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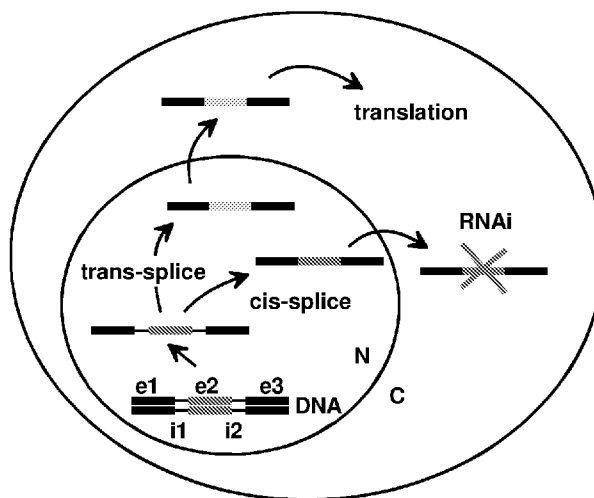
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(54) Title: FUNCTIONAL GENE REPLACEMENT THERAPY

Figure 2



(57) Abstract: The invention concerns a method for effecting functional gene replacement therapy using a combination of RNA *trans*-splicing and RNAi; at least one vector and/or at least one nucleic acid molecule for use in said method; and a therapeutic or pharmaceutical composition also for use in said method including said at least one vector and/or at least one nucleic acid molecule.

Functional Gene Replacement Therapy

Field of the Invention

The invention concerns a method for effecting functional gene replacement therapy; at least one vector and/or at least one nucleic acid molecule for use in said method; and a therapeutic or pharmaceutical composition also for use in said method including said at least one vector and/or at least one nucleic acid molecule. The invention has use in the medical and veterinary field.

Background of the Invention

Acquired and/or inherited genetic disorders are often characterized by the presence of defective or aberrant gene transcripts and gene products which can be caused by abnormalities in chromosomes, genes, and gene expression. Among others these disorders include cancers, storage disorders, asthma, diabetes, mental retardation, obesity, heart disease, autoimmune diseases, and infections with viruses such as HIV and HPV which integrate their genome into the host's genome. These disorders currently are treated symptomatically i.e. by administration of drugs which treat the symptoms rather than the genetic causes of the diseases although it is acknowledged that a causal treatment, i.e. one that addresses the cause of the disorder, is the only chance of a cure. A causal treatment implies the usage of genetic tools suitable to resolve the genetic defects.

One conventional gene therapy approach is based on complementing a defect gene function with an intact gene function. Alternatively, toxic or carcinogenic gene functions are preferably targeted with antagonistic drugs or RNA interference (RNAi). Whereas the former functional complementation implies the coexistence of the correct and the defective gene function in the same cell, the blocking of a defective function using RNAi results in functional loss. Thus, both of these approaches yield suboptimal results.

RNA *trans*-splicing, on the other hand, allows one to repair genetic defects at the RNA level by replacing a defect with an intact function (Figure 1). *Trans*-splicing is a special form of RNA processing in eukaryotes where exons from two different primary RNA transcripts are joined end to end and ligated. In other words, *trans*-splicing results in an RNA transcript that comes from multiple RNA polymerases on the genome. Thus, as opposed to cellular *cis*-splicing which occurs within one pre-mRNA transcript, *trans*-splicing describes splice reactions between two different pre-mRNAs.

In eukaryotes RNA *trans*-splicing is undertaken by the spliceosome which is a large and complex molecular machine composed of five small nuclear RNAs (snRNA), and a range of associated protein factors. The spliceosome removes introns from a transcribed pre-mRNA segment. This process is generally referred to as splicing. Spliceosome-mediated RNA *trans*

-splicing, in the following referred to as RNA *trans*-splicing or *trans*-splicing, is a gene therapy approach that uses a cell's spliceosome to combine two distinct pre-mRNAs to produce a chimeric mature mRNA. Thus *trans*-splicing typically replaces a disease causing gene portion with a wild-type portion.

RNA *trans*-splicing represents a technology that is able to repair defective gene expression at the level of precursor messenger RNA (pre-mRNA); without any need to interfere with genomic DNA [1-3]. *Trans*-splicing may occur naturally, however for therapeutic purposes an artificial *trans*-splicing RNA (tsRNA) is created to target a deleterious cellular pre-mRNA. A *trans*-splicing RNA is composed of three functional domains: (i) an antisense binding domain that is specific for the respective target message, (ii) a coding domain expressing a recombinant therapeutic gene or exon, and (iii) a splicing domain that includes all functional sequences required for spliceosomal splicing such as splice sites, a branch point, a polypyrimidine tract, splice enhancers etc. Depending on the design of the *trans*-splicing RNA, RNA *trans*-splicing can be used for 5' terminal (5'ER), internal (iER), or 3' terminal (3'ER) exon replacement. The *trans*-splicing technology allows molecular-surgical repair of defective gene functions and, hence, *trans*-splicing has high therapeutic potential compared with RNAi technology.

RNA interference (RNAi) also called post transcriptional gene silencing (PTGS) represents an evolutionary conserved mechanism of post-transcriptional gene silencing in higher eukaryotes including mammals and humans [4-6]. RNAi is undertaken by microRNAs (miRNAs), siRNAs, shRNAs, and piRNAs any of which can be endogenously expressed within or exogenously delivered into the target cells. Endogenous expression usually starts with the transcription of nuclear precursor molecules which are then exported into the cytoplasm where they are processed to finally trigger the formation of RNA-induced silencing complexes (RISC). RISC contains the so-called guide RNA sequence and provided that is complementary to a messenger RNA target the RISC can trigger target gene knockdown.

Despite the appeal of *trans*-splicing technology, so far, studies in mice indicate few *trans*-splicing activities are suitable for only a few disease phenotypes. One reason for low observed *trans*-splicing rates is based on the fact that intermolecular *trans*-splicing has to compete with intramolecular *cis*-splicing reactions. As a corollary, a substantial fraction of the target message is still spliced in *cis*, exported to the cytoplasm and translated into the aberrant gene function. Many aberrant gene products have carcinogenic or toxic activities. In these cases, we have considered a successful therapy requires both restoration of the defective gene function and quantitative elimination of the deleterious phenotype.

10 Statements of the Invention

According to a first aspect of the invention there is provided a method of gene therapy comprising:

- a) delivering into a target cell, at least one *trans*-splicing RNA molecule having
 - i) at least one sequence of nucleotides complementary to a target nuclear transcript comprising pre-mRNA;
 - ii) at least one wild-type therapeutic gene or exon or at least one gene or exon encoding an apoptotic/death signal; and
 - iii) at least one functional sequence required for spliceosomal splicing; and
- b) delivering into said same target cell, at least one RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of said target nuclear transcript; whereby
- c) *trans*-splicing of said pre-mRNA by said *trans*-splicing RNA molecule takes place to restore the function of a protein encoded thereby or to trigger death of the transcript or its product; and
- d) RNA interference of said cellular version of said target nuclear transcript takes place to prevent the function of a protein encoded thereby or to support the death of the pre-mRNA transcript or its product.

Reference herein to a *trans*-splicing RNA molecule is a molecule that interacts with a target precursor mRNA molecule and mediates a *trans*-splicing event to generate a novel chimeric mRNA that can be processed in the cell to yield a protein product.

In a preferred method said target nuclear transcript encodes a defective gene product, typically a defective protein and so said target nuclear transcript is a target defective nuclear transcript.

Reference herein to a target defective nuclear transcript is reference to a transcript of a defective gene which transcript carries or encodes the said defect whereby a defective gene protein product is ultimately produced following cellular translation.

- 5 Reference herein to a cellular version of said target nuclear transcript, defective or otherwise, is reference to the same or a modified version of said target (defective) nuclear transcript when outside the cell nucleus.

10 In a preferred method of the invention said molecules are delivered to said target cell using conventional delivery technologies well known to those skilled in the art including but not restricted to transfection, lipofection, electroporation, nucleofection, jet-injection, gene gun, and needle injection.

15 In another preferred method of the invention said molecules are delivered to said target cell using conventional delivery routes well known to those skilled in the art including but not restricted to topical, intravenous, intramuscular, oral, cutaneous, subcutaneous, intraperitoneal, nasal, intra-tracheal, systemic, and intratumoral.

20 Additionally or alternatively, said delivery is undertaken using a viral vector that encodes said *trans*-splicing RNA molecule or RNAi molecule and that can transfect or transform said cell and in either case said vector is suitably equipped to ensure the *trans*-splicing RNA molecule or RNAi molecule is manufactured in said cell following transfection or transformation.

25 Additionally or alternatively, said delivery is undertaken using a viral vector and/or plasmid that encodes said *trans*-splicing RNA molecule or RNAi molecule and that can transfect or transform said cell and in either case said vector is suitably equipped to ensure the *trans*-splicing RNA molecule or RNAi molecule is manufactured in said cell following transfection or transformation. In the instance where said viral vector is used it includes, but is not restricted to, retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated
30 viral vectors (AAV).

35 Additionally or alternatively, said delivery is undertaken using a non-viral vector that encodes said *trans*-splicing RNA molecule or RNAi molecule and that can transfect or transform said cell and in either case said non-viral vector is suitably equipped to ensure the *trans*-splicing RNA molecule or RNAi molecule is manufactured in said cell following transfection or transformation. In the instance where said non-viral vector is used it includes, but is not

restricted to, liposomes, nanoparticles, polymer capsules, and conjugates containing peptides including cell penetrating peptides, proteins including antibodies or receptors, sugars, lipids, nucleic acids, and steroids.

- 5 Additionally or alternatively, said delivery is undertaken using a naked nucleic acid vector that encodes said *trans*-splicing RNA molecule or RNAi molecule and that can transfect or transform said cell and in either case said vector is suitably equipped to ensure the *trans*-splicing RNA molecule or RNAi molecule is manufactured in said cell following transfection or transformation. Said naked nucleic acid vector includes but is not restricted to plasmids,
10 cosmids, DNA minicircles, dumbbell-shaped DNA vectors, and RNA.

In yet a further method of the invention a single one of the above referenced vectors is used to deliver both said *trans*-splicing RNA molecule and said RNAi molecule and so said single vector encodes both said *trans*-splicing RNA molecule and RNAi molecule. Said single
15 vector comprises either two separate expression cassettes or a single expression cassette to express the *trans*-splicing RNA and the RNAi molecule. Said single expression cassette includes but is not restricted to a gene expressing a *trans*-splicing RNA that harbours an intronic miRNA or shRNA or a miRtron.

- 20 In yet a further preferred method of the invention a single vector is used to deliver the *trans*-splicing RNA, the RNAi molecule, and as a third component the recombinant wild-type therapeutic gene.

Most preferably said *trans*-splicing RNA molecule comprises a binding domain that is
25 specific for the target pre-mRNA and so comprises a sequence of nucleotides that recognizes or is complementary to a sequence encoding a selected region of a gene, typically having a genetic defect, ideally said region is upstream or downstream of a particular exon to be spliced and so most preferably said region comprises intronic DNA. Those skilled in the art will appreciate that where one or more exons are to be inserted
30 between upstream and downstream exons, said *trans*-splicing RNA molecule comprises two binding domains complementary to regions either side of the exon(s) to be spliced.

Most preferably also said *trans*-splicing RNA molecule comprises a wild-type therapeutic gene or exon which when spliced into a target defective nuclear transcript restores the wild-
35 type function of said transcript and so enables the production of an effective gene protein product. Preferably said wild-type therapeutic gene or exon comprises recombinant RNA.

Alternatively, said *trans*-splicing RNA molecule comprises a sequence of nucleotides encoding an apoptotic signal leading to the selective destruction of said protein encoded by said transcript and ultimately the target cell. In this latter context the synergism of *trans*-splicing and RNAi can be relevant for suicide gene therapy where, for example, oncogene transcripts can be targeted by *trans*-splicing with apoptotic/death signals to ultimately selectively destroy/kill for example cancer cells. This effect is boosted when co-targeting the oncogene transcript with RNAi because many cancer cells require oncogene expression and so suppression of oncogene function often triggers cell death. Thus use of a *trans*-splicing RNA molecule having at least one apoptotic/death signal that triggers death of the corresponding transcript or its product and the co-use of a RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of the target nuclear transcript to prevent the function of a protein encoded thereby supports the death of the target cell.

More preferably still, said *trans*-splicing RNA molecule comprises functional sequences required for spliceosomal splicing such as splice sites, a branch point, a polypyrimidine tract, and splice enhancers. Depending on the design of the *trans*-splicing RNA, RNA *trans*-splicing can be used for 5' terminal (5'ER), internal (iER), or 3' terminal (3'ER) exon replacement. All these variations form part of the invention and are well known to those skilled in the art.

Most preferably still, said RNAi molecule is a micro RNA (miRNAs) and/or a siRNA either of which can be endogenously expressed within or exogenously delivered into the target cells. Endogenous expression usually starts with the transcription of nuclear precursor molecules (e.g. DNA molecules) which are then exported into the cytoplasm where they are processed to finally trigger the formation of RNA-induced silencing complexes (RISC). Alternatively, said RNAi molecule is an endogenously transcribed small hairpin (sh)RNA precursor which is processed by Dicer only after nuclear export within the cytoplasm triggering the formation of RISC.

In yet a further preferred method of the invention said RNAi molecule comprises a sequence of nucleotides that is complementary to a defective cellular version of said target nuclear transcript. The RNAi used is therefore specifically targeting the defect/mutated exon, not its functionally intact counterpart. More desirably still, said RNAi molecule comprises a sequence of nucleotides that provides for perfect pairing with said target nuclear transcript or said defective cellular version of said target nuclear transcript.

In yet a further preferred method of the invention said *trans*-splicing RNA molecule and said RNAi molecule are co-delivered exogenously into target cells using various delivery technologies. Additionally or alternatively, either or both said *trans*-splicing RNA molecule and said interfering RNAi molecule are transcribed endogenously from a vector or plasmid encoding same and, ideally, a single vector or plasmid.

In a yet further preferred embodiment of the invention said vector and/or plasmid further encodes a selected recombinant therapeutic gene with a view to treating the disorder that ensues when said target defective nuclear transcript is present.

Notably, since *trans*-splicing occurs in the nucleus and RNAi is effective in the cytoplasm of cells, we believe it is appealing to combine both technologies because this inherent spatial separation means RNAi will not target unspliced transcripts (Figures 2 and 3) thus both corrective mechanisms can be used at the same time to achieve a dual purpose which is either i) to restore gene function and prevent the effect of any defective genes or ii) to destroy cells expressing aberrant, including oncogenic, gene functions.

According to a second aspect of the invention there is provided a vector or plasmid comprising:

- a) at least one nucleic acid molecule encoding at least one *trans*-splicing RNA molecule having i) at least one sequence of nucleotides complementary to a target nuclear transcript comprising pre-mRNA, ii) at least one wild-type therapeutic gene or exon or at least one gene or exon encoding an apoptotic/death signal and iii) at least one functional sequence required for spliceosomal splicing; and
- b) at least one nucleic acid molecule encoding at least one RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of said target nuclear transcript.

In a preferred vector or plasmid said target nuclear transcript is defective.

According to a third aspect of the invention there is provided a therapeutic for treating a gene defect disorder comprising said vector or plasmid.

According to a further aspect of the invention there is provided a therapeutic for treating a gene defect disorder comprising:

- a) at least one nucleic acid molecule encoding at least one *trans*-splicing RNA molecule having i) at least one sequence of nucleotides complementary to a target nuclear transcript comprising pre-mRNA, ii) at least one wild-type therapeutic gene or exon or at least one gene or exon encoding an apoptotic/death signal and iii) at least one functional sequence required for spliceosomal splicing; and
- b) at least one nucleic acid molecule encoding at least one RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of said target nuclear transcript.

10 According to a yet further aspect of the invention there is provided a pharmaceutical composition comprising a therapeutic according to the invention and at least one carrier.

In a preferred embodiment of the invention said therapeutic or pharmaceutical composition is formulated for mammalian and ideally human use.

15

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprises”, or variations such as “comprises” or “comprising” is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

20

All references, including any patent or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art.

25

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

30

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

35

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

- 5 Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.
- 10 An embodiment of the present invention will now be described by way of example only with reference to the following wherein:

Figure 1 shows a schematic depiction of different gene therapy approaches. Displayed are cross sections through mammalian or human cells. Nuclear genomic DNA is being transcribed to single-stranded messenger RNA (mRNA) which is subsequently being spliced and exported into the cytoplasm where protein synthesis takes place. A defect (red) gene can lead to a defect mRNA and finally code for a defect or gene function/protein. The classical gene therapy approach is based on **gene complementation** of the defect with an intact (green) gene function. As a result, targeted cell carry both the defect and the recombinant intact function (**lower part**). Some defect proteins are pathogenic or carcinogenic. In such a case it is more promising to block the aberrant function using antisense or RNAi technologies i.e. **RNAi-based inhibition (upper part)**. In the consequence however targeted cells lose a gene function. RNA **trans-splicing based repair**, allows repairing the defect function or exon on the pre-mRNA level. In that case the corrected gene function replaces the defect function which is basically the most desirable outcome (**middle part**). Abbr.: e1-3: exons; i1,2: introns; e2: defect exon 2; e2*: corrected exon 2. Figure modified according to [7].

Figure 2 shows enhancement of apparent *trans*-splicing efficacy by RNAi. Compartmental separation of splicing and RNAi allows for selective cytoplasmic destruction of aberrant *cis*-spliced transcripts which escaped therapeutic nuclear *trans*-splicing. C: Cytoplasm; N: Nucleus; e: exon; i: intron. Red: defect sequence; green: intact sequence. *Trans*-splicing RNAs providing the intact sequences and siRNAs are not depicted in this figure.

Figure 3 shows examples how an siRNA effector molecule can be co-delivered together with a *trans*-splicing RNA expression vector. A, Co-delivery of a chemically synthesised

siRNA. B, Co-delivery of a separate shRNA expression vector. C, Co-delivery as independent shRNA expression cassette implemented into the *trans*-splicing RNA expression vector. D, Co-delivery as miRtron implemented into the *trans*-splicing RNA expression cassette. Splicing of the miRtron will generate the shRNA or miRNA precursor which can then be exported into the cytoplasm in a Drosha- and Exportin-5-independent manner.

Figure 4 shows a detailed molecular illustration of synergies between RNA *trans*-splicing and RNA interference. **A**, for *trans*-splicing-based 5' exon replacement; **B**, for *trans*-splicing-based 3' exon replacement; **C**, for *trans*-splicing-based internal exon replacement. The siRNA used is specifically targeting the defect/mutated exon, not its functionally intact counterpart. Due to the subcellular compartmentalization, the siRNA can exert its silencing potential only in the cytoplasm, i.e. target the defect/mutated exon exclusively on the level of the *cis*-spliced cytoplasmic target message but not on the level of the nuclear un-spliced pre-mRNA. That is, RNAi cannot negatively interfere with *trans*-splicing but instead support *trans*-splicing-based replacement of aberrant gene functions.

Figure 5 shows experimental proof of synergies (no negative interference) between RNA *trans*-splicing-based 3' exon replacement and RNAi. The RNAi effector was co-delivered as exogenously synthesised siRNA (**scenario A, Figure 3**). In this experimental setup, the alpha-fetoprotein (AFP) pre-mRNA was targeted with a *trans*-splicing RNA that replaces the 3' terminal exons 6 to 14 of the endogenous transcript or exon 6 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the herpes simplex virus thymidine kinase (HSVtk). The HepG2 target cells were co-transfected with a vector expressing AFP exons 3 to 6, a vector expressing the *trans*-splicing RNA, and the siRNA which was directed against AFP exon 6 using lipofection. Total cellular RNA was isolated 24 hours post transfection and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using real-time reverse transcriptase (rtRT-)PCR. **A**, knockdown of *cis*-spliced AFP mRNA by the exon 6-targeting siRNA. **B**, dose response curve of knockdown in A. **C**, knockdown of the *cis*-spliced AFP message illustrated by ΔCt values. **D**, the siRNA has no effect on *trans*-spliced AFP mRNA levels as depicted by ΔCt values. $\Delta Ct = Ct_{cis-splicedAFP} - Ct_{\beta-actin}$. Data represent mean values of three independent experiments. Error bars indicate mean deviations from the averages. P values were calculated using a student's t-test. *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$.

Figure 6 shows experimental proof of synergies (no negative interference) between RNA *trans*-splicing-based 5' exon replacement and RNAi. The RNAi effector was co-delivered as exogenously synthesised siRNA (**scenario A, Figure 3**). In this experimental setup, the AFP pre-mRNA was targeted with a *trans*-splicing RNA that replaces the 5' terminal exons 1 to 3 of the endogenous transcript or exon 3 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the HSVtk. The HepG2 target cells were co-transfected with a vector expressing AFP exons 3 to 6, a vector expressing the *trans*-splicing RNA, and the siRNA which was directed against AFP exon 3 using lipofection. Total cellular RNA was isolated 24 hours post transfection and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using rtRT-PCR. **A**, knockdown of *cis*-spliced AFP mRNA by the exon 3-targeting siRNA. **B**, dose response curve of knockdown in **A**. **C**, knockdown of the *cis*-spliced AFP message illustrated by ΔCt values. **D**, the siRNA has no effect on *trans*-spliced AFP mRNA levels as depicted by ΔCt values. $\Delta Ct = Ct_{cis\text{-spliced AFP}} - Ct_{\beta\text{-actin}}$. Data represent mean values of three independent experiments. Error bars indicate mean deviations from the averages. P values were calculated using a student's t-test. *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$.

Figure 7 shows an exemplary suggested design of gene therapy vectors. A single vector contains two (b and c) or three (a, b, and c) functional elements which support the replacement of a defect by an intact gene function: a, the recombinant therapeutic version of the defect endogenous gene the expression of which complements the defect with the intact gene function; b, a gene expressing a *trans*-splicing RNA suitable to repair the defect target message on the pre-mRNA level in the nucleus; and c, a gene coding for siRNA precursor, a small hairpin (sh)RNA, specifically targeting the defect/mutated exon of the target message in the cytoplasm. After transcription, shRNAs are exported from the nucleus into the cytoplasm recruiting the exportin-5 dependent pathway and will then be recognised and processed in the cytoplasm by dicer to generate the siRNA, trigger RISC formation, and knockdown of *cis*-spliced target messages that escaped nuclear *trans*-splicing. The combination of functional elements b and c allows partly replacement of the defect by an intact gene function with simultaneous elimination of the defect gene function on the RNA level. The combination of functional elements a to c can in addition either fully restore native expression levels of the intact target gene or trigger its over-expression.

Figure 8 shows experimental proof of synergies (no negative interference) between RNA *trans*-splicing-based 3' exon replacement and RNAi. In this experimental setup, the *trans*-splicing RNA and the RNAi effector (shRNA) were encoded on separate plasmid vectors

(**scenario B, Figure 3**). The alpha-fetoprotein (AFP) pre-mRNA was targeted with a *trans*-splicing RNA that replaces the 3' terminal exons 6 to 14 of the endogenous transcript or exon 6 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the herpes simplex virus thymidine kinase (HSVtk). The HepG2 target cells were co-transfected with 500ng a vector expressing AFP exons 3 to 6, 500ng of a vector expressing the *trans*-splicing RNA, and 500ng of a vector expressing an shRNA which was directed against AFP exon 6 using lipofection. Total cellular RNA was isolated 24 hours post transfection and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using real-time reverse transcriptase (rtRT-)PCR. **Left panel**, knockdown of *cis*-spliced AFP mRNA by the exon 6-targeting shRNA. **Right panel**, the endogenously expressed shRNA has no negative effect on *trans*-spliced AFP mRNA levels and even seems to support RNA-*trans*-splicing. Data represent mean values of three independent experiments. Error bars indicate mean deviations from the averages. P values were calculated using a student's t-test. *: p<0.05, **: p<0.01; ***: p<0.001.

Figure 9 shows experimental proof of synergies (no negative interference) between RNA *trans*-splicing-based 5' exon replacement and RNAi. In this experimental setup, the *trans*-splicing RNA and the RNAi effector (shRNA) were encoded on the same plasmid vector (**scenario C, Figure 3**). The alpha-fetoprotein (AFP) pre-mRNA was targeted with a *trans*-splicing RNA that replaces the 5' terminal exons 1 to 3 of the endogenous transcript or exon 3 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the HSVtk. The HepG2 target cells were co-transfected with 500ng of a vector expressing AFP exons 3 to 6 and 500ng of a vector expressing both the *trans*-splicing RNA and the shRNA which was directed against AFP exon 3 using lipofection. Total cellular RNA was isolated 24 hours post transfection and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using real-time reverse transcriptase (rtRT-)PCR. **Left panel**, knockdown of *cis*-spliced AFP mRNA by the exon 3-targeting shRNA. **Right panel**, the endogenously expressed shRNA has no significant negative effect on *trans*-spliced AFP mRNA levels. Data represent mean values of three independent experiments. Error bars indicate mean deviations from the averages. P values were calculated using a student's t-test. *: p<0.05, **: p<0.01; ***: p<0.001.

Figure 10 shows experimental proof of synergies between RNA *trans*-splicing-based 3' and 5' exon replacement and RNAi. In this experimental setup, the *trans*-splicing RNA and the RNAi effector (shRNA) were encoded by the same transcription cassette with the shRNA implemented as a miRtron into the HSVtk mRNA (**scenario D, Figure 3**). The miRtron has

no negative effect on *trans*-splicing but instead enhances the *trans*-splicing activity. HepG2 target cells were co-transfected with 500ng a vector expressing AFP exons 3 to 6 and a vector expressing the *trans*-splicing RNA together with an AFT-targeting miRtron using lipofection. **A**, Co-transfection of 500ng of the 3'EL construct. **B**, Co-transfection of 150ng of the 5'EL construct. **C**, Co-transfection of 500ng of the 5'EL construct. Total cellular RNA was isolated 24 hours post transfection and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using real-time reverse transcriptase (rtRT-)PCR. **Left panel**, no significant effect of the miRtron on *cis*-spliced AFP mRNA levels. **Right panel**, co-expression of the miRtron enhances RNA *trans*-splicing. Data represent mean values of three independent experiments. Error bars indicate mean deviations from the averages. P values were calculated using a student's t-test. *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$.

Figure 11 comparing the efficiency of shRNAs in knocking down the AFP mini-gene target with real time RT-PCR using (a) 3'AFP probe and (b) 5'AFP probe. The shRNAE3a and shRNAE3b targets AFP exon 3 and shRNAE6a and shRNAE6b targets AFP exon 6. The "a" denotes conventional perfect pairing miRNA hairpin precursor and the "b" contains two bulges in the guide sequence. $n=3$, mean $\pm$ SEM, test for significance used was one-way ANOVA with Dunnett post-hoc. Relative mRNA expression was calculated in terms of fold change ($2^{-\Delta\Delta Ct}$) where $\Delta Ct = Ct_{cis\text{-spliced AFP}} - Ct_{\beta\text{-Actin}}$. * $p < 0.05$.

Figure 12 relative mRNA expression checking the levels of escaped *cis*-spliced AFP and *trans*-splicing when specific shRNAs are introduced as separate vector. The effect of co-transfection of 250 ng AFP mini-gene vector plus 250 ng shRNA vectors E6a and E6b along with 250 ng *trans*-splicing vector using Lipofectamin 3000 on (a) 3' *cis*-splicing and (b) 3' *trans*-splicing. The effect of co-transfection of 250 ng AFP mini-gene vector plus 250 ng shRNA vectors E3a and E3b along with 250 ng *trans*-splicing vector using Lipofectamin 3000 on (c) 5' *cis*-splicing and (d) 5' *trans*-splicing. In the negative controls total DNA was topped up with 250 ng empty pSuper vector. $n=3$, mean $\pm$ SEM, test for significance used was one-way ANOVA with Dunnett post-hoc. Relative mRNA expression was calculated in terms of fold change ($2^{-\Delta\Delta Ct}$) where $\Delta Ct = Ct_{cis/trans\text{-spliced AFP}} - Ct_{\beta\text{-Actin}}$. * * $p < 0.01$.

Figure 13 relative mRNA expression checking the levels of escaped *cis*-spliced AFP and *trans*-splicing when specific shRNAs are cloned into the *trans*-splicing cassette as a single vector system. The effect of shRNAs E6a and E6b fused with *trans*-splicing vector on (a) 3' *cis*-splicing and (b) 3' *trans*-splicing. The effect of shRNAs E3a and E3b fused along with *trans*-splicing vector on (c) 5' *cis*-splicing and (d) 5' *trans*-splicing. Cells were co-transfected

with 250 ng of each plasmid using Lipofectamin 3000. In the negative controls total DNA was topped up with 250 ng empty pSuper vector. n=3, mean $\pm$ SEM, test for significance used was one-way ANOVA with Dunnett post-hoc. Relative mRNA expression was calculated in terms of fold change ($2^{-\Delta\Delta Ct}$) where $\Delta Ct = Ct_{cis/trans\text{-}spliced\ AFP} - Ct_{\beta\text{-}Actin}$. * p<0.05.

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Figure 14 relative mRNA expression checking the levels of escaped *cis*-spliced AFP and *trans*-splicing when specific shRNAs (mirtrons) are cloned into the HSV-tk coding region of *trans*-splicing cassette as a single vector system. The effect of shRNAs E6a and E6b mirtron fused with *trans*-splicing vector on (a) 3' *cis*-splicing and (b) 3' *trans*-splicing. The effect of shRNAs E3a and E3b mirtron fused along with *trans*-splicing vector on (c) 5' *cis*-splicing and (d) 5' *trans*-splicing. Cells were co-transfected with 250 ng of each plasmid using Lipofectamin 3000. In the negative controls total DNA was topped up with 250 ng empty pSuper vector. n=3, mean $\pm$ SEM, test for significance used was one-way ANOVA with Dunnett post-hoc. Relative mRNA expression was calculated in terms of fold change ($2^{-\Delta\Delta Ct}$) where $\Delta Ct = Ct_{cis/trans\text{-}spliced\ AFP} - Ct_{\beta\text{-}Actin}$. * p<0.05.

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Materials & Methods

siRNA Design: siRNAs targeting exon 3 of AFP and exon 6 of AFP were designed following the protocols proposed previously [8,9]. siRNA targeting luciferases was previously described [8]. The selected siRNA sequence was summarized in Table 1 and ordered from Dharmacron Thermo Scientific.

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siRNA_E6: sense 5'-AUUAAGAGAAAGCAGCUUGdTdT-3' (SEQ ID NO:1); antisense 5'-CAAGCUGCUUUCUCUUAUUC-3' (SEQ ID NO:2)

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siRNA_E3: sense 5'-AGUCUUCAGGGUGUUUAGAdTdT-3' (SEQ ID NO:3), antisense 5'-UCUAAACACCCUGAAGACUGU-3' (SEQ ID NO:4)

Cell Culture: HepG2 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin- streptomycin.

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Cell Transfection: HepG2 cells in log growth phase were plated in 24 well plate at 10^5 cells / well. 500 ng of pVax1- AFP, 500 ng of pVax1- PTM, and siRNA of various concentration (0, 0.1, 1, 10, 100 pmol/uL) were co-transfected 14 hours after plating using lipofectamine 2000 TM or 3000 TM (Introvigen) in Opti-MEM (Introvigen) following the manufacturer's protocol. Alternatively, various amounts of *trans*-splicing constructs, pVAX1-AFP, shRNA, combined *trans*-splicing-shRNA constructs or mirtron constructs were co-transfected. In the

negative controls total DNA was topped up with empty pSuper vector to transfect all cells with the same total amount of DNA. Successfully transfected Hep G2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin (P-S) overnight.

5

Design of Target mRNA constructs: Target mRNA was previously designed by S. Poddar according to endogenously express part of the alpha fetoprotein (AFP) from a DNA minigene plasmid. The AFP minigene consisted of exon 3, intron 3, linked exons 4 and 5, intron 5 and exon 6. It was introduced into the pVAX1 plasmid which harbors a Kanamycin resistance

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Design of *trans*-splicing RNA constructs: *Trans*-splicing RNA constructs 3'PAR and 5'PAR for 3' exon replacement (ER) or 5' ER were designed including a target binding domain, a splicing domain, and a coding domain (which in this case was a gene for HSVtk expression), and synthesized by GeneArt® (Life Technologies). 3'PAR interacts with AFP minigene mRNA (target mRNA) and undergoes 3'exon labeling (3'EL) by excising and replacing exon 6 with the domain which encodes the Herpes simplex virus thymidine kinase (HSV-TK). It was designed with:

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- i. A binding domain in antisense orientation to intron 5 of the AFP minigene,
- ii. A splicing domain with a 3' active splice signal, a branch point consensus and a
- 20 polypyrimidine tract,
- iii. A coding domain which encodes the HSV-TK but a deletion of the translational start codon to prevent translation in the absence of *trans*-splicing,
- iv. A poly-adenosine tail to confer correct processing of the *trans*-splice product.

5'PAR was designed for 5'exon labeling (5'EL) in which exon 3 is excised and replaced with

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coding domain of HSV-TK and it consists of:

- i. A splicing domain containing a 5' splice site,
- ii. A coding domain which encodes the HSV-TK,
- iii. A binding domain consisting of 42 complementary bases pairing to intron 3
- iv. A polyA tail

Design and cloning of shRNA: Polycistronic shRNA plasmids obtained from the GeneArt® Gene synthesis service (Life Technologies). Two 21-nucleotides shRNA guide strands, targeting exon 3 and exon 6 of AFP were generated. Selection of guide sequence candidates were done according to the criteria for *in silico* selection of siRNA (Patzel, 2007).

5 Selected shRNA guide strands were further extended with additional two nucleotides at both 3' and 5' ends, forming 25-nucleotide elongated shRNA guide strands. 25-nucleotide elongated shRNA guide strands in this article were selected to fold an open secondary structure with high Gibbs free energy, ΔG predicted by Mfold (Zuker, 2003). Both guide strands were used to replace mature miRNA sequences within human precursor miRNA (pre-miRNA) structures selected from the miRBase namely hsa-miR-4699, hsa-miR-3116-1, hsa-miR-106b and hsa-miR-20a [10-14], forming Dicer substrate shRNAs. Exon 3 targeting guide strand replaced hsa-miR-4699 and hsa-miR-106b, while exon 6 targeting guide strand replaced hsa-miR-3116-1 and hsa-miR-20a. The shRNA constructs derived from the replacement of mature sequences of human miRNAs by guide strands were labeled as shRNA_E3_a (or shRNA1), shRNA_E3_b (or shRNA3), shRNA_E6_a (or shRNA2) and shRNA_E6_b (or shRNA4) respectively. The passenger strand of shRNAs were modified such that identical secondary structures to original miRNAs were maintained.

shRNA_E3_a: 5'-AGCAAGAACAGUCUUCAGGGUGUUUAGAAAUGAUUAAGAAUUUUC
GUAAACACCCUGAAGACUGUUCUUGCU-3' (SEQ ID NO:5)

20 shRNA_E6_a: 5'-CUUUUAUAGAAUUAAGAGAAAGCAGCUUGUUUGGUAUCGUAGAAC
AAGCUGCUUUCUCUUAUUCUUAUAAAG-3' (SEQ ID NO:6)

shRNA_E3_b: 5'-CCUGCCGGGUUUCUAAACACCCUGAAGACUGUUCUGGUCCUCUCC
UUGGAAACUCUUCAGGGUGUUUAUCUAAUCCAGCAGG-3' (SEQ ID NO:7)

shRNA_E6_b: 5'-GUAGCAUAACAAGCUGCUUUCUCUUAUUCUGUUUAGUCAUGAAUA
25 AGAGAAAGCAGCUUCAUUAUACUGC-3' (SEQ ID NO:8)

Design and cloning of combined *trans*-splicing RNA molecules and shRNA plasmid:

To increase ease of delivery of the combined *trans*-splicing RNA constructs and RNA interference therapy for future applications, a combined *trans*-splicing RNA molecule and shRNA plasmids in a single plasmid was designed.

Design and cloning of the mirtron: The design of an artificial mirtron considers rules (i) for the design of a spliceable RNA that (ii) generates an RNAi effector molecule [15]. The miR-1224 was chosen from the miRBase due to its existence in mammalian cells as an endogenous mirtron (Sibley et al., 2011). Mmu-miR-1224 was originally designed as introns within the eGFP plasmid in Sibley *et al.*'s experiment [16]. In this article, miR-1224 served as

the model structure for the design of a mirtron. The structure of miR-1224 was predicted by Mfold [17] and RNAfold [18]; both predicted structures were identical. Two shRNA guide strands were designed to replace the seed region of miR-1224. The passenger strands of mirtron-RNAs were modified such that the original secondary structures of the miRNAs were maintained. Final constructs were labelled as mirtron_E3 (or mirtron1) and mirtron_E6 (or mirtron2).

miR-1224: 5'-GUGAGGACUCGGGAGGUGGAGGGUGGUGCCGCCGGGGCCGGGCGCU
GUUUCAGCUCGCUUCUCCCCCACCUCUCUCUCCUCAG-3' (SEQ ID NO:9)

miRtron_E3: 5'-GTCTAAACACCCTGAAGACTGTTTCAGCATCATTAGAGCCAGAGTTCTGT
CTCAGCTAACACTCCCCGTCTTTAGGGACTTTAGAG-3' (SEQ ID NO:10)

miRtron_E6: 5'-GTAACAAGCTGCTTTCTCTTAATTCGCATCATTAGAGCCAGAGTTCTGT
CTCAGCGATTACTCCCCAGAGAAAGTACGTTGTTAG-3' (SEQ ID NO:11)

The cloned mirtron constructs were termed as 5'PAR-mirtron_E3 (or 5'mir1) and 3'PAR-mirtron_E6 (or 3'mir2).

General cloning strategies: Approximately two µg of the basis vector constructs were incubated with 1µl each of two specific restriction enzymes and 10X Fast Digest Buffer (Thermo Scientific) in a 50µl reaction at 37°C for two hours, then 80°C for 15 minutes to inactivate the restriction enzymes. Following the digestion, the entire 50 µl digestion mixtures were loaded onto 1% agarose gels for gel electrophoresis at 90V for one hour. Two bands were observed under UV illumination and desired bands were cut and weighed. The DNA was extracted from the gels using GeneJET Gel Extraction Kit (Thermo Scientific). The vector DNA and the insert DNA were then ligated with 1µl T4 DNA ligase (Thermo Scientific) at a concentration ratio of one to three (vector to insert) in 20µl reaction mixture which consists of 10X T4 ligase buffer. The ligation step was carried out at 22°C for four hours and the T4 ligase was inactivated at 70°C for 15 minutes. A total of 10 µl of the ligation mixture was then transformed into chemically-competent *E. coli* strain DH5α using a standard transformation protocol. Successful clones (three to five from each transformation set) were selected for colony PCR using forward and reverse primers designed and were verified for the correct amplification band size before they were sent for sequencing (AITBiotech) to confirm for successful cloning.

Cloning of pVAX1-shRNA and pSUPER-shRNA

30µg of shRNA and 2µg cloning plasmid, pVAX1 or pSUPER was digested with 1µl each of restriction enzymes as summarized in the following table (**Table 1**). After digestion, ligation and transformation steps were done as mentioned above in Section 2.6.

- 5 **Cloning of 3'PAR-shRNA_E6a and 5'PAR-shRNA_E3a:** Cloning of the combined hybrid plasmid was done by insertion of *trans*-splicing RNA expression cassettes of constructs 3'ER and 5'ER into previously cloned pSUPER-shRNA constructs using SpeI and BamHI restriction sites. The resulting constructs were termed 3'ER-shRNA2 and 5'ER-shRNA1. 2µg of *trans*-splicing RNA constructs, 3'ER or 5'ER and 30µg of cloning plasmid, pSUPER-shRNA1 or pSUPER-shRNA2 were digested with 1µl each of restriction enzymes as summarized in the following table (**Table 2**).
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- Cloning of pVAX1-mirtron:** The parental *trans*-splicing constructs, 3'PAR and 5'PAR having two SphI (or PaeI) restriction endonuclease cleavage sites at the backbone, couldn't be used for mirtron cloning. Therefore prior to the introduction of mirtron, the two SphI sites must be diminished. Four primers were designed for PCR of the entire *trans*-splicing constructs using Pfu DNA polymerase (Thermo Scientific) by introducing a mismatch in one SphI sequence or a total replacement of the SphI restriction site with a BamHI restriction site. FP_pVAX_large and RP_pVAX_large were designed for the PCR of the bigger fragment between two SphI sites while FP_pVAX_small and RP_pVAX_small were used to PCR amplify the smaller fragment between two SphI sites. Primers were phosphorylated prior to the PCR. Blunt end ligation was carried out after PCR with the addition of PEG4000. Successful clones, 3'PAR_mod and 5'PAR_mod were picked for the cloning of mirtron. Mirtron_E3 and mirtron_E6 were first isolated from the synthesized mirtron_E3_E6 plasmid by GeneArt® with following restriction enzymes: Table 3.
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- Digested mirtron constructs, mirtron1 or mirtron2 and 2µg of cloning plasmid, *trans*-splicing RNA constructs, 5'PAR_mod or 3'PAR_mod respectively was digested with 1µl each of restriction enzymes of SphI and SacI. Positive clones were selected via colony PCR and then verified by sequencing. Cloned plasmids were labelled as 3'mir2 and 5'mir1.
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RNA isolation and first-strand cDNA synthesis: Total RNA from transfected HepG2 cells was isolated using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol

24 hours or 48 hours post-transfection depending on conditions to be tested. First strand cDNA was synthesized using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Approximately 500 ng of total cellular RNA was incubated with 1µl each of random hexamer and dNTPs at 65°C for five minutes in a 14µl reaction. After one minute chill snap, 6µl of master mix consisting 4µl of buffer, 0.1M DTT (1µl), RNase OUT (0.5µl) and Superscript III reverse transcriptase (0.5µl) were added and incubated at 25°C for five minutes then 50°C for two hours, followed by inactivation of the enzyme at 70°C for 15 minutes.

First-strand cDNA synthesis with specific primers for detection of shRNA and mirtron

processing efficiency: First strand sequence specific cDNA was synthesized using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Approximately 500 ng of total cellular RNA was incubated with 1.5 µl of specific stem loop primer and 1µl of dNTPs at 65°C for five minutes in a 14µl reaction. After one minute chill snap, 6µl of master mix consisting 4µl of buffer, 0.1M DTT (1µl), RNase OUT (0.5µl) and Superscript III reverse transcriptase (0.5µl) were added and incubated at 50°C for two hours, followed by inactivation of the enzyme at 70°C for 15 minutes.

Real-time Polymerase Chain Reaction (qPCR): Quantitative real-time PCR was done using TaqMan probe as fluorescence reporter. Quencher probes were designed for each set of constructs (probes 3'AFP and 5'AFP) both targeting on exon 5 and exon 4 of the AFP mini gene respectively. Forward and reverse primers (3'FP and 3'RP_*cis* for the 3'AFP probe and 5'FP_*cis* and 5'RP for the 5'AFP probe) were designed to detect escaped *cis*-spliced AFP mRNA while 3'FP and 3'RP_*trans* for the 3'AFP probe and 5'FP_*trans* and 5'RP for the 5'AFP probe were designed to detect successful *trans*-spliced AFP/HSV-tk chimeric mRNA. Approximately 20ng of first-strand cDNA incubated with 5µl of TaqMan Universal PCR Master Mix buffer (Applied Biosystem), 0.5µl probe, 0.5µl forward primer and 0.5µl reverse primer in a 10µl reaction in 96-well PCR plate (Applied Biosystem). The PCR cycling conditions were as below: an initial 10 minutes denaturation step at 95°C was followed by 40 cycles of denaturation and annealing (95°C for 15 seconds and 60°C for 1 minute). For the quantification of processing efficiency, real time qPCR was carried out according to the universal TaqMan-based RT-PCR protocol for cost-efficient detection of small noncoding RNA developed [19]. Primers were designed using Primer Express (Applied Biosystems) with preference for open secondary structures and melting temperatures at around 60 °C. RT primers (stem loop primers) were designed with 6 nucleotides complementary with 3' ends of miRNAs. Forward primers (FP) recognizing remaining nucleotides that were 2 nucleotides away from the 6 nucleotides mentioned above on miRNA were designed with

additional nucleotides at 5' ends for stability and to achieve melting point of 58 to 60 °C qPCR cycle condition were retained for the determination of C_t value.

Data and statistical analysis: ΔC_t values were calculated by subtracting the C_t value of β -actin (endogenous control) from the C_t value obtained from real-time qPCR. $\Delta\Delta C_t$ was then obtained by subtracting ΔC_t (construct) by ΔC_t (control). A fold change was calculated with the equation of $2^{-\Delta\Delta C_t}$. This fold change value was then represented as relative RNA expression in all charts shown in this report. Data shown as 3 experiments made in triplicates. Error bar represents SEM. All statistical analysis were done in GraphPad Prism 5 (GraphPad Software) using two-tailed unpaired t test for comparison between two constructs and two way ANOVA (multiple comparison) for comparison among multiple constructs against control construct.

Trans-splicing RNA vectors and exogenously synthesised siRNAs were co-delivered into target cells using various transfection technologies including lipofectamine 2000 or 3000. Alternatively, the *trans*-splicing RNA and interfering RNA, were transcribed endogenously from a single plasmid or DNA-vector (Figure 3).

Results and discussion

In the following study we investigated of RNA *trans*-splicing and RNAi using alpha-fetoprotein (AFP) a protein whose elevated levels are a known marker for Hodgkin's disease and various types of liver disease such as chronic liver disease and liver cancer.

More specifically, we investigated a combination of RNA *trans*-splicing and RNAi using *trans*-splicing RNA triggering either 5'ER or 3'ER. For 3'ER, the alpha-fetoprotein (AFP) pre-mRNA was targeted with a *trans*-splicing RNA that either replaces the 3' terminal exons 6 to 14 of the endogenous transcript or exon 6 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the herpes simplex virus thymidine kinase (HSVtk). For 5'ER the AFP pre-mRNA was targeted with a *trans*-splicing RNA that replaces the 5' terminal exons 1 to 3 of the endogenous transcript or exon 3 of an exogenously delivered recombinant transcript encompassing only the exons 3 to 6 by a sequence coding for the HSVtk. In both cases, HepG2 target cells were co-transfected with a vector expressing AFP exons 3 to 6, a vector expressing the *trans*-splicing RNA, and the siRNA which was directed against AFP exon 6 or exon 3 using lipofection. Total cellular RNA was isolated and both, the *cis*-spliced and the *trans*-spliced AFP message was quantified using real-time reverse transcriptase (rtRT-)PCR. The results demonstrated for 5'ER and 3'ER that RNAi-based knockdown of a target gene (AFP) does not negatively

interfere with *trans*-splicing-based reprogramming (the fusion with the HSVtk) in the same cell (Figures 5-6 and 12-14).

The data in Figure 11 prove that all investigated shRNAs trigger knockdown of the *cis*-spliced target message, regardless of their precursor structures.

Further the mode of delivery of the *trans*-splicing RNA and RNAi does not appear to be crucial to success (Figures 12-14) thus suggesting some flexibility in the nature of the delivery used e.g. a single vector; a single vector wherein said *trans*-splicing RNA and said RNAi are remotely located/separated; a single vector wherein said *trans*-splicing RNA and said RNAi are next to each other/joined; or a vector for each type of RNA i.e. one for the *trans*-splicing RNA and one for the RNAi. However, clearest results were obtained when siRNAs were used as RNAi effector molecules. In addition, the use of a miRtron even enhances *trans*-splicing which might be due to the fact that the miRtron functions like an intron and recruits the spliceosome for its own *cis*-splicing which subsequently facilitates the *trans*-splice reaction as well.

Notably, it will also be possible to combine the investigated modes of delivery, i.e. generating a vector that expresses a miRtron-containing *trans*-splicing RNA and a shRNA, in order to maximize knockdown of the *cis*-spliced defective RNA on the one hand and to boost *trans*-splicing on the other hand.

The fact that both, the *trans*-splicing molecule and the RNAi effector can be encoded by a single DNA vector facilitates the use of any state-of-the-art delivery system including the use of viral delivery vectors which can carry only a single recombinant viral genome. That is important for therapeutic applications in vivo since it would be very unlikely that a single cell can be successfully targeted with two different genetic vectors.

We have demonstrated herein that *trans*-splicing RNA and RNAi act synergistically to replace a defect and so restore normal gene function and prevent any defective products being translated into protein products, respectively. This combined strategy is essential to compensate for incomplete *trans*-splicing activities and so to successfully target deleterious transcripts thus preventing any related diseases. This invention describes a significant improvement of spliceosome-mediated RNA *trans*-splicing by combining it with RNAi technology. The amalgamation of RNA *trans*-splicing with the RNAi technology triggers

clinically relevant efficiencies of functional genetic repair enabling the treatment of previously incurable genetic disorders.

Major embodiments of this invention are:

- 5 1. The amalgamation of two technologies which are commonly used separately, namely spliceosomal RNA *trans*-splicing and RNA interference, to achieve a significant improvement and broaden/expand applications of RNA *trans*-splicing towards previously incurable inherited and acquired genetic disorders. The technology can be applied but is not restricted to (i) genetic disorders that are associated with point mutations of cellular genes. Examples
- 10 are color blindness, cystic fibrosis, hemochromatosis, hemophilia, phenylketonuria, polycystic kidney disease, sickle cell disease, Tay-Sachs disease, and various cancers. A complete list can be found under <http://www.genome.gov/10001204> and includes:

Achondroplasia
 Alpha-1 Antitrypsin Deficiency
 Antiphospholipid Syndrome
 Autism
 Autosomal Dominant Polycystic Kidney Disease
 Breast cancer
 Charcot-Marie-Tooth
 Colon cancer
 Cri du chat
 Crohn's Disease
 Cystic fibrosis
 Dercum Disease
 Down Syndrome
 Duane Syndrome
 Duchenne Muscular Dystrophy
 Factor V Leiden Thrombophilia
 Familial Hypercholesterolemia
 Familial Mediterranean Fever
 Fragile X Syndrome
 Gaucher Disease
 Hemochromatosis
 Hemophilia
 Holoprosencephaly
 Huntington's disease
 Klinefelter syndrome
 Marfan syndrome
 Myotonic Dystrophy
 Neurofibromatosis
 Noonan Syndrome

Osteogenesis imperfecta
 Parkinson's disease
 Phenylketonuria
 Poland Anomaly
 Porphyria
 Progeria
 Prostate Cancer
 Retinitis Pigmentosa
 Severe Combined Immunodeficiency (SCID)
 Sickle cell disease
 Skin Cancer
 Spinal Muscular Atrophy
 Tay-Sachs
 Thalassemia
 Trimethylaminuria
 Turner Syndrome
 Velocardiofacial Syndrome
 WAGR Syndrome
 Wilson Disease

It can further be applied to (ii) diseases associated with the expression of specific disease markers including oncogenes, cancer genes, and viral transcripts (e.g. HIV, HPV).

2. Physical structures or molecules, i.e. improved vectors for genetic therapy which compartmentally or molecularly unify the features of the two technologies, RNA *trans*-splicing and RNA interference (Figure 7). These vectors can be any state of the art gene delivery system that contains or allows synthesis of therapeutic *trans*-splicing RNA and RNAi effector molecules.

3. Applications of the embodiments under 1 and 2 in a therapeutic context.

Conclusion

We have demonstrated that *trans*-splicing RNA and RNAi act synergistically, we suggest because they are compartmentally separated in cells, to replace a genetic defect with an intact gene function.

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Table 1. Summary of the cloning strategies for shRNA constructs.

shRNA	Cloning Plasmid	Restriction enzyme 1	Restriction enzyme 2	Labeling
shRNA1	pVAX1	AflIII	EcoRI	pVAX1-shRNA1
	pSUPER	BglIII	HindIII	pSUPER-shRNA1
shRNA2	pVAX1	HindIII	KpnI	pVAX1-shRNA2
	pSUPER	HindIII	Sall	pSUPER-shRNA2
shRNA3	pVAX1	KpnI	XhoI	pVAX1-shRNA3
	pSUPER	Sall	XhoI	pSUPER-shRNA3
shRNA4	pVAX1	XhoI	Apal	pVAX1-shRNA4
	pSUPER	XhoI	DraII (EcoO109I)	pSUPER-shRNA4

Table 2. Summary of cloning strategies for 3'ER-shRNA_E6_a and 5'ER-shRNA_E3_a.

<i>Trans-splicing constructs</i>	Cloning Plasmid	Restriction enzyme 1	Restriction enzyme 2	Labeling
3'ER	pSUPER-shRNA2	SpeI	BamHI	3'ER-shRNA2
5'ER	pSUPER-shRNA1	SpeI	BamHI	5'ER-shRNA1

Table 3. Summary of cloning strategies for 3'PAR-shRNA_E6_a and 5'PAR-shRNA_E3_a.

Mirtron	Restriction enzyme 1	Restriction enzyme 2
Mirtron1	BamHI	BglII
Mirtron2	Sall	EcoRI

Claims

1. A method of gene therapy comprising:
 - a) delivering into a target cell, at least one *trans*-splicing RNA molecule having:
 - 5 i) at least one sequence of nucleotides complementary to a target nuclear transcript comprising pre-mRNA,
 - ii) at least one wild-type therapeutic gene or exon or at least one gene or exon encoding an apoptotic/death signal and
 - iii) at least one functional sequence required for spliceosomal splicing; and
 - 10 b) delivering into said same target cell, at least one RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of said target nuclear transcript; whereby
 - c) *trans*-splicing of said pre-mRNA takes place to restore the function of a protein encoded thereby or to trigger death of the transcript or its product; and
 - 15 d) RNA interference of said cellular version of said target nuclear transcript takes place to prevent the function of a protein encoded thereby or to support the death of the pre-mRNA transcript or its product.
2. The method according to claim 1 wherein said target nuclear transcript is defective.
3. The method according to claim 1 or claim 2 wherein said molecules are delivered to
 - 20 said target cell using conventional transfection technologies.
4. The method according to any one of claims 1 - 3 wherein said delivering is undertaken using a vector and/or plasmid that encodes said *trans*-splicing RNA molecule or RNAi molecule and that can transfect or transform said target cell whereby said *trans*-splicing RNA molecule or RNAi molecule is manufactured in said
 - 25 cell following transfection or transformation.
5. The method according to any one of claims 1- 4 wherein a single vector is used to deliver both said *trans*-splicing RNA molecule and said RNAi molecule and so said single vector encodes both said *trans*-splicing RNA molecule and RNAi molecule.
6. The method according to claim 5 wherein said RNAi is contained as a miRtron in said
 - 30 vector.
7. The method according to any one of the preceding claims wherein said *trans*-splicing RNA molecule comprises at least one binding domain that is specific for the target pre-mRNA and so comprises at least one sequence of nucleotides that recognizes or is complementary to said pre-mRNA.
- 35 8. The method according to any one of the preceding claims wherein said *trans*-splicing RNA molecule comprises at least one wild-type therapeutic gene or exon which when

spliced into said target nuclear transcript provides a gene capable of producing an effective or wild type protein product or restores the wild-type function of a defective gene product and so enables the production of an effective or wild type protein product.

9. The method according to any one of the preceding claims wherein said apoptotic/death signal comprises at least one cell death gene or exon(s) which when spliced into said target nuclear transcript encodes a cell death signal that results in destruction of the transcript or its product or the cell expressing said target nuclear transcript.

10. The method according to any one of claims 1 - 8 wherein said *trans*-splicing RNA molecule comprises at least one wild-type therapeutic gene or exon.

11. The method according to any one of the preceding claims wherein said *trans*-splicing RNA molecule comprises recombinant RNA.

12. The method according to any one of the preceding claims wherein said *trans*-splicing RNA molecule comprises functional sequences required for spliceosomal splicing.

13. The method according to any one of the preceding claims wherein said *trans*-splicing RNA molecule comprises functional sequences required for 5' terminal (5'ER), or internal (iER), or 3' terminal (3'ER) exon replacement.

14. The method according to any one of the preceding claims wherein said RNAi molecule is a micro RNA (miRNA) and/or a siRNA and/or shRNA.

15. The method according to claim 14 wherein said RNAi is delivered as a precursor molecule and the RNAi is endogenously expressed within the target cell.

16. The method according to any one of claims 4 -15 wherein said vector and/or plasmid further encodes a selected recombinant therapeutic gene.

17. The method according to any one of the preceding claims wherein said RNAi molecule comprises a sequence of nucleotides that is complementary to a defective cellular version of said target nuclear transcript.

18. The method according to any one of the preceding claims wherein said RNAi molecule comprises a sequence of nucleotides that provides for perfect pairing with said target nuclear transcript.

19. A vector or plasmid comprising:

a) at least one nucleic acid molecule encoding at least one *trans*-splicing RNA molecule having:

i) at least one sequence of nucleotides complementary to a target nuclear transcript comprising pre-mRNA,

- ii) at least one wild-type therapeutic gene or exon or at least one gene or exon encoding an apoptotic/death signal and
 - iii) at least one functional sequence required for spliceosomal splicing; and
 - b) at least one nucleic acid molecule encoding at least one RNAi molecule having a sequence of nucleotides that is complementary to a cellular version of said target nuclear transcript.
20. A vector or plasmid according to claim 19 wherein said target nuclear transcript is defective.
21. A vector or plasmid according to claim 19 or claim 120 wherein said RNAi is contained as a miRtron in said vector.
22. The vector or plasmid according to any one of claims 19-21 wherein said RNAi molecule comprises a sequence of nucleotides that is complementary to a defective cellular version of said target nuclear transcript.
23. The vector or plasmid according to any one of claims 19-22 wherein said RNAi molecule comprises a sequence of nucleotides that provides for perfect pairing with said target nuclear transcript.
24. A therapeutic for treating a gene defect disorder comprising the vector or plasmid according to any one of claims 19 - 23.
25. A pharmaceutical composition comprising a therapeutic according to claim 24 and at least one carrier.
26. The pharmaceutical composition according to claim 25 wherein said pharmaceutical composition is formulated for mammalian use.
27. The therapeutic according to claim 24 wherein said therapeutic composition is formulated for mammalian use.
28. The pharmaceutical composition according to claim 26 or the therapeutic according to claim 27 wherein the pharmaceutical composition or therapeutic is formulated for human use.
29. A method according to any one of claims 1-18, a vector or plasmid according to any one of claims 19-23, a therapeutic according to any one of claims 24, 27 and 28, or a pharmaceutical composition according to any one of claims 25-26 and 28 as substantially herein described and with reference to the accompanying figures.

Figure 1

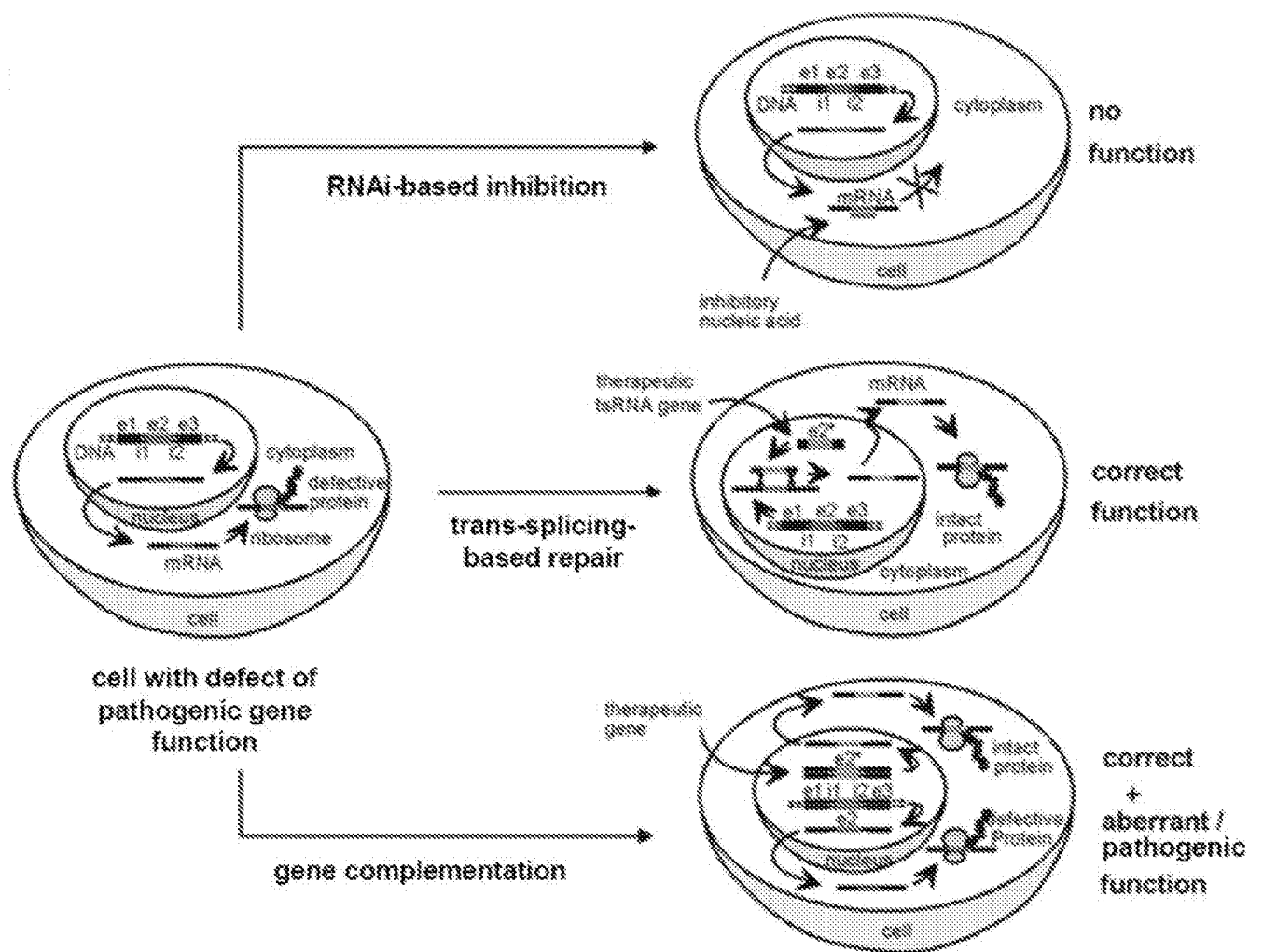


Figure 2

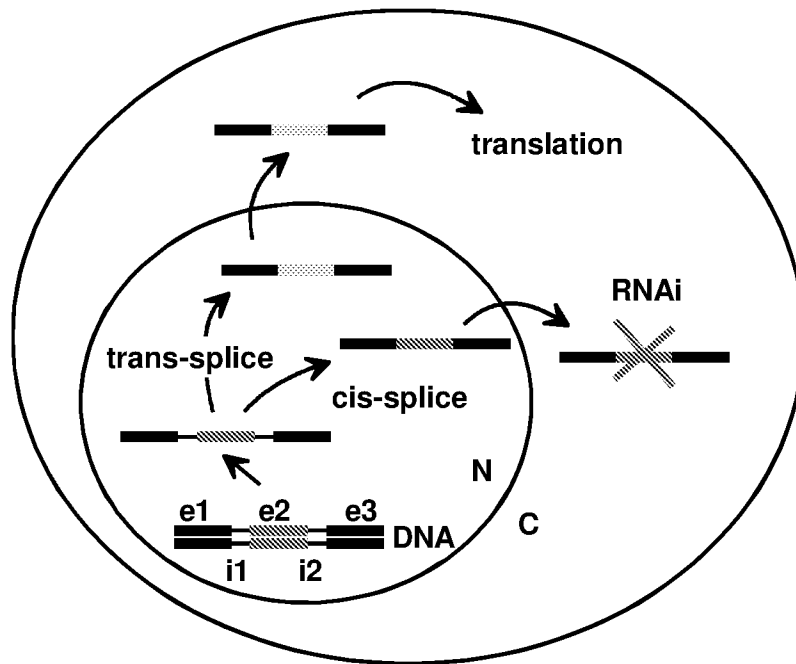


Figure 3

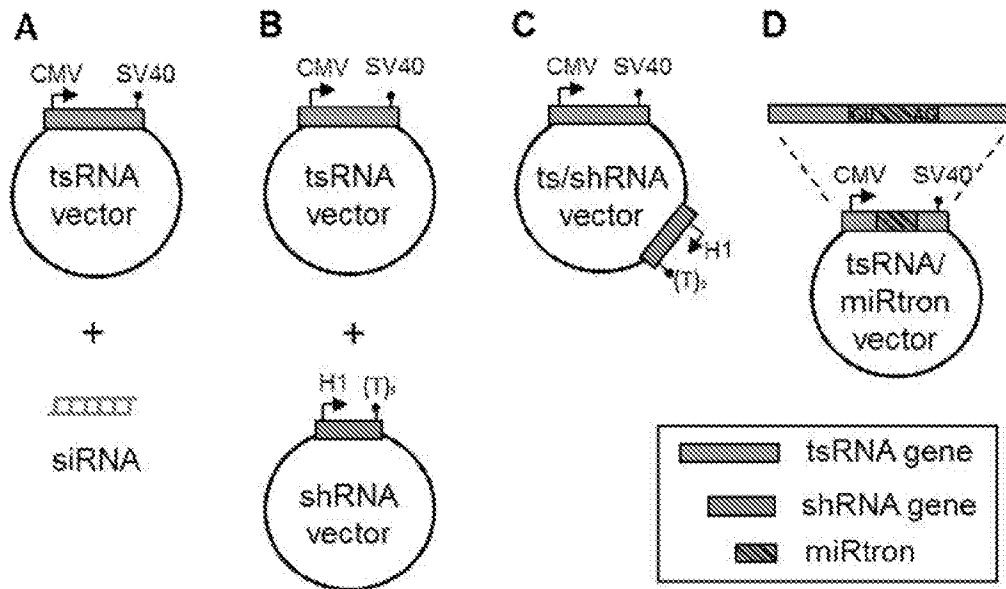
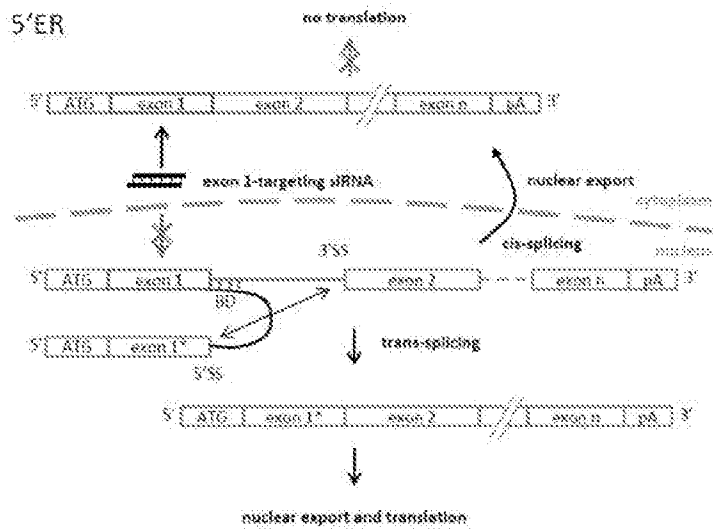
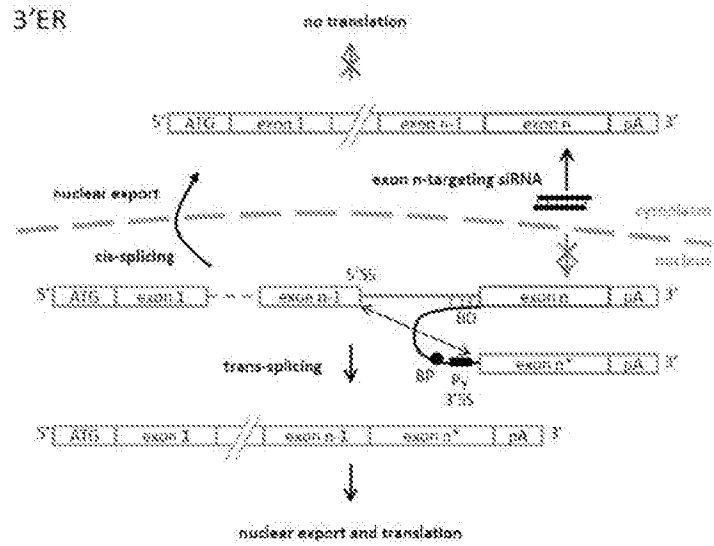


Figure 4

A



B



C

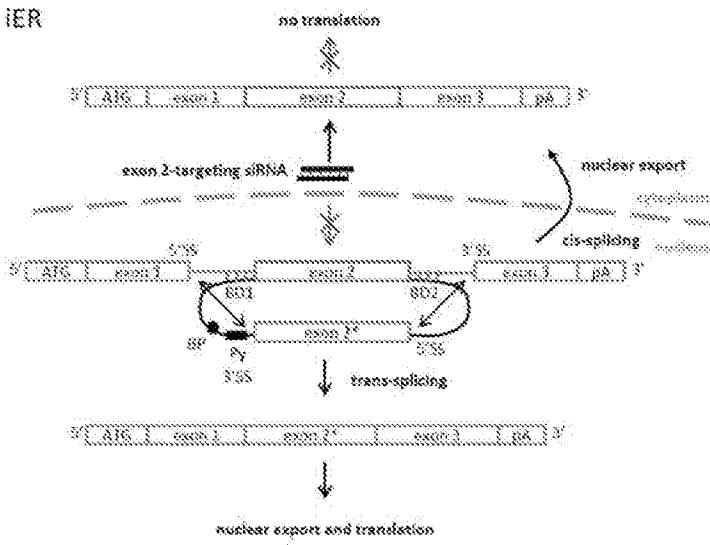
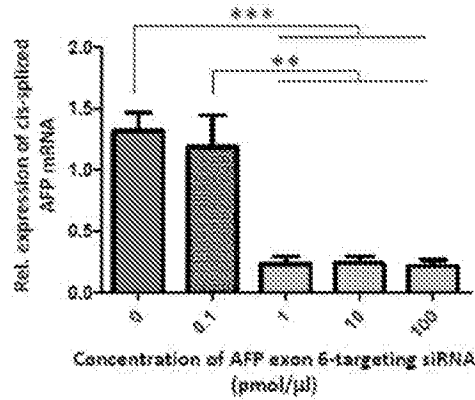
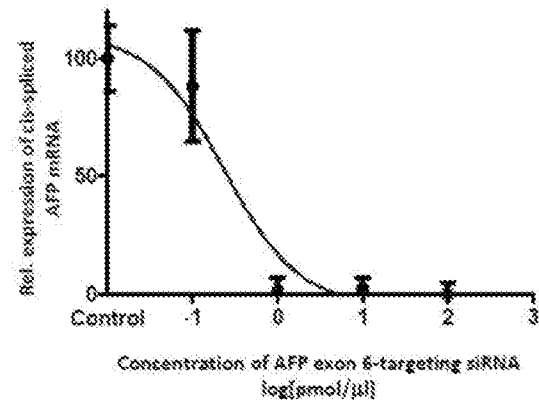


Figure 5

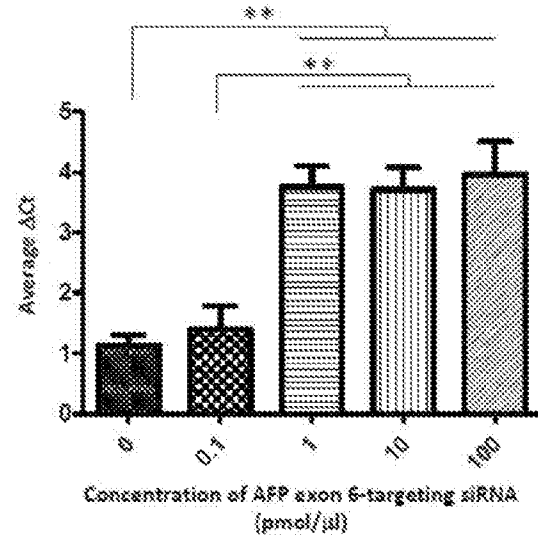
A Knockdown of cis-spliced AFP mRNA by exon 6-targeting siRNA



B Knockdown of cis-spliced AFP mRNA by exon 6-targeting siRNA



C Knockdown of cis-spliced AFP mRNA by exon 6-targeting siRNA



D No effect of exon 6-targeting siRNA on trans-spliced AFP mRNA levels

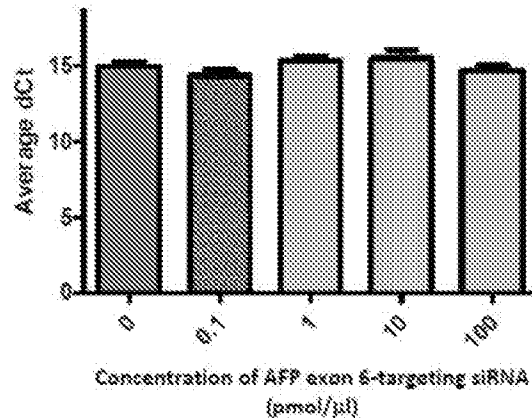


Figure 6

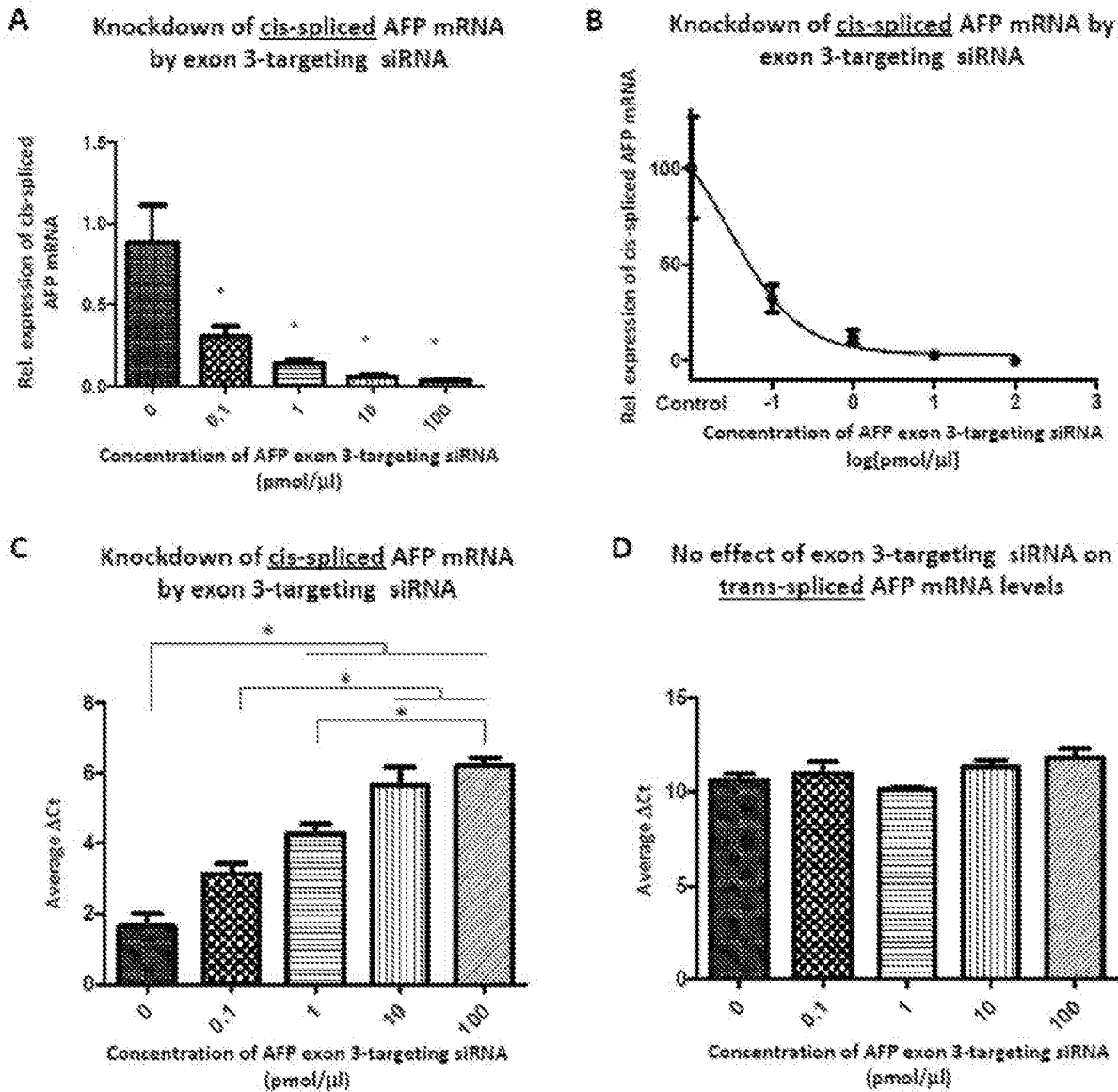


Figure 7

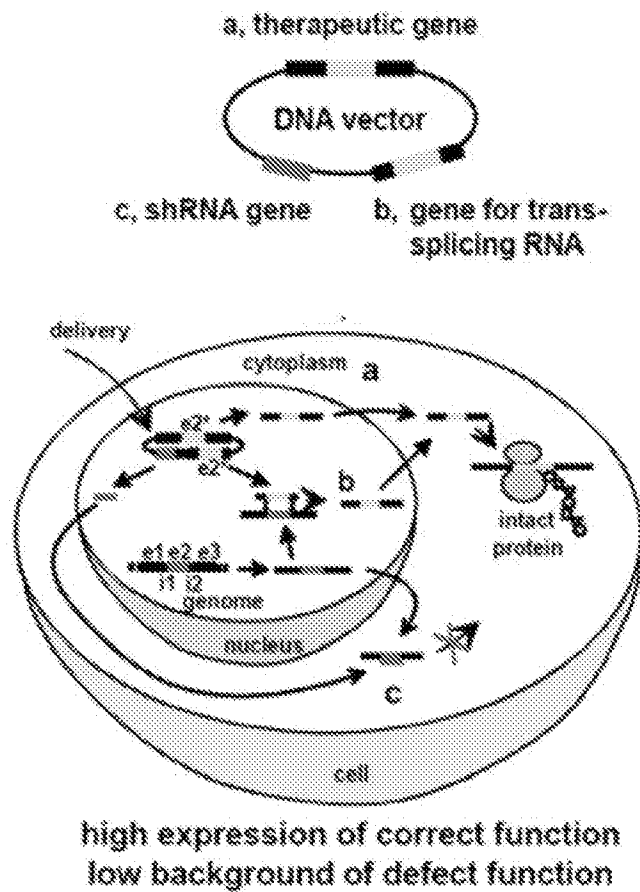


Figure 8

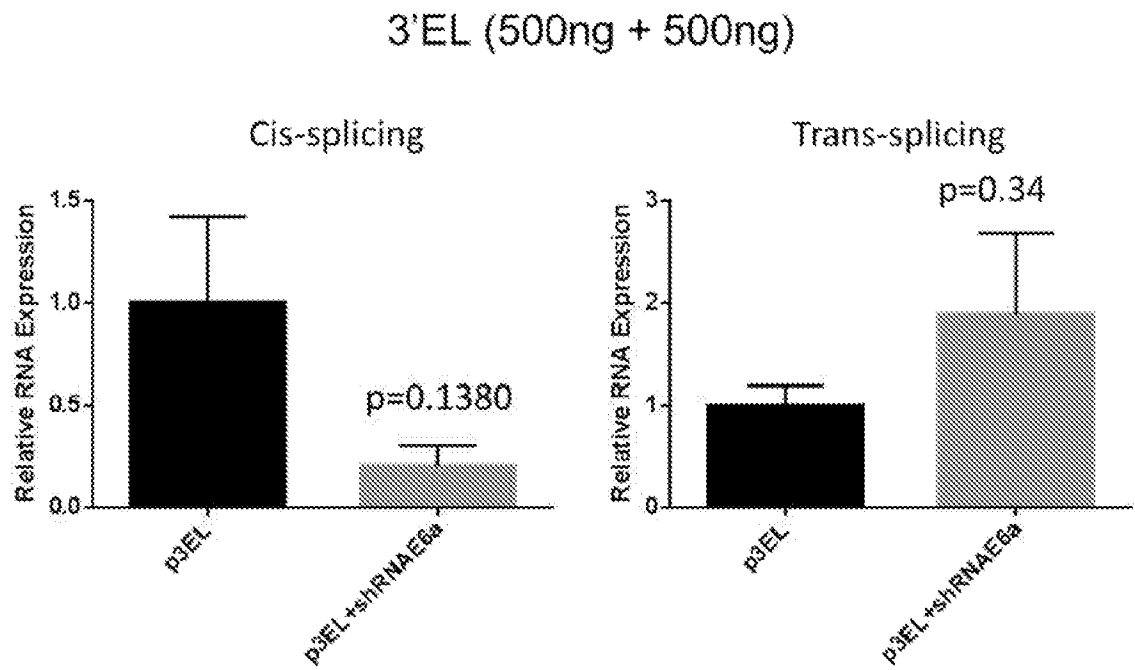


Figure 9

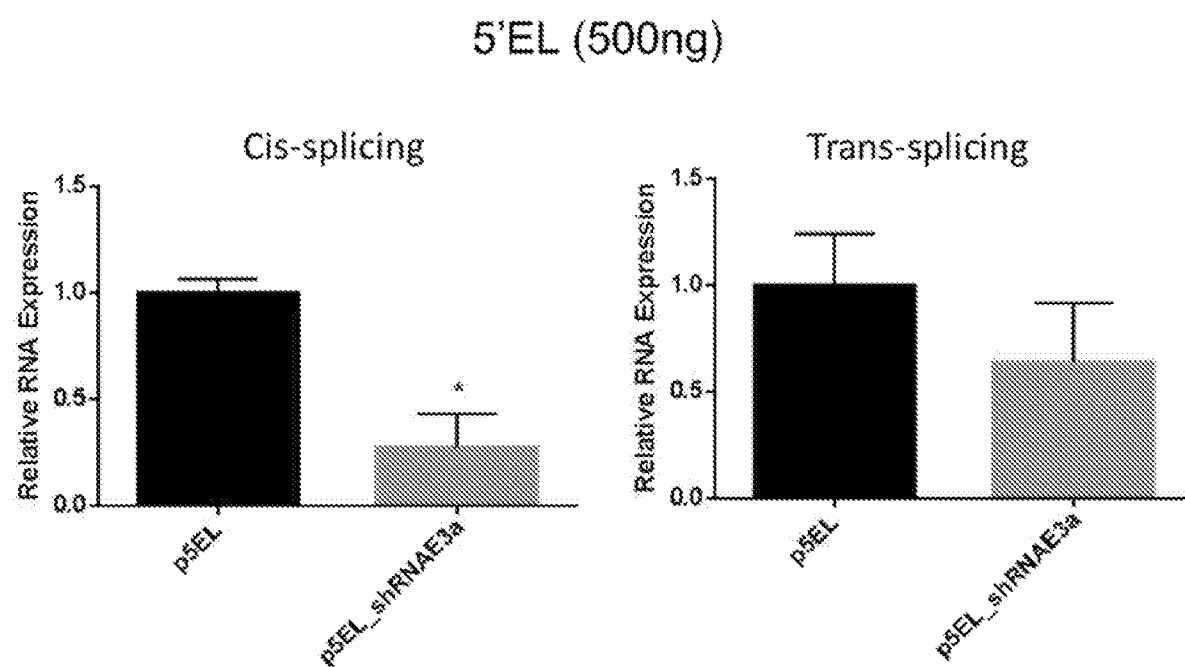


Figure 10

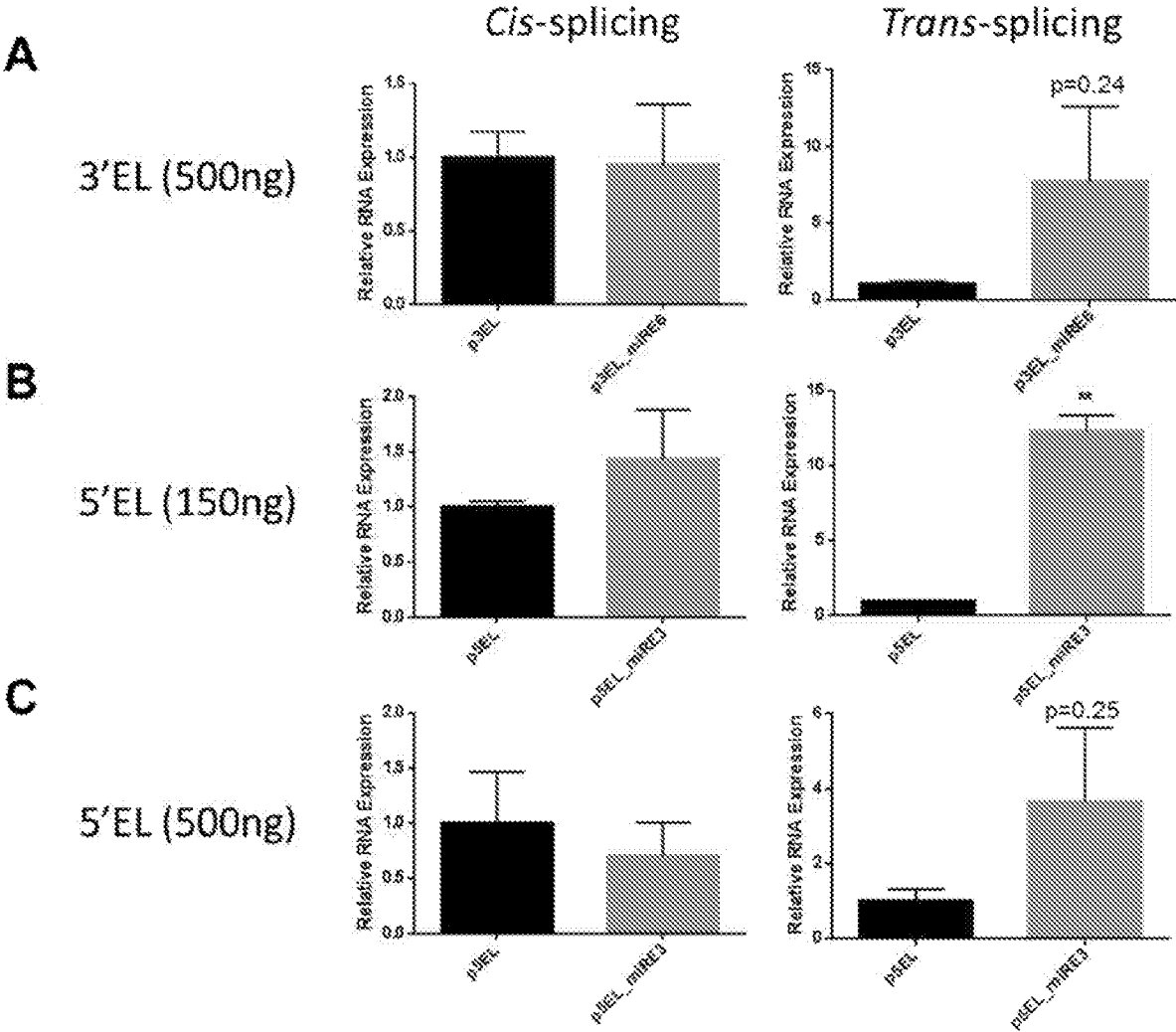


Figure 11

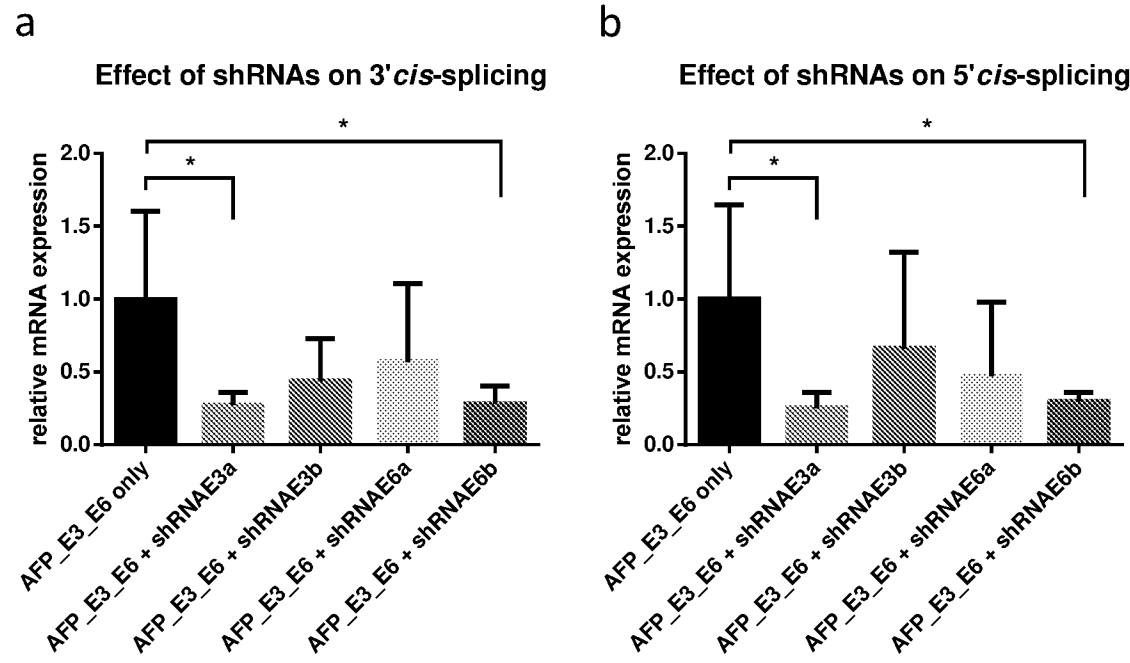


Figure 12

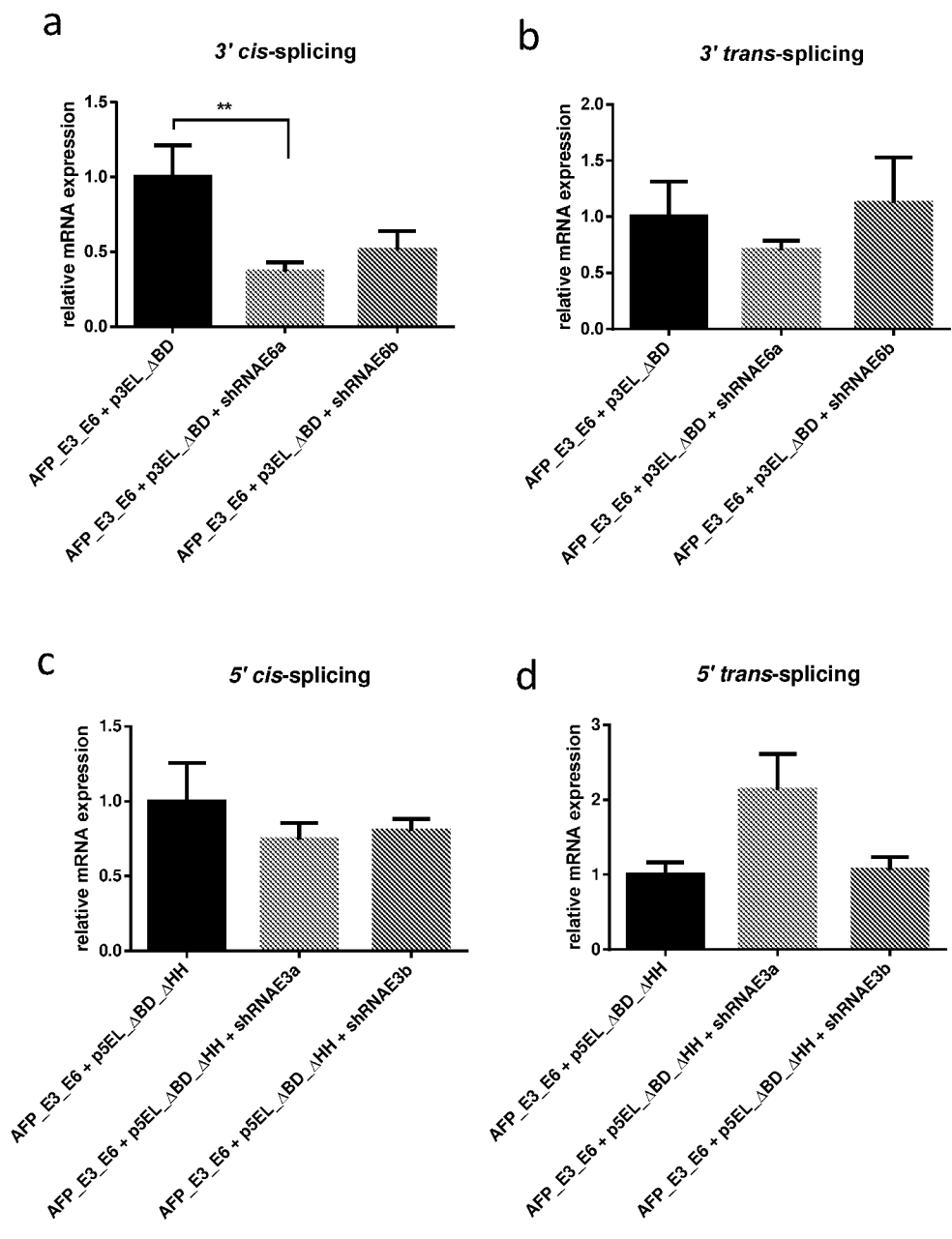


Figure 13

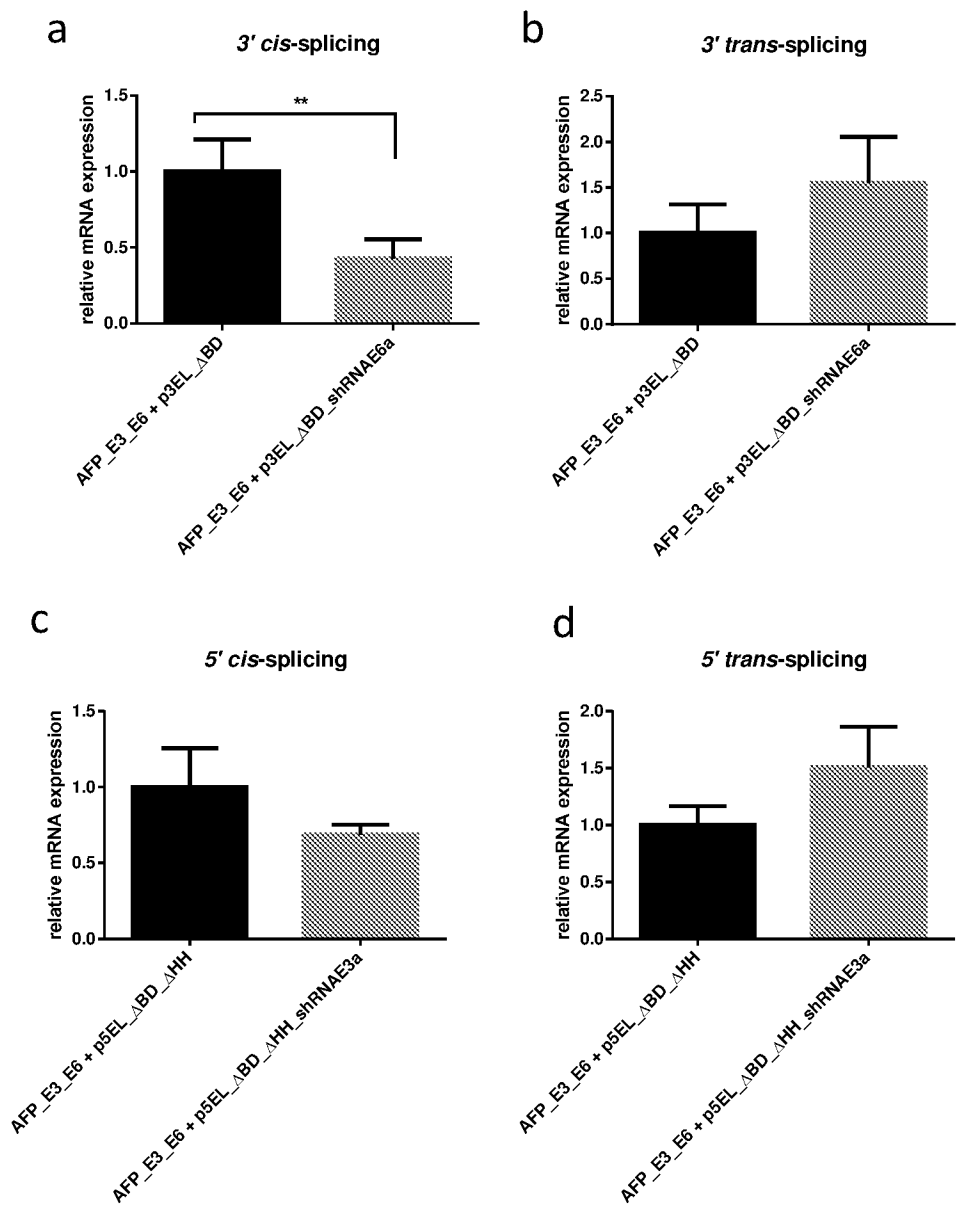
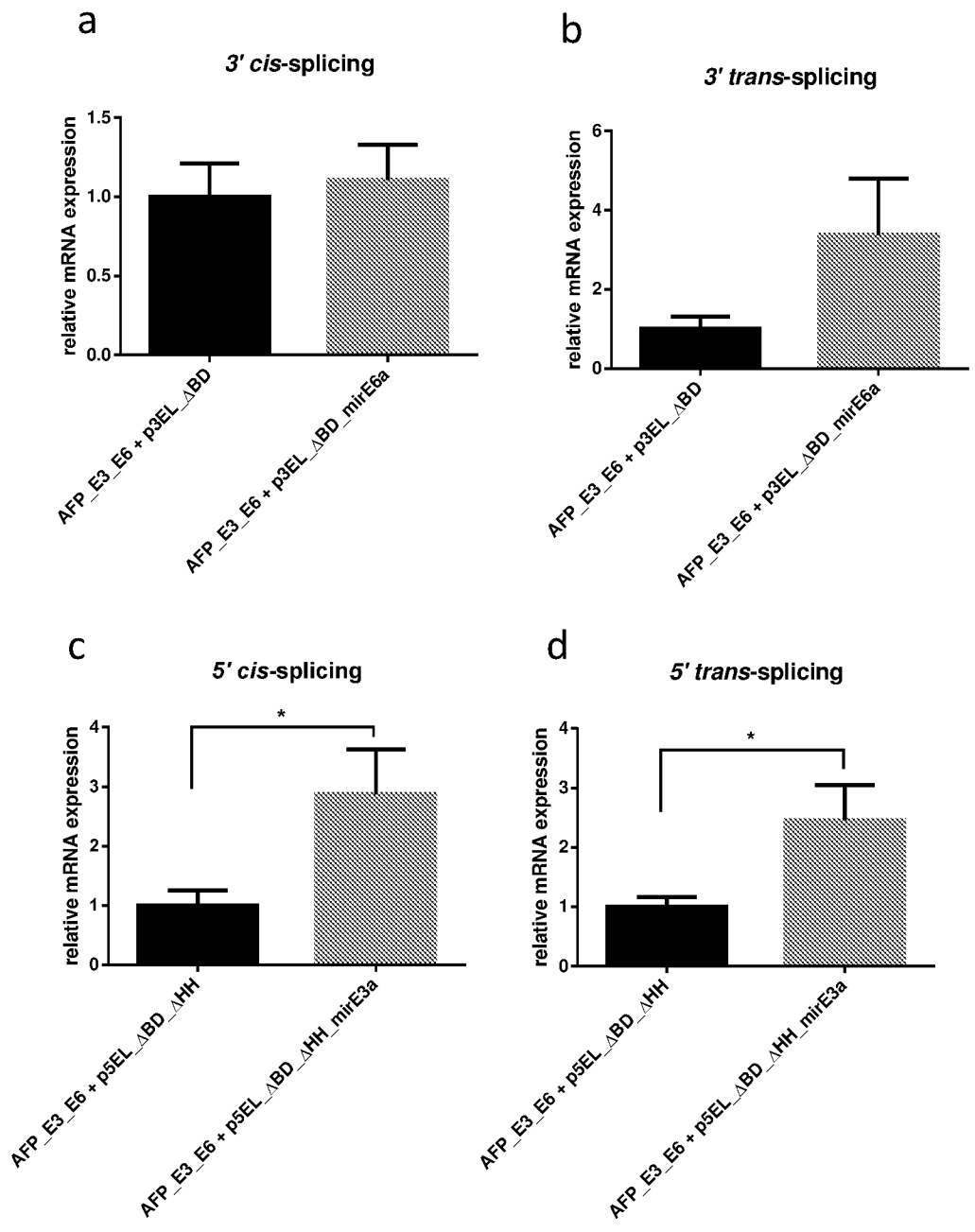


Figure 14



INTERNATIONAL SEARCH REPORT

 International application No.
PCT/SG2016/050350

A. CLASSIFICATION OF SUBJECT MATTER

A61K 48/00 (2006.01) C12N 15/09 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, BIOSIS, WPIAP, EPODOC, ESP@CE, GOOGLE; trans-splicing, smart, tsrna, RNAi, interfering RNA, siRNA, shRNA, antisense, ASO, gene silencing, gene therapy, Patzel and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 25 October 2016		Date of mailing of the international search report 25 October 2016	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Anita Cochrane AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832118	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2016/050350
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2005/070948 A1 (INTRONN, INC.) 04 August 2005 Abstract, pages 5, 9, 13 & 15 As above	1-8, 10-28 9
Y	WO 2010/012472 A1 (MITCHELL, LLOYD, G.) 04 February 2010 Abstract, pages 7, 12-13 & 22-23	9
X Y	KOLLER U et al, "Trans-Splicing Improvement by the Combined Application of Antisense Strategies", International Journal of Molecular Sciences, 6 Jan 2015, 16: 1179-1191 Abstract, pages 1182 & 1187 As above	1-8, 10-28 9
X Y	SHABABI M et al, "Combination of SMN Trans-Splicing and a Neurotrophic Factor Increases the Life Span and Body Mass in a Severe Model of Spinal Muscular Atrophy", Human Gene Therapy, 2011, 22: 135-144 Abstract, pages 138 and Figure 1 As above	1-8, 10 -28 9
P,A	BERGER A et al, "mRNA trans-splicing in gene therapy for genetic diseases", WIREs RNA, 2016, 7: 487-498 Abstract, Figure 1	

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☒ Claims Nos.: **29**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Supplemental Box
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of Box II**

The claim does not comply with Rule 6.2(a) because it relies on references to the description and/or drawings.

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/SG2016/050350	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2005/070948 A1	04 August 2005	WO 2005070948 A1	04 Aug 2005
		US 2006234247 A1	19 Oct 2006
		US 8053232 B2	08 Nov 2011
WO 2010/012472 A1	04 February 2010	WO 2010012472 A1	04 Feb 2010
		EP 2151248 A1	10 Feb 2010
		EP 2320952 A1	18 May 2011
		EP 2320952 B1	04 May 2016
		JP 2011529333 A	08 Dec 2011
		JP 5735912 B2	17 Jun 2015
		US 2013059901 A1	07 Mar 2013
		US 8735366 B2	27 May 2014
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			