least 12 genes are known to underlie EB, which render structural and mechanical stability of the skin (Fine et al., 2008). The characteristic blister formation occurs on the level of the basement membrane zone and the basal keratinocytes, depending on the gene, which is mutated. In EB simplex (EBS) the split formation in the skin occurs due to cytolysis of the basal keratinocytes, in junctional EB (JEB) within the lamina lucida and in dystrophic EB (DEB) on the dermal aspect of the basement membrane zone. Due to the clinical and genetic heterogeneity of this disease the development of a gene therapy is challenging, since every type of EB has to be targeted separately. Furthermore, the different subtypes differ in their mode of inheritance. Especially for basal EB simplex, where mutations in the keratin 5 (*K5*), keratin 14 (*K14*) and plectin (*PLEC*) genes may cause the clinical phenotype, dominant mutations are frequent. But also in dystrophic EB, where mutations in the collagen VII gene (*COL7A1*) are causative for the disease, dominantly inherited subtypes are known. Another characteristic feature of the involved genes is that many of them are very large and exceed the packaging capacities of commonly used (viral) vectors (e.g. *COL7A1*: ~9,3kb, *PLEC*: ~14.8kb). Functionally, all genes involved in EB have structural significance. Whereas keratin 5 and keratin 14 are major components of the cytoskeleton in basal keratinocytes, plectin is a cytolinker protein, connecting the actin filament network with the microtubules, the intermediate filaments and the hemidesmosomes and desmosomes. Isoforms of plectin are expressed in many cell types and always have "networking" functions. This is the reason why epidermolysis bullosa triggered by mutations in this gene goes along with muscular dystrophy and pyloric atresia. 12 plectin isoforms, differing in their 5' transcript portion have been identified up to now, most of them differing in their first exon. However, the structure and mode of function is comparable between all isoforms (Rezniczek et al., 2003). Structurally, plectin consists of two globular domains, flanking a central rod domain. The 5' globular domain is encoded by exons 1 to 30 and is over 4kb in length, whereas the rod is encoded by one single 3.38kb

exon. Plectin peptides form homodimers with a central coiled-coil of alpha helices and two globular domains. Within the globular domains the binding sites for its various binding partners are situated. Therefore, mutations in the rod cause mainly EBS with muscular dystrophy, whereas EBS with pylorus atresia is caused by mutations outside exon 31 (Natsuga et al., 2010). In this chapter we describe a *trans*-splicing based gene therapeutic approach on the example of epidermolysis bullosa simplex, caused by mutations in the *PLEC* gene. Based on the first proof-of-principle publication from 2008 we developed a screening method to correct patient fibroblasts with a high efficiency and specificity, rendering this approach a potential candidate for future *in vivo* studies.

2. Gene therapy in EB

For many devastating diseases gene therapy is the only hope for a curative intervention. Many efforts have been made in a spectrum of gene therapeutic strategies, resulting in a small range of gene therapeutic approaches available *in vitro*, each having its advantages and limitations.

A very straight-forward approach is gene supplementation therapy, in which a wildtype cDNA copy of a given mutated gene is brought into a cell to revert the phenotype. This gene complementation approach results in the rescue of the phenotype by providing the functional protein. Full-length cDNA therapy can be used for most recessively inherited diseases, where the transgene does not exceed the size of the packaging capacity of the vector of choice. In EB, cDNA therapy was already used for collagen 17 (*COL17A1*), laminin beta 3 (*LAMB3*) (Dellambra et al., 2000; Dellambra et al., 1998), integrin beta 4 (*ITGB4*) (Dellambra et al., 2001) and collagen 7 (*COL7A1*) (Siprashvili et al., 2010). In 2006, De Luca and his group applied *ex vivo* gene therapy for a patient suffering from recessive dystrophic EB for the first time (Mavilio et al., 2006). Patient's epidermal stem cells were transduced with a *LAMB3* expressing retrovirus. Expanded skin sheets were transplanted onto the anterior parts of the patient's legs, showing complete epidermal regeneration after eight weeks. Molecular analysis confirmed the correct integration of the *LAMB3* chain in the laminin-332 protein, rendering integrity to the transplanted skin section.

Even though great achievements were made also for other genes, cDNA therapy has limitations. In the case of the *LAMB3* approach described above, some case specific characteristics facilitated the success of this application. These were the autosomal recessive mode of inheritance, the limited size of the *LAMB3* coding region, the residual 5% gene expression of the endogenous *LAMB3* alleles (avoiding immune rejection) and the nature of LAMB3 in making part in a trimer, thereby avoiding an excess of the functional protein, as the amount of available laminin-332 is limited by laminin alpha 3 and laminin gamma 2. This last point is crucial as also overexpression of the transgene can have major impact on the therapeutic outcome.

For other genes the starting situation is different. Mutated genes can be very large or dominantly inherited, or they need strict expression control due to their negative interference when overexpressed. Quite contrary, it was found that low levels of functional protein can be sufficient to render a normal phenotype. For this reason cDNA therapy is often complemented with knockdown approaches like RNA interference (RNAi) or antisense oligonucleotides (ASOs) to knock down the mutated gene and minimizing an excess of the transcript.

But not only complementary, but also standing alone such alternative strategies were developed, many of them acting on RNA level. Therapy on RNA level has the advantage that cell targeting does not have to be as strict, since the interfering process can only take place in cells actively expressing the targeted gene. This also regulates the amount of interference, thus avoiding the overexpression of the respective gene. A widely applied approach is the use of interfering RNAs (RNAi). There, specifically designed short-hairpin RNAs (shRNAs) or short interfering RNAs (siRNA) are introduced into cells of interest. After processing, specific 21bp RNA fragments are incorporated in an RNA induced silencing complex (RISC), specifically hybridizing to an allele harbouring a mutation. This leads to the cleavage of the target mRNA (Siomi and Siomi, 2009).
For keratin 14, RNAi was used for characterization studies of EBS (Werner et al., 2004; Russell et al., 2009). However, the drawback of RNAi is that even though theoretically any gene and any mutation can be targeted, the risk of haploinsufficiency has to be considered. To meet this problem, rescue approaches are conducted by introducing the wildtype cDNA, leading to the limitations discussed above.
Another approach on RNA level is the use of ribozymes. Ribozymes are RNA molecules, which can specifically cleave themselves, a DNA or RNA molecule at a specific site, mediating degradation or ligation of yonder. The mostly used ribozymes are hammerhead ribozymes and group-I-intron self-splicing ribozymes. Both have recognition sites specifically hybridizing to a target mRNA, mediating cleavage or splicing. Group-I-self splicing ribozymes can be altered to introduce a desired cDNA portion to be *trans*-spliced 3′ of the endogenous target site. Besides the fact that recognition sites of ribozymes are rather short (~ 30nt for group-I-self splicing introns) and therefore increasing the probability of hybridizing unspecifically or off-target, only downstream gene portions can be replaced. For 5′ mRNA stretches this approach is not applicable. Regarding EB, McLean I and Terron A engineered three ribozymes mediating specific cleavage of EBS underlying keratin 14 (McLean & Terron, 2002). Cleavage of more than 90% of endogenous keratin 14 in cultured keratinocytes was achieved upon transfection. However, no differentiation between wildtype and mutated alleles was possible, resulting in a nearly complete loss of *K14* mRNA, necessitating rescue by ribozyme resistant wildtype keratin 14. Given the limitations of the above mentioned approaches we assume that Spliceosome Mediated RNA *Trans*-splicing (SMaRT) is a promising tool for gene therapy.

2.1 Spliceosome Mediated RNA *Trans*-Splicing

Spliceosome Mediated RNA *Trans*-Splicing (SMaRT) is a gene therapeutic approach, taking advantage of the cell's spliceosome to recombine two distinct pre-mRNAs to result in one mature mRNA. Pre-mRNA splicing is a naturally occurring process during mRNA maturation, which was first seen in trypanosomes, nematodes and recently also in humans (Murphy et al., 1986; Flouriot et al., 2002; Davis et al., 1995). During *trans*-splicing, the spliceosome ligates a 5′ exon from one pre-mRNA with a 3′ exon from another pre-mRNA, thereby producing an "alternative" mature mRNA, composed of exons derived from two different precursors. For SMaRT, this process is utilized to replace a disease causing gene portion by its wildtype copy. With this technology, any coding region of interest can be *trans*-spliced to any targeted, endogenous pre-mRNA (Puttaraju et al., 1999). Depending on the gene portion to be replaced, 5′ exon replacement, 3′ exon replacement and internal exon replacement (IER) are distinct (Figure 1).

Commonly, SMaRT is applied as a therapeutic tool, replacing gene portions harbouring a disease relevant mutation. Such settings were reported for epidermolysis bullosa (*COL7A1, KRT14, PLEC*) (Murauer et al., 2010; Wally et al., 2010; Wally et al., 2008), Duchenne muscular dystrophy (Lorain et al., 2010), cystic fibrosis (Liu et al., 2002; Song et al., 2009), frontotemporal dementia with parkinsonism (Rodriguez-Martin et al., 2009), severe combined immunodeficiency (Zayed et al., 2007), spinal muscular atrophy (Coady et al., 2007), sickle cell anemia and ß-thalassemia (Kierlin-Duncan and Sullenger, 2007). First *in vivo* assays showed the functionality of SMaRT in a mouse model of spinal muscular atrophy (Coady et al., 2008; Coady and Lorson, 2010). However, SMaRT has also been shown to be functional for a number of other approaches like *in vivo* imaging (Walls et al., 2008), antibody and therapeutic protein production (Wang et al., 2009; Iwasaki et al., 2009) and suicide therapy in squamous cell carcinoma (Gruber et al., 2011).

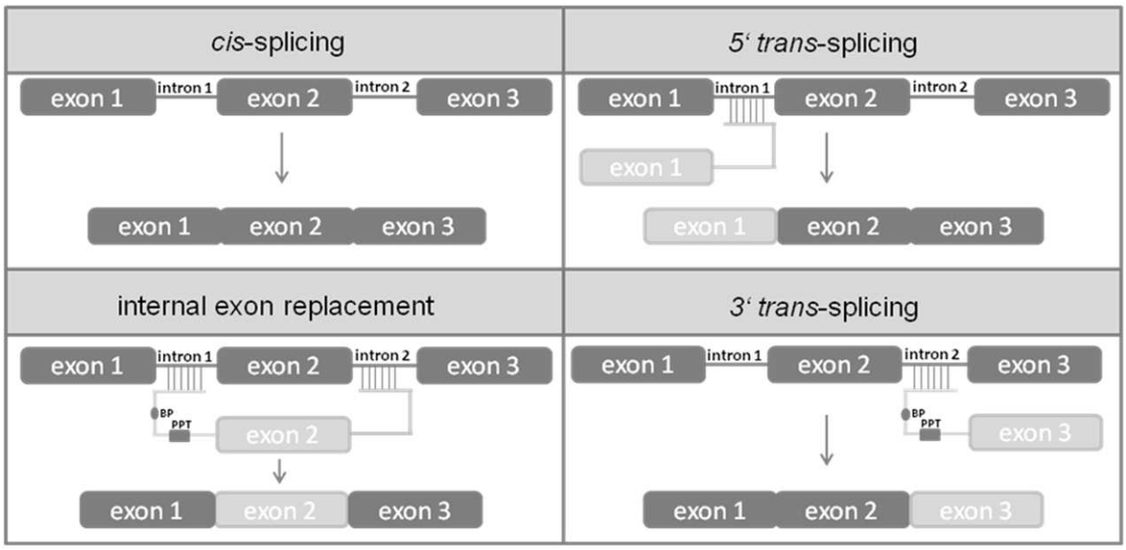

Fig. 1. RNA *trans*-splicing can be performed to replace one or more (a) 5′, (b) 3′ or (c) internal exons.

Technically, SMaRT is triggered by RNA-*trans*-splicing molecules (RTMs), which are specifically engineered for targeting a selected endogenous mRNA. Depending on the type of *trans*-splicing to be applied (5′, 3′ or IER), RTMs have to comprise certain features facilitating the *trans*-splicing process to take place. These are (a) The coding region to be replaced or integrated, (b) a hybridization/binding domain (BD), (c) a spacer region for sterical reasons and (d) splicing elements like a branch point (BP), polypyrimidine tract (PPT) and functional splice sites (SS). For 5′ *trans*-splicing the RTM comprises the 5′ (wildtype) coding sequence to be replaced, a spacer region and a binding domain. The binding domain hybridizes to the targeted intron, facilitating the generation of a mature mRNA mediated by the cell's spliceosome, comprising the 5′ exons from the RTM and the 3′ exons from the endogenous target. For 3′ *trans*-splicing a PPT and a BP have to be included for spliceosome assembly (for 5′ *trans*-splicing PPT and BP are provided by the endogenous target intron). Internal exon replacement is a combination of 5′ and 3′ *trans*-splicing (Figure 1).

Recent studies have shown that the binding domain is crucial for the efficiency, functionality and specificity of the replacement process. The binding domain is cloned

reverse and complementary and usually targets the intron adjacent to the most central exon to be replaced. Recent studies showed that slight variations in the sequence can render an RTM from a highly functional to a non-functional or very weak RTM. The mechanisms to consider when rationally designing a binding domain are not yet clearly understood.

3. SMaRT for EBS-MD

Correction of *PLEC* by spliceosome mediated RNA *trans*-splicing was first demonstrated 2008 (Wally et al., 2008). In brief, a rationally designed RTM was engineered, targeting intron 9 to replace the upstream coding sequence including exon 9 of the endogenous *PLEC* mRNA. EBS-MD patient fibroblasts, harbouring a 3bp insertion in exon 9 (1287ins3) (Bauer et al., 2001) were transduced with the RTM and *trans*-splicing was monitored on RNA level, protein level and by immunofluorescence microscopy. An increase of full-length plectin protein by 58,7% was detected in transfected fibroblasts, whereas untreated fibroblasts showed hardly any expression. Even though the whole amount of plectin was still less than in wildtype fibroblasts, characteristic cytoplasmatic plectin-specific staining was detected by immunofluorescence microscopy. On RNA level, semi-quantitative real-time PCR (SQRT-PCR) revealed an increase of 83,42% of plectin mRNA expression. The rationally designed RTM used in this study was designed to cover the 3' exon 9/intron 10 junction of the plectin pre-mRNA. Even though these results were promising, potential improvement by the variation of the binding domain was likely to be achieved. We therefore established a method to identify highly efficient binding domains for any gene of interest from a large pool of RTMs with randomly generated binding domains. From these libraries highly potent RTMs can be isolated and much information about the design of yonder can be obtained.

4. Principles of a fluorescence based RTM screen

The studies mentioned above showed that the binding domain is crucial for the efficiency and specificity of the *trans*-splicing process. Even minor variations in binding position, length and composition of the BDs result in significantly different efficiencies of the *trans*-splicing process. Rational design and evaluation of RTM binding domains can be an arduous process as there are no convincing criteria for the design of the binding domain. To simplify the process of RTM design we have developed a screening system that can be used to select the most efficient RTMs from a library containing a diversity of RTMs with complementary binding domains (BDs) for a certain target region of a pre-mRNA of interest. Using this method we can test a high number of RTMs for the efficiency of randomly generated binding domains. Binding domains are obtained by sonication or restriction digest of the targeted exonic and/or intronic region of the gene of interest. The resulting fragments have a length between 50 and 400bp (depending on the target region of choice) and are cloned into a fluorescence based RTM backbone. This random cloning results in the inclusion of one or more binding domains in a sense or antisense orientation, thereby resulting in about 50% of RTMs with a binding domain in a correct orientation for target hybridization, which is complementary. The vector backbone consists of a fluorescence reporter gene (e.g. dsRed) as transfection control and a 5' or 3' portion of a second fluorescence reporter gene (e.g. acGFP) respectively, mimicking the target gene portion to be *trans*-spliced to, therefore being the *trans*-splicing reporter. The generated RTM libraries are co-transfected with a corresponding target vector, harbouring the targeted full-

length exonic and/or intronic region and the respective 5′ or 3′ reporter gene portion. Crucial is the inclusion of functional splice sites at the junctions of the split fluorescence reporter to facilitate *trans*-splicing. Functional *trans*-splicing upon co-transfection results in cells double positive for both reporter genes. The ratio between the transfection control reporter and the *trans*-splicing reporter gives information about the quality of the RTM. Cells transfected by highly functional RTMs show a proportional transfection control expression (dsRed) in relation to the *trans*-splicing reporter (acGFP), whereas RTMs with low *trans*-splicing efficiencies show a high amount of cells expressing exclusively the transfection control and less cells expressing the *trans*-splicing product (i.e. acGFP) as well.

4.1 RTM library construction for plectin and screening for highly functional molecules

For plectin we cloned two 5′ *trans*-splicing libraries. *Trans*-splicing of the RTM to the target results in the restoration of the open reading frame of acGFP, leading to the expression of the full-length protein in RTM and target co-transfected cells. Based on the previously described correction of *PLEC* by 5′ *trans*-splicing (Wally et al., 2008) we selected the target region exon/intron 9 (199bp in length) of *PLEC,* using the acGFP split reporter and dsRed as reporter for RTM transfection.

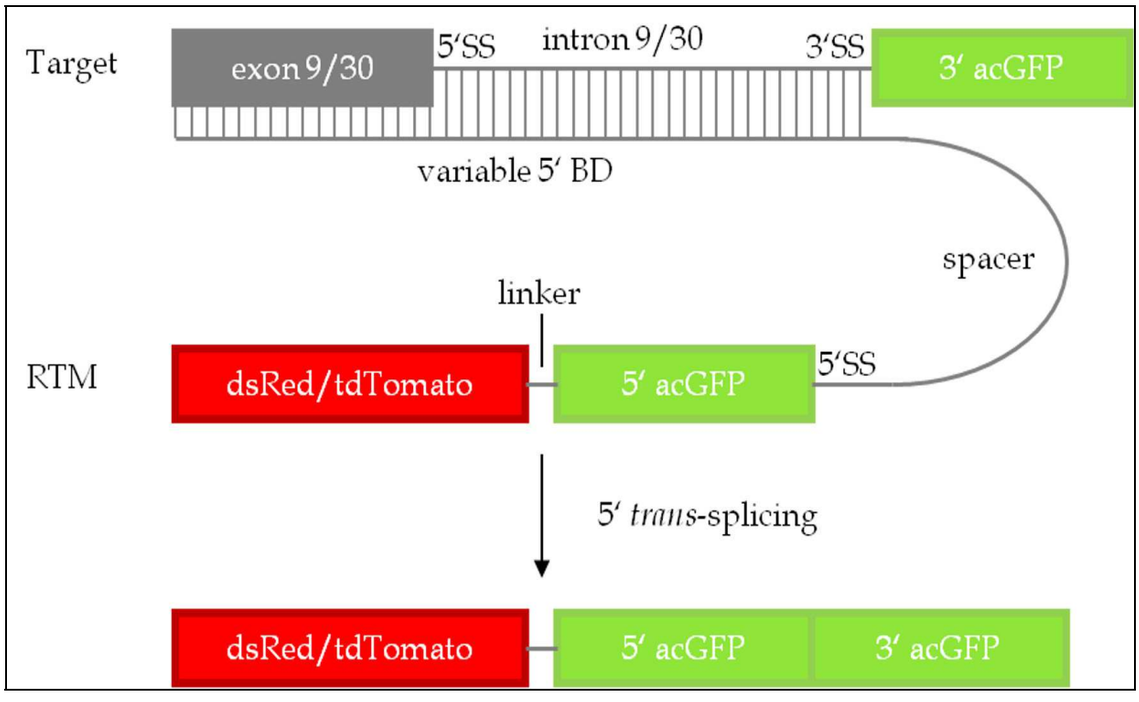

Fig. 2. Schematic depiction of the fluorescence based RTM screening system for the plectin gene.

To widen the spectrum of mutations included in the upstream coding region of *PLEC*, we constructed another library and its respective target molecule for exon/intron 30 (998bp in length) in parallel.

The RTM backbone for the exon/intron 30 specific BD library includes the more intense tdTomato instead of dsRed as RTM transfection control. The RTM screening system requires cells expressing an RTM and a plectin specific target molecule. Interaction between the RTM

and target pre-mRNAs by RNA *trans*-splicing leads to the fusion of both split parts of acGFP, thus restoring the full-length coding sequence and expression of acGFP. The target molecule harbors the 3′ acGFP part flanked by a 3′ splice site (SS) and the genomic region of *PLEC* (exon/intron 9 or 30). The RTM contains the 5′ portion of acGFP linked in frame with the reporter gene tdTomato (for exon/intron 30) or dsRed (exon/intron 9) respectively. The 5′ acGFP part is flanked by a functional 5′SS, a short spacer sequence and a binding domain randomly created by fragmenting the *PLEC* target region (Figure 2).

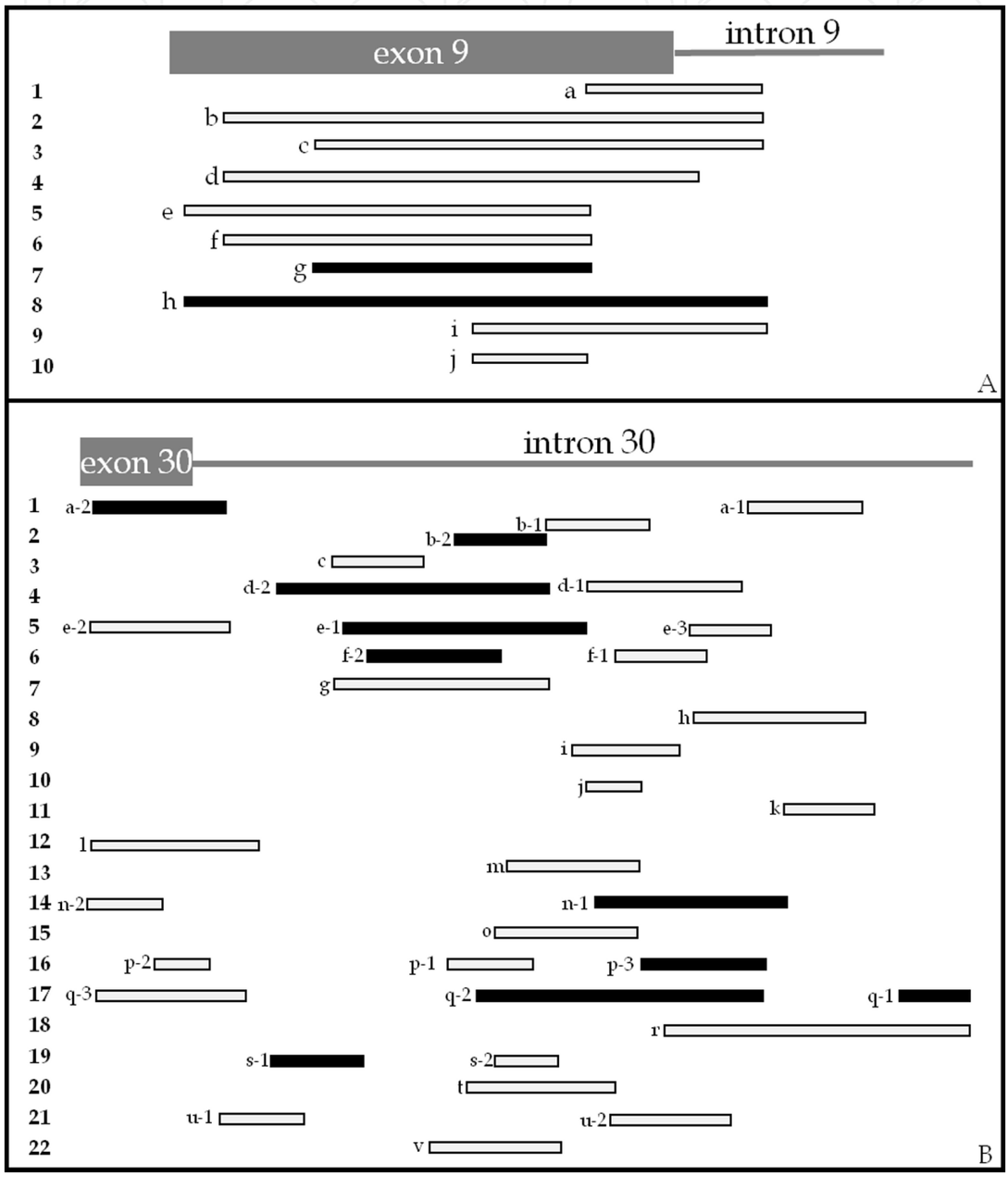

Fig. 3. Composition of the binding domains of all RTMs tested for exon/intron 9 (A) and exon/intron 30 (B).

The 5′ *trans*-splicing arm of the RTM backbone, downstream of the 5′ portion of acGFP, contains a donor (5′) splice site, a short spacer region and a random binding domain created by fragmentation of the respective *PLEC* target region. A high diversity of RTMs with different binding properties to the targeted exon/intron regions was constructed either by sonication (exon/intron 30) or CviJI* digestion (exon/intron 9) of the PCR amplified target region. CviJI* produces blunt end DNA fragments, cleaving between a G and a C of the sequence 5′-PuGCPy-3′. The randomized CviJI* digested, as well as the blunt end repaired sonicated fragments were cloned into the RTM backbone. To show the robustness of the screening system, 5′ BD libraries consisting of about 10^2 to 10^4 individual clones were constructed and several randomly picked clones were sequenced to select those with the correct (complementary) orientation. 10 RTMs from the exon/intron 9 library (Figure 3A) and 22 RTMs from the exon/intron 30 library (Figure 3B) harboring one or more parallel and/or complementary binding domains were tested for their *trans*-splicing efficiency, quantifying GFP expression by semi-quantitative real time PCR (SQRT-PCR) on mRNA level (for exon/intron 30 specific RTMs) and by flow cytometry on protein level (exon/intron 9 and 30 specific RTMs).
For all RTMs (1-10 for ex/in9 and 1-22 for ex/in 30) the binding domains were characterized and mapped (Figure 3). BDs in a complementary orientation (white bars) as well as BDs in a parallel orientation (black bars) were identified. Some RTMs had more than one BD included. BDs were named a to j and a to v respectively. Numbers next to the alphabetic characters indicate their order from 5′ to 3′.

4.1.1 Screening for efficient 5' RTMs targeting exon/intron 9 of the plectin gene

Ten individual RTMs from a 5′ BD library were selected to show the potential and robustness of the RTM screening system. Eight RTMs contain complementary BDs to the target region exon/intron 9, inducing the *trans*-splicing reaction between target and RTM pre-mRNAs. RTMs 7 and 8 harbor a BD parallel to the target region and are therefore included as negative controls in the experiments. Co-transfection of target expression plasmids along with either one of the ten RTMs resulted in a diverse expression profile of dsRed and acGFP (Figure 4). Weak RTMs (1, 7-10) show only low expression levels of dsRED - acGFP protein in comparison to the highly efficient RTMs 2-6. RTMs 7 and 8, harboring parallel BDs, produce a background expression of acGFP in less than 1% of all analyzed cells. The most efficient RTMs 2-6 show functional acGFP expression in 44-67% of all treated cells with a geometric mean of acGFP expression ranging from 6 (RTM 3) to 8 (RTM 6) (Figure 4B). These values reflect the average acGFP expression of all analyzed GFP positive cells. Geometric mean calculations after flow cytometric analysis were performed using the FlowJo software (Treestar). Flow cytometric analysis of RTM 6, 7 and 9 transfected HEK293AD cells show a significant difference in the amount of acGFP expression and fluorescence intensity (Figure 4A). RTM 6 contains a complementary BD, binding exon 9 only, but close to the exon/intron junction, maybe influencing the 5′ splice site on the target molecule. This might be the reason why RTM 6 is significantly more efficient in comparison to RTM 7 or RTM 9, harboring a parallel (RTM 7) or a short antisense BD (RTM 9). No acGFP expression was detected in HEK293AD cells transfected with either target or RTM alone. As the binding position of RTMs 2-6 on the target molecule is similar, there was not much difference in reporter gene expression. Masking almost the whole exon 9 with complementary BDs seems to increase the *trans*-splicing efficiency significantly. RTMs 1, 9 and 10 contain either short BDs specific for a short stretch of the target exon (RTM 10) or for

a short portion containing the 5' exon/intron boundary (RTM 1 and 9). These RTMs induced acGFP expression in less than 14% of transfected cells. Additionally, the intensity of acGFP expression was much lower (Figure 4).

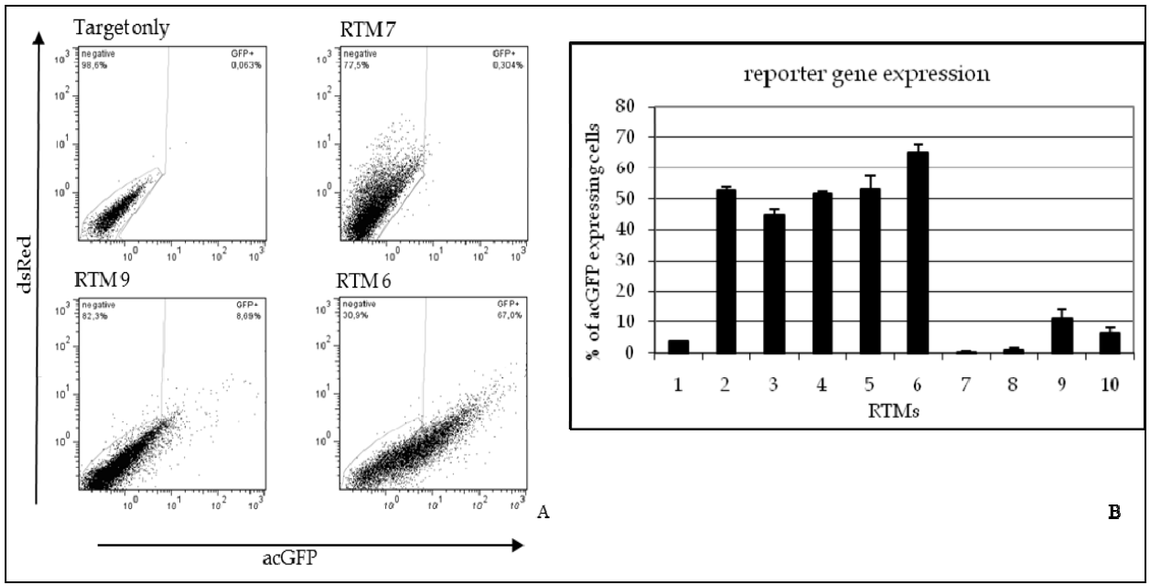

Fig. 4. Exon/intron 9: Expression of the fluorescence reporter genes acGFP and dsRed and the percentage of acGFP positive cells.

4.1.2 Screening for efficient 5' RTMs targeting exon / intron 30 of the plectin gene

The same experiments as described for exon/intron 9 were conducted for exon/intron 30. This intron was chosen to cover a maximum 5' *PLEC* gene portion and thereby including more known mutations. Again, flow cytometric analysis was performed with HEK293AD cells co-transfected with RTM and target plasmids. For each RTM two independent co-transfections were performed. Two days after co-transfection, the subset of acGFP expressing cells and the expression level of acGFP was quantified by flow cytometry. The number of acGFP positive cells and the relative amount of acGFP expressed reflect the functionality of RTMs and their respective binding domains. Between 5.10^3-2.10^4 HEK293AD cells were analyzed for each transfection.

After transfecting each of the 22 RTMs, harboring at least one complementary binding domain, together with the target molecule into HEK293AD cells, flow cytometry was performed. The geometric mean of acGFP expression ranged from 1.3 to 3.2 according to the RTM introduced into the target cells. The highly efficient RTMs 12, 13 and 17 showed acGFP expression in over 20% of all transfected cells and had a calculated geometric mean of over 2.6. RTMs with a weak *trans*-splicing efficiency (e.g. RTMs 1, 4, 11) showed a high amount of cells expressing the RTM transfection control gene tdTomato only and less cells expressing acGFP as well (Figure 5B). By setting the highly efficient RTMs 5, 12 and 17 in relation to the weak RTM 22, significant differences in their reporter gene expression were observed (Figure 5A). RTMs 5, 12 and 17 produced significantly higher levels of acGFP. RTM 22 generated only a background expression of acGFP in about 1% of analyzed HEK293AD cells.

Besides flow cytometry, all 22 RTMs with BDs ranging from 57 to 355 nucleotides, complementary to essentially all regions of the exon 30/intron 30 target (Figure 3B), were

individually tested for *trans*-splicing induced restoration of acGFP expression by SQRT-PCR. A *PLEC* specific 5′ *trans*-splicing RTM is able to recombine with a target pre-mRNA by a specific 5′ *trans*-splicing reaction. Thus the exon on the target molecule is replaced by the 5′ part of acGFP, leading to the expression of the reporter molecule acGFP in RTM treated cells. *Trans*-splicing between the RTM and target molecule was detected by semi-quantitative real-time PCR (SQRT-PCR). For that, an acGFP specific primer pair was used to quantify the amount of full-length acGFP transcripts present in HEK293AD cells co-transfected with the target molecule plasmid harboring exon/intron 30 of *PLEC* along with either one of the 22 RTMs (Figure 6). This demonstrates that the acceptor (3′) and donor (5′) splice sites on the target molecule and the RTM were recognized by the endogenous splicing machinery and that both acGFP parts were connected by 5′ RNA *trans*-splicing, leading to full-length acGFP expression. By analyzing the amount of expressed acGFP transcripts in co-transfected HEK293AD cells, the influence of different RTM binding domains on *trans*-splicing efficiency can be shown on mRNA level. As a reference gene, GAPDH was used. For the evaluation of the results all RTMs were referred to the weakest RTM 11 (set to 1), producing a low level of acGFP. As shown in Figure 6, RTMs 5, 12 and 17, accomplishing the exon/intron boundary of the target molecule by their complementary BDs, showed a high *trans*-splicing efficiency. RTM 12 achieved an up to 90 fold expression of acGFP transcripts in comparison to RTM 11. Masking the 5′ splice site on the target molecule may direct the ratio of the splicing reactions from *cis* to *trans,* since the binding of splicing factors might be disturbed.

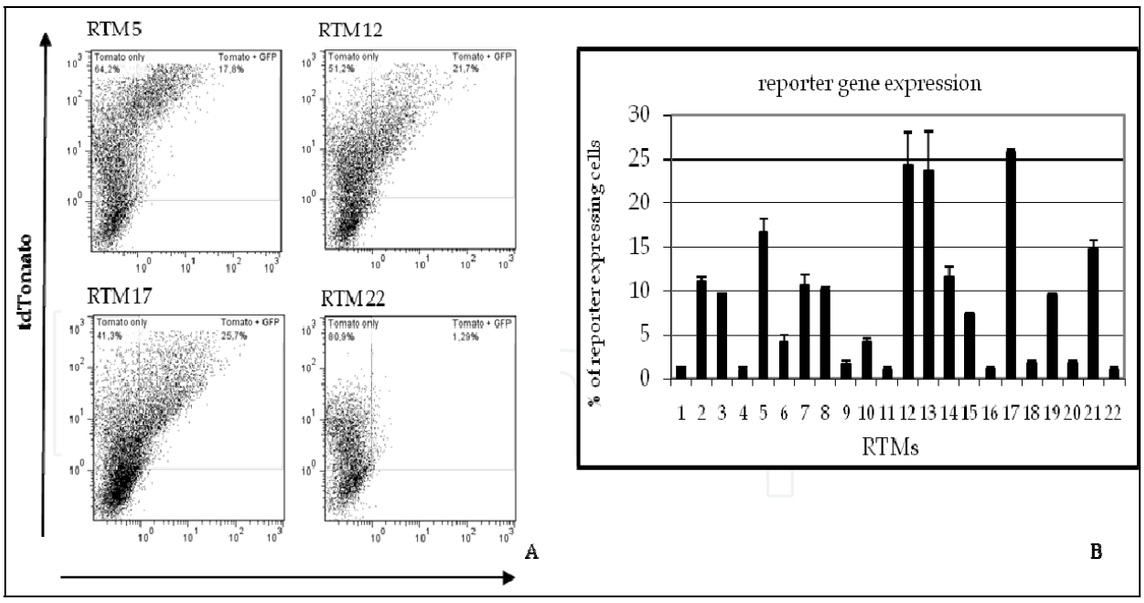

Fig. 5. Exon/intron 30: Expression of the fluorescence reporters acGFP : tdTomato was analyzed using flow cytometry (A) Four RTMs with different BDs show varying ratios of acGFP : tdTomato (B) The percentage of acGFP positive cells is summarized for all RTMs tested.

The data created by flow cytometric analysis (Figure 5) correlates with those obtained from semi-quantitative real-time PCR (SQRT-PCR). RTMs 5, 12 and 17, hybridizing to the exon/intron junction of the target molecule showed high *trans*-splicing efficiencies on mRNA (SQRT-PCR) and protein (flow cytometry) level, indicating the reliability of the RTM screening system. Since the highly efficient RTMs 5, 12 and 17 have similar complementary binding domains (exon/intron junction of the target molecule), this region seems to be the ideal binding position for efficient RNA *trans*-splicing in the case of the *PLEC* gene region exon/intron 30. The blockage of the exon/intron boundary of the target molecule, accomplished by these RTMs, may interfere with recognition of the exon 30 5′ splice site and facilitate *trans*-splicing.

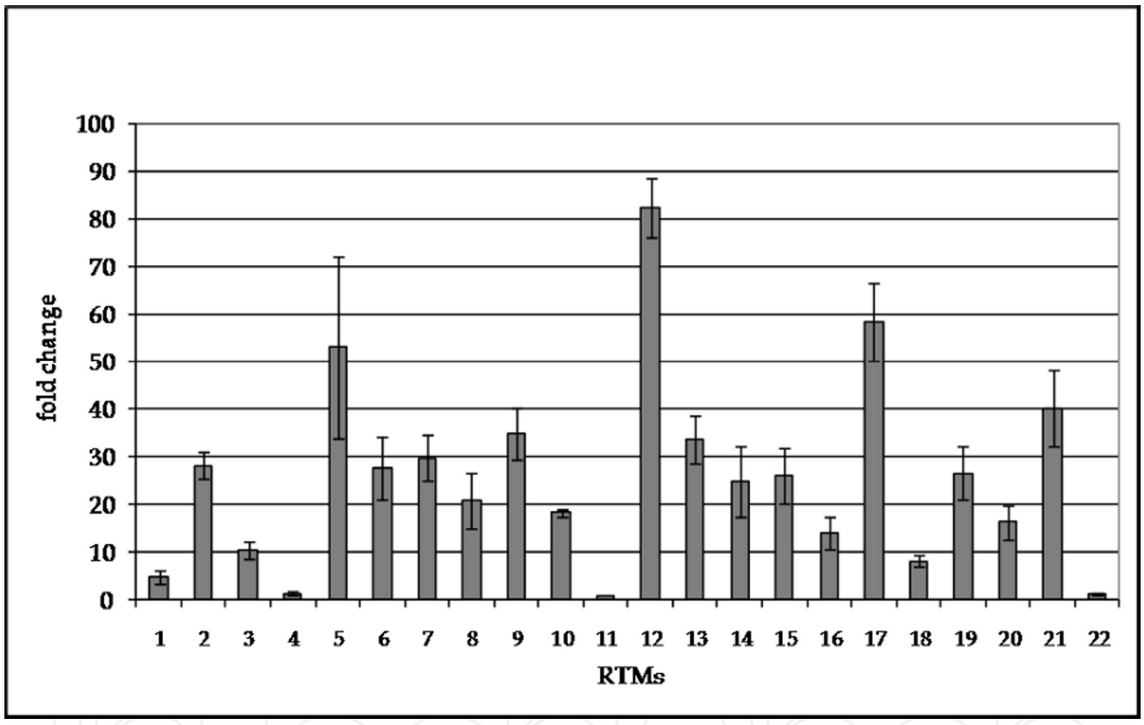

Fig. 6. Exon/intron 30: acGFP expression on mRNA level.

4.2 *Trans*-splicing with endogenous *PLEC* transcripts

One day after transfection of single RTMs into HEK293AD cells, endogenous *trans*-splicing was detected by RT-PCR. Specific *trans*-splicing between the RTM and the endogenous target pre-mRNA of *PLEC* resulted in the fusion of the 5′ portion of acGFP to exon 10 or 31 of *PLEC*. The fusion mRNA was amplified by including an acGFP specific forward primer and an exon 10 or 31 specific reverse primer into the polymerase chain reaction. After gel electrophoresis the 5′acGFP-exon 10/31 *PLEC* fusion PCR product was visible on an agarose gel at a size of 413bp and 542bp respectively (Figure 7). The DNA band was gel purified and sequenced. All RTMs introduced into HEK293AD cells (RTMs specific for exon/intron 9: 1-6, RTMs specific for exon/intron 30: 7, 12, 13), induced endogenous 5′ *trans*-splicing.

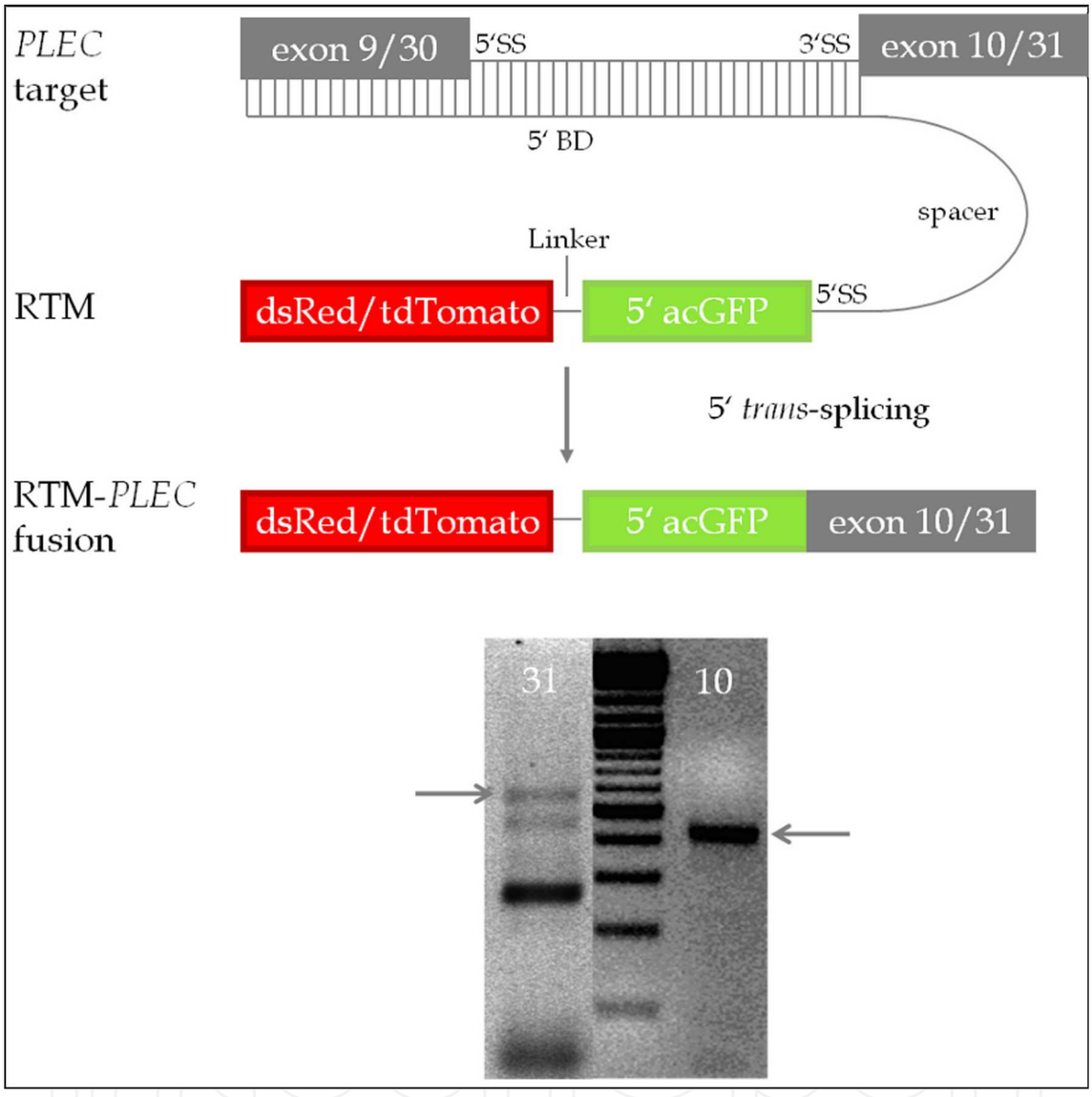

Fig. 7. Single transfection of selected RTMs (exon/intron 9 or 30 RTM screen) into HEK293AD cells resulted in the fusion of the 5′ portion of acGFP of the RTM to the respective endogenous target exon of *PLEC* (exon 10 or exon 31) on pre-mRNA level.

5. Discussion

During the last years it was shown that SMaRT is a functional tool for the correction of disease causing mutations of many genes on mRNA level. Several approaches in different settings (i.e. reporter gene based, endogenous *trans*-splicing, *in vivo* application) have

corroborated this assumption (Dallinger et al., 2003; Coady and Lorson, 2010; Wally et al., 2010). Also the applicability of SMaRT beyond mRNA repair was shown, using it for *in vivo* imaging and antibody and protein production. For the latter, therapeutic protein encoding sequences were *trans*-spliced to the highly abundant albumin mRNA, resulting in the expression of the therapeutic molecule (Wang et al., 2009). For epidermolysis bullosa, SMaRT was applied for a number of underlying genes, including *PLEC* (Wally et al., 2008), *COL7A1* (Murauer et al., 2010), *K14* (Wally et al., 2010) and *COL17A1* (our unpublished results). Whereas for *PLEC* and *COL7A1* the binding domains of the RNA–*trans*-splicing molecules (RTMs) were designed empirically, based on preliminary data, the binding domains for *K14* and *COL17A1* resulted from the evaluation of a number of randomly cloned BDs. Using a fluorescence based screening system, we saw that binding domains are crucial for the efficiency and specificity of the *trans*-splicing process. In this reporter-based assay, BDs were tested independently from the influence of gene characteristics and variations in splicing domains. This facilitated a direct comparison of the BDs, revealing a high impact of (a) localization within the targeted exon/intron, (b) composition and (c) length. Comparing obtained data, hints on principles for BD design were extracted. These are: (a) highly functional BDs have a length between 90 and 170bp, (b) masking of the competitive exon/intron junction is mostly beneficial, (c) targeting of a large intron results in more potential and diverse *trans*-splicing results since more binding sites are available. Furthermore, these facts underlay a hierarchy, with length ranging before splice site masking. Best BDs identified in the exon/intron 30 screen had a length of around 140 - 170bp AND masked the respective target splice site. An optimal target intron is not always available, restricting optimization in this aspect. However, screening for BDs also revealed exceptions from the rule. For example binding domains from RTMs 5 and 6 for exon/intron 9 exclusively bind within the exon. Even though we know that also splicing domains and spacer sequences influence *trans*-splicing success, regarding the BDs, it is not yet clearly understood what further influences *trans*-splicing efficiency and specificity. The first published BD for *PLEC* (Wally et al., 2008) masked the target splice site to be used for *trans*-splicing, resulting in reasonable *trans*-splicing rates. However, we still recommend using the fluorescence based screening system, as this is rather facile as soon as vector backbones are cloned. Backbones can be used for any gene of interest and any targeted intron. Within the *K14* gene, the applicability of SMaRT for dominant diseases was shown. The concomitant knock-down of the mutated and the increase of the wildtype allele multiply the effect of correction. Even though endogenous efficiencies are not high level, *trans*-splicing rates can be enough to revert a disease phenotype. Werner et al. showed in an *in vitro* model that dominance is limited and depends on the ratio of wildtype versus mutated *K14* molecules (Werner et al., 2004). Also, for dominant *COL7A1* mutations, overexpression of the wildtype protein can rescue the RDEB phenotype (Fritsch et al., 2009). Finally, Cao et al. showed that dominance is dependent on a certain ratio of wildtype to mutant alleles; in their *in vivo* mouse model a neomycin-resistance gene inserted in intron 1 of *K14* led to a 50% reduction of the mutated *K14* expression, resulting in phenotypically normal pups which showed no blistering (Cao et al., 2001). In view of this ameliorative effect, therapeutic approaches able to reduce the expression of a mutant gene product by 50% could be sufficient. Functional assays with RTM treated patient cells showed a transition of migratory behaviour and invasiveness versus wildtype in respective tests.

An improvement of the screen will be the sorting of analysed cells, resulting in a faster and more restricted identification of highly functional RTMs. Isolation of cells showing a certain

ratio of the two fluorescence reporters expressed will increase the number of potential binding domains characterized. Single cell expansion and plasmid extraction will provide highly potential BDs to integrate into an endogenous setting of spliceosome mediated RNA *trans*-splicing.

The development of an RTM selection system has a high impact on *trans*-splicing efficiency and specificity. Further improvements can be made regarding codon usage, nuclear retention signals, promoter selection and mode of delivery. Inclusion of nuclear retention signals might have great influence on the *trans*-splicing efficiency, but also regarding minimization of side effects and background expression of unspliced RTMs. Codon optimization and the use of weaker promoters and enhancers can be used to increase the safety of retrovirally introduced RTMs (Baum and Schambach, 2011; Fath et al., 2011).

Currently, a limiting fact is the lack of mouse models meeting the exigencies of a SMaRT approach. Many mouse models are generated by the inclusion of intron-free genes, making them unfeasible *trans*-splicing experiments. However, mouse models are becoming easier available and creatable. This will pave the way to *in vivo* studies as the basis for clinical trials.

6. Prospects and visions

Bringing spliceosome mediated RNA *trans*-splicing toward clinics is the aim of presumably all conducted studies for mRNA correction. This technology also gives hope to patients suffering from EB subtypes which cannot be treated by commonly used approaches like full-length cDNA based therapy. Patients regarding this have alterations in very large genes like *PLEC*, *COL7A1* and *COL17A1*, and also those harbouring mutations in dominantly inherited genes, like *K14* and *K5*. The next step will be to show *in vivo*, that *trans*-splicing can convert a disease phenotype to wildtype. The generation of viable, intron-posessing mouse models is a task that is being gone about currently. Looking further, *ex vivo* gene therapy is the currently most favoured approach for epidermolysis bullosa patients. Transplantation of skin sheets derived from autologous epidermal stem cells that were transduced with a respective corrective molecule is the current state of the art. This approach was successfully conducted for full-length cDNA therapy in junctional epidermolysis bullosa in 2006 (Mavilio et al., 2006). There, a 50cm² area was replaced on the patient legs. Even five years after transplantation the transplanted areas have not shown any blisters at all and are indistinguishable from healthy skin (De Luca, personal communication).

However, systemic application of SMaRT is the technology to go for, which is not available yet. Delivery of (any) gene therapeutic advice is a problem common to all approaches. Increasing the safety of lenti- and retroviral vectors is being pushed forward with good results. Also the use of transposons like sleeping beauty (Hackett et al., 2010) and piggyBack (Yusa et al., 2011) are promising tools. Finally, targeting is another challenge to be overcome. However, in this case SMaRT has the advantage of being functional only in cells expressing the target gene, therefore minimizing off-target effects in other cell types.

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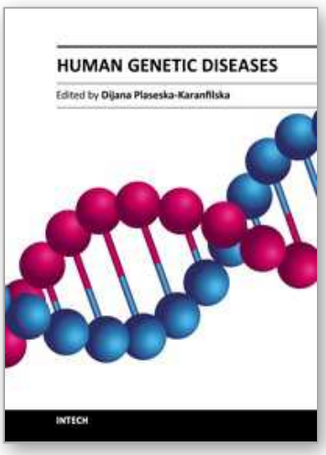

Human Genetic Diseases

Edited by Dr. Dijana Plaseska-Karanfilska

ISBN 978-953-307-936-3
Hard cover, 286 pages
Publisher InTech
Published online 30, September, 2011
Published in print edition September, 2011

The genetics science is less than 150 years old, but its accomplishments have been astonishing. Genetics has become an indispensable component of almost all research in modern biology and medicine. Human genetic variation is associated with many, if not all, human diseases and disabilities. Nowadays, studies investigating any biological process, from the molecular level to the population level, use the â€œgenetic approachâ€ to gain understanding of that process. This book contains many diverse chapters, dealing with human genetic diseases, methods to diagnose them, novel approaches to treat them and molecular approaches and concepts to understand them. Although this book does not give a comprehensive overview of human genetic diseases, I believe that the sixteen book chapters will be a valuable resource for researchers and students in different life and medical sciences.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Verena Wally, Ulrich Koller and Johann W. Bauer (2011). High-Throughput Screening for Highly Functional RNA-Trans-Splicing Molecules: Correction of Plectin in Epidermolysis Bullosa Simplex, Human Genetic Diseases, Dr. Dijana Plaseska-Karanfilska (Ed.), ISBN: 978-953-307-936-3, InTech, Available from: http://www.intechopen.com/books/human-genetic-diseases/high-throughput-screening-for-highly-functional-rna-trans-splicing-molecules-correction-of-plectin-i

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