Progress toward therapy with antisense-mediated splicing modulation

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Abstract

Antisense oligonucleotides (AO) or antisense RNA can complementarily bind to a target site in pre-mRNA and regulate gene splicing, either to restore gene function by reprogramming gene splicing or to inhibit gene expression by disrupting splicing. These two applications represent novel therapeutic strategies for several types of diseases such as genetic disorders, cancers and infectious diseases. In this review, the recent developments and applications of antisense-mediated splicing modulation in molecular therapy are discussed, with emphasis on advances in antisense-mediated splice targeting, applications in diseases and systematic delivery.

Keywords

Alternative splicing; antisense oligonucleotide; cancer; genetic disorders; molecular therapy; splicing modulation

Introduction

Pre-mRNA splicing is an essential step in eukaryotic gene expression, and has become a novel target for drug design. Splicing-targeted antisense approaches can lead to potent modulation of disease-related gene expression, either to restore gene function by reprogramming gene splicing or to inhibit gene expression by disrupting splicing. Therefore, antisense-mediated splicing modulation potentially represents a novel therapeutic strategy for several types of diseases such as genetic disorders, cancers and infectious diseases.

Antisense-mediated splicing modulation has generally included the use of antisense oligonucleotides (AOs). An alternative approach to this method uses expression vectors to produce antisense RNA inside the cell. AOs or antisense RNA could complementarily bind to a target site in pre-mRNA, and regulate the splicing process. 2′-*O*-Methyl (2′-O-Me) and 2′-*O*-methoxyethyl (2′-MOE) phosphorothioate oligomers are the two most widely used, early-generation, splicing modulation AOs. A newer generation of AOs has been developed, mainly by chemical modifications of the furanose ring of the nucleotide, to further enhance target affinity, biostability and pharmacokinetics [1]. Three of the most promising types of new-generation AOs are: (i) phosphoroamidate morpholino oligomer (PMO); (ii) peptide nucleic acid (PNA); and (iii) locked nucleic acid (LNA) [2–5].

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This review discusses the most recent developments and applications of antisense-mediated splicing modulation in molecular therapy in which AOs are most commonly used, with an emphasis on advances in antisense-mediated splicing targeting, applications in diseases, systematic delivery of AOs, as well as the possible future directions for this area of research.

Antisense-mediated splice targeting

AOs or antisense RNA can be used in two contrasting ways to manipulate pre-mRNA splicing: reprogram pre-mRNA splicing or knockdown gene expression (Figure 1).

Antisense-mediated splicing reprogramming is primarily used to restore the function of the deficient gene in genetic diseases. The reprogramming blocks cryptic splicing sites, created by an inherited mutation, to redirect splicing back to the correct SSs (Figure 1A). This approach has been successfully used to correct splicing mutations in several genes, such as *CFTR* (cystic fibrosis transmembrane conductance regulator), *HBB* (β-globin), *tau*, *LMNA* (lamin A/C) and *Oa1* (osteoarthritis QTL 1) [6–8]. AOs can also be used to induce exon skipping to delete a region of pre-mRNA that contains a mutation (Figure 1B). For example, this approach has been evaluated for the treatment of Duchenne muscular dystrophy (DMD) [9]. Furthermore, AOs can be used to regulate alternative splicing, such as in *Bcl-x*, *IL-5*, *SMN2* (survival motor neuron 2), *FGFR1* (fibroblast growth factor receptor 1) and *MyD88* (myeloid differentiation primary response gene 88) [6,10,11]. In eukaryotes, in addition to the correct recognition of exon-intron junctions, accurate splicing is also dependent on regulatory sequence elements within exons and introns, such as exonic splicing enhancers and silencers (ESEs and ESSs, respectively). Recently, these regulatory elements have been targeted by antisense approaches to modulate splicing outcome (Figure 1C). Moreover, some efforts have been made to improve the efficiency of splicing modification by combining different AOs, or AOs and siRNAs; some recent advances are discussed below. Lastly, AOs or antisense RNA can also be used to knockdown gene expression by interrupting splicing, such as inducing exon skipping (Figure 1B), and several strategies have been developed for this purpose.

Targeting the regulatory elements of splicing

Spinal muscular atrophy (SMA) is caused by the homozygous loss of *SMN1*. *SMN2*, an almost identical copy of the *SMN1* gene, is present in all patients with SMA, but is nonfunctional due to exon 7 skipping. Exon 7 skipping in SMN2 is mainly caused by a single nucleotide substitution in the affected exon [12]. This exon is included in a small proportion of SMN2 transcripts, thus producing low levels of functional SMN2 protein. In patients with SMA, the low level of full-lengthSMN2 protein is not sufficient to compensate for the loss of SMN1, and thus cannot maintain the viability of α-motor neurons; the loss of α-motor neurons leads to disease development [13]. Theoretically, if exon 7 could be reintroduced, SMN2 would be functional. The reintroduction of exon 7 represents a potential treatment approach for patients with SMA [14,15]. Hua *et al* identified an essential core sequence in exon 7 of SMN2 that is surrounded by two inhibitory regions containing ESSs [16]. Blocking or masking any part of the central core sequence promoted skipping of exon 7, whereas blocking either of the two ESS-containing regions that flank exon 7 promoted efficient exon 7 inclusion. If combined with blocking of the intronic splicing silencers (ISSs) within intron 7 of SMN2 [17,18], these elements could provide promising molecular targets for treating SMA.

Cystic fibrosis (CF), caused by mutations in the *CFTR* gene, is a disease model that could be used to study therapeutic approaches targeting ISSs. The approximately 150 nt ISS of CFTR exon 9 promotes the exclusion of exon 9 in the mature mRNA, and exon 9 exclusion is related to the occurrence of monosymptomatic and full forms of CF. Buratti *et al* successfully mapped the binding sites of serine/arginine-rich protein (SR) trans-acting factors responsible for the exclusion of exon 9 into an approximately 40-nt long region [19]. It would be interesting to

determine whether blocking these SR protein binding sites using AOs could restore exon 9 inclusion in the mature CFTR mRNA.

AO-mediated exon skipping to restore a disrupted reading frame caused by mutations has been tested in DMD [9]. AO-blocking of ESEs efficiently induced exon skipping of the dystrophin gene. A collection of AOs targeting putative ESE sites effectively induced the specific skipping of 38 of the 79 different DMD exons [20]. However, this approach is limited to genes with highly repetitive exons, such as DMD.

ESEs could also provide appropriate targets for silencing disease-causing gene expression [21]. Khoo *et al* used different sequence-targeting AOs, including AOs against ESEs and splice sites, to induce apolipoprotein B (apoB) exon 27 skipping to interrupt the reading frame and silence the expression of apoB [22]. AOs against ESEs induced skipping of exon 27, but much less efficiently than AOs against splice sites [22]. These results suggested that downregulation of apoB100 by exon skipping could be a potential therapeutic approach for lowering circulating LDL and cholesterol levels.

The use of combinations of AOs for modifying splicing

Generally, a well-designed AO can reprogram or silence pre-mRNA splicing. However, efficiency needs to be improved to achieve therapeutic effects in many situations. The use of cocktails or combinations of AOs against different sites in pre-mRNA has been investigated. For example, specific combinations of 2′-O-Me AOs improved exon skipping by targeting two putative splicing regulatory sequences within one exon in DMD. Such double targeting was effective even for the distal 'unskippable' exons 47 and 57 [23]. AO cocktails have also been tested with different chemistries, 2′-O-Me and PMOs, in DMD [24]. In the *apoB* gene, AOs targeting a routine splice site in combination with AOs against a branch-point sequence were the most effective at inducing exon 27 skipping [22].

Combining splicing modification with siRNA-mediated gene knockdown

In patients with β-thalassemia, a mutated β-globin gene causes deficient synthesis of functional β-chain and, consequently, leads to excessive free α-globin chains precipitating at the erythrocyte membrane, resulting in hemolytic anemia. Theoretically, rebalancing the α/β-globin expression ratio could alleviate the disease. Xie *et al* combined two different strategies to rebalance α/β-globin expression in single-cell zygotes of a β^{654} mouse model of β-thalassemia: siRNA to knockdown α-globin mRNA, and AOs to correct splicing-deficient β-globin [25]. Three sets of transgenic mice with short hairpin RNA, antisense RNA or combined constructs were analyzed. Significant restoration of β-thalassemia was observed in all transgenic mice. The F1 progeny of these treated mice also demonstrated significant restoration of β-thalassemia, particularly in those receiving combined constructs, implying the utility of the combined strategy for gene therapy.

Gene knockdown by antisense-mediated splicing modulation

Pre-mRNA splicing also provides a target for knockdown of disease-related gene expression. Some recent examples of this approach are discussed.

Antisense-induced exon skipping can disrupt reading frames—Disrupting the reading frame of a gene leads to the premature termination of protein synthesis, and could also trigger the degradation of mRNA through a mechanism known as nonsense-mediated decay [26]. Theoretically, disrupting the reading frame provides an alternative method for downregulating gene expression. Antisense-induced exon skipping was used to inhibit HIV-1 multiplication by targeting the HIV-1 regulatory proteins Tat and Rev. Asparuhova *et al* used antisense U7 RNAs to induce a partial skipping of the Tat and Rev internal exons, and repress

the expression of these proteins [27]. HIV-1 multiplication in lymphocytes was inhibited by the most efficient constructs, suggesting a new therapeutic approach in the treatment of HIV.

Inhibition of RNA splicing via branch-point modification—The 2′-OH group of the branch point adenosine is a key moiety that initiates the splicing reaction by attacking the phosphate at the 5′ exon-intron junction [28]. Most eukaryotic box C/D small nucleolar RNAs (snRNAs) guide base-pairing with target RNAs and direct site-specific 2′-*O*-methylation [29]. Zhao *et al* designed artificial box C/D snRNAs to target 2′-*O*-methylate in *Saccharomyces cerevisiae* pre-mRNAs at the adenosine branch point, resulting in efficient blocking of splicing initiation [30]. This new approach could be applied to target other genes by changing the guide sequence in the C/D snRNAs. However, this system has only been tested in yeast; whether it would be successful in pre-mRNA modification in higher eukaryotic systems remains uncertain.

Antisense U7 snRNAs

To increase the longevity of AOs, an antisense U7 snRNA expression system was originally developed to produce antisense RNA in cells [31]. This method has been investigated in DMD, SMN2 and β-globin [32–34]. Because U7 snRNAs are nuclear, the design ensures the accumulation of antisense RNA in the nucleus; localization to the nucleus is especially useful for splicing modulation. Because the antisense U7 snRNAs are generated continuously by the expression vector within the cell, efficient modulation of splicing is persistent over many cell divisions, as long as the expression vectors are retained.

U7-snRNAs have been successfully used to block mutation-activated cryptic splicing sites in PTCH1, BRCA1 and CYP11A, and restore full-length protein [35]. Bifunctional U7 snRNAs also demonstrated more permanent correction effects than AOs in SMA [36]. When used for gene silencing, U7 antisense snRNA also improved the efficiency of splicing modulation. For example, U7-snRNA-mediated splicing disruption of Tat and Rev efficiently inhibited HIV multiplication [27]. However, this method requires vector expression systems, similar to conventional gene therapy approaches, and the systematic delivery and safety risk of the constructs remain major concerns.

Applications in disease

As alluded to above, splice-targeted antisense approaches have been tested in many genetic diseases, such as DMD, CF, SMA, β-thalassemia, Hutchinson-Gilford progeria syndrome, ocular albinism and ataxia-telangiectasia (A-T). These approaches have also been used in cancers associated with the misregulation of pre-mRNA alternative splicing of *Bcl-x* [37] and *FGFR1* [38]. The applications of this approach in DMD are the most advanced; the first clinical trial of AO therapy for DMD has been completed (see below). In addition, splice-targeted gene knockdown approaches have been developed for treating cancers and infectious diseases.

Genetic disorders for splice-targeted therapy

Ataxia-telangiectasia—A-T is a progressive autosomal recessive disorder resulting from mutations in the *ATM* (A-T mutated) gene. Approximately half of the unique ATM mutations are splicing mutations [39]. In one study, three types of splicing mutations for correction were selected: a 5′ exonic cryptic splice site variant, a 3′ exonic cryptic splice site variant, and a pseudo-exon inclusion variant. Antisense PMOs corrected ATM splicing, producing up to 20% functional full-length ATM protein that restored the radiosensitive cellular phenotype of A-T cells [40]. If modest increases in functional ATM protein levels could improve the disease phenotype, A-T may provide an exciting model for exploring splice-targeted therapeutic approaches.

Methylmalonic acidemia and propionic academia—In a study similar to the A-T study, Ugarte *et al* used PMOs to restore normal splicing caused by intronic mutations in the genes for methylmalonic acidemia and propionic acidemia [41]. Three point mutations were located in deep intronic regions of three genes, *MUT* (methylmalonyl coenzyme A mutase), *PCCA* (propionyl coenzyme A carboxylase, α polypeptide) and *PCCB* (propionyl coenzyme A carboxylase, β polypeptide); these mutations caused the introduction of pseudoexons into the transcripts. PMOs were designed to mask the 5′ or 3′ cryptic splice sites of the potential pseudoexonic regions. Restoration of correctly spliced mRNA led to effective protein synthesis, and the activities of the enzymes coded by these genes were consequently restored in the fibroblasts of patients.

Dystrophia myotonica—The neuromuscular disease myotonic dystrophy (DM) is caused by microsatellite repeat expansions in the *DMPK* (dystrophia myotonica-protein kinase) and *ZNF9* (CCHC-type zinc finger, nucleic acid binding protein) genes [42]. These nucleotide repeat expansions are associated with aberrant splicing in DM cells. In DM, an increase in the excitability of skeletal muscle leads to repetitive action potentials, stiffness and delayed relaxation. This constellation of features is associated with abnormal alternative splicing of the muscle-specific chloride channel (ClC-1) and reduced conductance of chloride ions in the sarcolemma [43]. Wheeler *et al* reported that targeting the 3′ splice site of ClC-1 exon 7a with PMOs could reverse the defect of ClC-1 alternative splicing in two mouse models of DM. This further led to expression of the full-length protein in the surface membrane, normalized muscle ClC-1 current density and deactivation kinetics, and elimination of myotonic discharges [44], indicating the potential of AOs in correcting alternative splicing defects in DM.

Duchenne muscular dystrophy—Antisense-mediated exon skipping for the treatment of DMD can remove nonsense mutations or frame-shifting mutations from mRNA [9], and thus far presents the most promising therapeutic strategy for DMD. Recently, this strategy has been further validated.

Wilton *et al* concluded that every exon targeted could be removed from the dystrophin mRNA, not withstanding some variable efficiency for different exons. Interestingly, no single motif was identified as a universal target site of AOs [45]. The length of AOs can affect the efficiency of splicing modulation [46]. Furthermore, AO cocktails have proven to be more efficient than a single AO [23,24]. Gurvich *et al* identified several novel mutations that cause pseudoexon inclusion in patients with DMD, and concluded that aberrant splicing caused by pseudoexons could be corrected using AOs [47]. In an experimental model, McClorey *et al* extended the AO-meditated exon-skipping studies from animal models or cultured human muscle cells to human muscle explants, which more closely resemble *in vivo* conditions; similar results were achieved [48].

Perhaps the most interesting data are those from the first clinical trial of AOs for DMD [49]. A 2-′O-Me AO, PRO-051 (Prosensa Therapeutics BV), was evaulated in patients (n = 4) with DMD. PRO-051 (0.8 mg) efficiently restored dystrophin expression by the introduction of exon 51 skipping in treated muscle fibers of all four patients after a single injection into the tibialis anterior muscle of the leg. In a biopsy taken 4 weeks after the injection, novel dystrophin expression was observed in the majority of muscle fibers (64 to 75%), with protein levels of dystrophin that are expected to be clinically relevant (3 to 12% of the total protein extract of that found in the control specimen and from 17 to 35% of that of the control specimen in the quantitative ratio of dystrophin to laminin α2.). The robust expression of dystrophin protein after a single injection indicates the positive effects of the AO. This is the first clinical trial of an RNA-based therapeutic agent for DMD and, therefore, is an important step towards treatment of this disease. This therapy may represent the beginning of personalized mutation-based molecular medicine [50].

Prior to this clinical trial, only a single case report existed of a patient with DMD treated with an AO to restore the reading frame by skipping exon 19 [51]. The 10-year-old patient with an out-of-frame, exon 20 deletion in the dystrophin gene was treated with a 31-mer phosphorothioate oligonucleotide (0.5 mg/kg iv) against the splicing enhancer sequence of exon 19 for 4 weeks, at 1-week intervals. One week after the final infusion, exon 19-skipped, in-frame mRNA was identified in the muscle. The dystrophin protein was also detected in the sarcolemma of muscle cells after treatment. The AO-induced exon skipping levels in this study were low (≤6% correction in mRNA level) probably due to the low dose and the suboptimal chemistry of the AO.

Targeting diseases using antisense-mediated gene silencing

AO-mediated splice modulation provides a novel method for silencing or knocking down gene expression, and thus extends the clinical potential of AO therapy. As discussed previously, these approaches were developed to knockdown HIV regulatory proteins Tat and Rev [27], as well as to decrease LDL levels by inhibiting apoB expression [22].

Delivery of AOs

One of the major obstacles to the successful application of all RNA-based therapies is that of antisense delivery to target tissues; this obstacle is further complicated by the potential instability of antisense RNA in blood and degradation in cells. The lipidic nature of biological membranes is the major impediment to the intracellular delivery of macromolecules such as AOs. Furthermore, entrapment within endocytic vesicles and degradation in the lysosome also need to be avoided to maintain AO activity. Various carrier systems, such as cell-penetrating peptides (CPPs), liposomes, cationic lipids and polymers, and polymeric nanoparticles, have been developed and evaluated for efficient intracellular AO delivery. Some recent advances are discussed below.

Cell-penetrating peptides

CPPs are a class of small cationic peptides of approximately 10 to 30 amino acids that can be used as transmembrane drug delivery agents through various forms of endocytosis for low-molecular weight compounds, including drugs, imaging agents, oligonucleotides, peptides and proteins [52]. CPPs are also known as 'protein transduction domains'. Earlier-identified CPPs, such as Tat or penetratin, had been used to deliver neutral AOs, such as PNA and PMO; however, the entrapment of AOs within endocytic vesicles limited delivery efficiency [53]. Substantial progress in developing improved CPPs has been made recently.

Arginine-rich cell-penetrating peptides—Several novel arginine-rich CPPs have been developed for improved delivery of PNAs and PMOs. Morris *et al* described a peptide, Pep-3, with the potential for *in vivo* delivery of charged PNA and DNA mimics [54]. This peptide combines a tryptophan/phenylalanine domain with a lysine/arginine-rich hydrophilic motif, and can form stable nano-size complexes with both uncharged and charged PNAs. Pep-3 promoted the efficient delivery of PNAs into several cell lines, without any associated cytotoxicity. Pep-3 also successfully delivered PNA *in vivo* via intratumoral and intravenous administration in mice. Another arginine-rich peptide, M-918, derived from the tumor suppressor protein p14AR, also improved PNA delivery into several cell types, such as HeLa, human breast cancer cells and CHO [55].

Moreover, Abes *et al* developed two arginine-rich CPPs, $(RXR)_4XB$ and R(6)Pen [56,57], that allowed efficient nuclear delivery of splice-correcting PNAs and PMOs at micromolar concentrations. The $(RXR)_4XB$-PMO conjugate could avoid uptake by lysosomes and access the nuclear compartment and, therefore, more efficiently deliver PMO analogs. This $(RXR)_4$

CPP can deliver PMOs directly into primary murine leukocytes [58]. The *in vivo* efficacy of $(RXR)_4$-PMO conjugates has been demonstrated in a mouse model of DMD [59]. After intraperitoneal injection, this CPP enhanced cell uptake of the PMO, resulting in widespread dystrophin expression in all muscles examined, except for cardiac muscle.. *In vivo* disposition of $(RXR)_4$-PMO was also investigated in rats [60]. After intravenous administration, conjugation to CPP increased the uptake of PMO in all tissues, except brain tissue. $(RXR)_4$-PMO did not demonstrate any obvious toxicity at a dose of 15 mg/kg [60].

RVG-9R: The first CPP to cross the blood-brain-barrier—Kumar *et al* fused a short peptide derived from rabies virus glycoprotein (RVG) to a nine arginine siRNA packaging peptide (RVG-9R); this conjugate enabled the transvascular delivery of siRNA specifically to target neuronal cells expressing the nicotinic ACh receptor (nAChR) in the brain [61]. Intravenous treatment with RVG-9R-bound siRNA against flavivirus induced robust protection against fatal viral encephalitis in mice. This study was the first to demonstrate that the systemic delivery of macromolecular oligonucleotides can traverse the blood-brain barrier and specifically target the brain of adult mice. Considering that the blood-brain barrier precludes the entry of large molecules into the brain, this finding could make treating numerous neurological disorders possible.

Cationic lipids

Cationic lipids and cationic polymers have been widely used for the delivery of charged AO analogs, such as 2′-O-Me or 2′-MOE. However, most of the available commercial formulations are toxic and unstable in the presence of serum proteins, and the delivery efficiency is also limited [62]. Resina *et al* designed a novel dioctadecyldimethylammonium chloride/ dioleoylphosphatidylethanolamine (DOGS/DOPE) liposome formulation, DLS, that mediated the efficient nuclear delivery of negatively charged 2′-O-Me and 2′-MOE in serum-supplemented cell culture [53]. DLS-packed 2′-O-Me or 2′-O-MOE induced more efficient splicing correction than newly-developed R-Ahx-R-conjugated PNAs *in vitro*.

Nanoparticles

Nanoparticles have been used for the delivery of nucleic acids (DNA, RNA and oligonucleotides) due to their ability to penetrate the cell wall and deliver biomolecules into living systems [63–65]. The majority of these applications were for the delivery of vectors and siRNA; however, nanotechonolgy has also been applied to the deliverly of AOs. The nano-sized dendritic α,ε(-poly(L-lysine)s (DPL) can efficiently deliver DPL-2′-OMe complexes and correct pre-mRNA splicing in cells [66]. A Pep-3-based nanoparticle system can efficiently deliver PNA mimics into living cells and animal tumor models [54]. Seferos *et al* have described LNA nanoparticle conjugates that form stable duplexes with complementary target sequences, and readily enter cells [67]. Moreover, PMOs were also used to prepare a MORF/ streptavidin/tat nanoparticle to improve efficiency of cellular delivery [68]. Taken together, these reports suggest promise for the delivery of the three main AOs (PNA, PMO and LNA) to be delivered as nanoparticles.

Conclusion

Antisense-mediated splicing modification can manipulate gene expression in two ways, either to restore gene function by reprogramming gene splicing or to knockdown gene expression by interfering with normal splicing. These approaches present novel therapeutic strategies for a variety of diseases. Although significant progress has been achieved, the clinical application of antisense-mediated splicing modulation remains limited by the specificity and efficiency of pre-mRNA targeting, systemic delivery, and the largely untested *in vivo* safety of antisense agents. The novel systemic delivery systems need to be evaluated for *in vivo* delivery efficiency,

especially after long-term administration of AOs. The potential off-target effects caused by unspecific AO targets must also be considered and investigated.

Considering that the majority of human genes undergo alternative splicing and an increasing number of diseases are found to be caused by alternative splicing [69–71], many more potential applications of AOs can be expected. Also, knocking down gene expression by antisense approaches may have potential for treating certain cancers and infectious diseases. Therefore, the fields of targeting strategy, AO chemistry [72–74], and systematic delivery of AOs have a promising future in splice-targeted antisense-mediated therapy.

References

•• of outstanding interest

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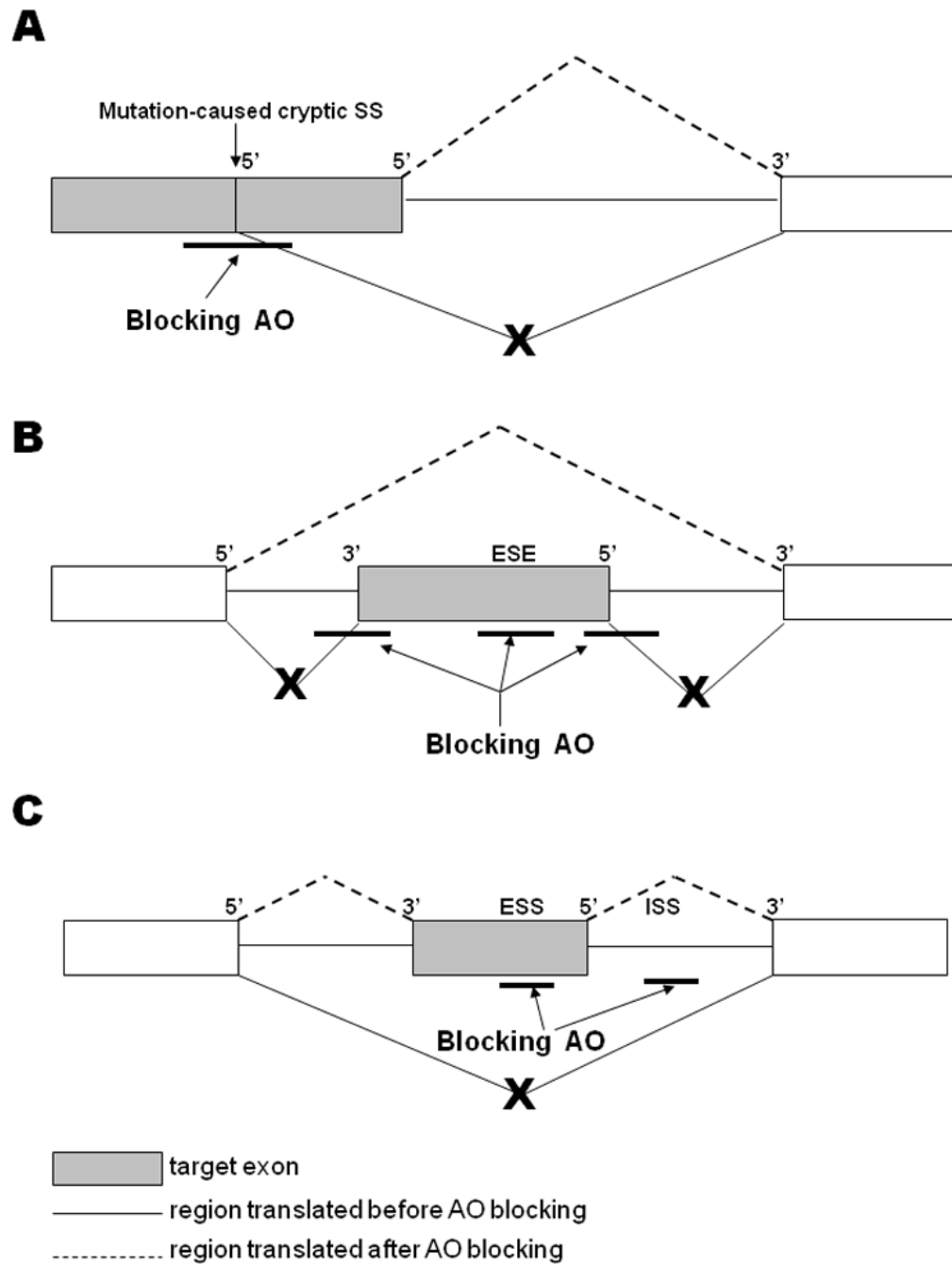
Figure 1. Schematic demonstration of antisense-mediated splicing modulation
(**A**) Antisense oligonucleotide (**AO**) blocking of a mutation-induced cryptic 5′ splicing site
(**SS**). (**B**) AO blocking of exon-intron junctions and/or exonic splicing enhancer (**ESE**) site to
induce exon skipping. (**C**) AO blocking of exonic splicing silencer (**ESS**) or intronic splicing
silencer (**ISS**) sites to enhance exon inclusion.