RNA interference: a potential therapeutic tool for silencing splice isoforms linked to human diseases

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Alternative splicing of precursor messenger RNAs (pre-mRNAs) is one of the most important sources of protein diversity in vertebrates. An estimated 35%–70% of human genes generate transcripts that are alternatively spliced, and defects in this process are linked to numerous human genetic diseases and various forms of cancer. The discovery that 21–23 nucleotide RNA duplexes, known as small interfering RNAs (siRNAs), can knockdown the homologous mRNAs in mammalian cells has revolutionized many aspects of drug discovery including down-regulation of disease-associated splicing isoforms. In addition, RNA interference (RNAi)-mediated silencing of splicing regulators has the potential to define the complex network of alternative splicing regulation and to analyze gene function. In this review, I first provide a brief introduction to mRNA splicing and its relationship to human diseases. This is followed by a brief overview of RNAi. Finally I discuss the therapeutic potential of RNAi in targeting disease-linked splicing isoforms.

INTRODUCTION

The majority of metazoan protein-coding genes contain intervening sequences (introns) whose removal from precursor messenger RNAs (pre-mRNAs) is an essential step in gene expression. The excision of these noncoding sequences (i.e., introns) and joining of coding sequences (exons) is catalyzed by a large macromolecular complex called the spliceosome, which is composed of five small nuclear RNAs (U1, U2, U4, U5, and U6) and numerous small nuclear ribonucleoprotein (snRNP) and non-snRNP proteins (1,2). Three *cis* acting elements within the pre-mRNA, known as the 5' and 3' splice sites and the branchpoint sequence (BPS) direct the assembly of the spliceosome. Thus, the spliceosome performs two functions: recognition of the intron/exon boundaries and catalysis of the intron removal with simultaneous joining of the exons.

Pre-mRNAs can also undergo alternative splicing, a precisely regulated process in which differential joining of 5' and 3' splice sites of a single

pre-mRNA generates variant mRNAs with diverse, and often antagonistic, functions (3,4). Remarkably, the *Drosophila* Down syndrome cell adhesion molecule (*Dscam*) gene can potentially generate more than 38,000 isoforms by alternative splicing (5–7). There is growing evidence that both extra- and intracellular signaling regulate alternative splicing, which can lead to the skipping or inclusion of exons, retention of introns, shortening or lengthening of exons, and in some cases, inclusions of exons in the mRNA in a mutually exclusive manner (8–11). It has been estimated that 35%–70% of human genes generate transcripts that are alternatively spliced (12). In addition, 70%–90% of alternative splicing events result in the generation of proteins with diverse functions ranging from sex determination to apoptosis (3,13–15). Alternative splicing of pre-mRNA is now recognized as the most important source of protein diversity in vertebrates (16–18).

In general, the splicing signals of alternatively spliced exons do not

match with the consensus sequence (19,20). Although the underlying mechanisms that regulate alternative splicing are poorly understood, it has become increasingly clear that the auxiliary *cis* acting elements, known as exonic and intronic splicing enhancers (ESEs and ISEs, respectively) and exonic and intronic splicing silencers (ESSs and ISSs, respectively), play an important role in the recognition of exons surrounding the regulated splice sites (21). The function of auxiliary *cis* acting elements is to provide the binding surface for splicing regulators, which in cooperation with other factors communicate with the basal splicing machinery to enhance or inhibit splice-site selection (21,22) (Figure 1). The most common splicing regulators are RNA binding proteins that are members of heterogeneous nuclear ribonucleoprotein (hnRNP) (23,24) and serine/arginine-rich (SR) protein families (25,26). Thus, regulation of alternative splicing is a combinatorial phenomenon in which cooperative assembly of the activation and/or repression complexes near the regulated exons determine

whether a particular exon will be included in the mRNA.

ABERRANT SPLICING AND HUMAN DISEASES

The defective regulation of splice variant expression has been identified as the cause of several genetic disorders (27–33), and certain forms of cancer have been linked to unbalanced isoform expression of genes involved in processes ranging from cell cycle regulation to angiogenesis (34–39). In general, aberrant splicing is caused by mutations of the *cis* and *trans* acting elements. Whereas the mutations of the splice sites, the branchpoint, and auxiliary elements are categorized as *cis* mutations, *trans* acting mutations affect the components of the basal splicing machinery or proteins that regulate alternative splicing. Given that aberrant splicing is linked to numerous diseases, the emergence of new technologies for controlling mRNA splicing is not surprising (40–42). Importantly, tools that specifically destroy a disease-linked mRNA isoform will have far-reaching effects in medicine and biotechnology.

EMERGENCE OF RNAi AS A POTENTIAL THERAPEUTIC TOOL

RNA interference (RNAi) is a remarkable phenomenon by which eukaryotes modulate their gene expression at pre- and posttranscriptional levels (43–45). Although

there were some indications of the existence of RNAi in plants (46), the real breakthrough, that RNAi-mediated gene silencing is induced by double-stranded RNA (dsRNA), was demonstrated for the first time in the nematode *Caenorhabditis elegans*, in which worms injected with dsRNAs could silence homologous genes (47). However, dsRNAs induce immune responses that act as a defense mechanism against viral infection in mammalian cells, which presented a major roadblock to the use of dsRNA for gene silencing (48). To overcome this problem, Tuschl and colleagues designed a novel approach whereby transfection of mammalian cells with chemically synthesized short 21- to 22-nucleotide (nt) RNAs with 2-nt 3' overhangs could induce sequence-specific gene silencing without nonspecific inhibition of translation (49). This elegant work combined with the discovery that microRNAs (miRNAs) (50–52) act as endogenous triggers of the RNAi laid the foundation for in vivo expression of siRNAs using short hairpin RNAs (shRNAs) as mimics of miRNAs (53–58). The shRNAs varying in size and length of the stem can be expressed using Pol III promoter, such as U6, H1, and transfer RNA (tRNA) promoters. Alternatively, the Pol III system can be designed to express the sense and the antisense strand of siRNA in tandem using two independent Pol III promoters (reviewed in Reference 59). After transcription, intrinsic RNase III processes shRNAs into siRNA duplexes followed by incorporation of the siRNA strand into the RNA-induced silencing complex (RISC) (for

review see Reference 60). Therefore, both siRNAs and shRNAs are complementary approaches that can silence a targeted gene, and either could be used as method of choice depending on specific situation.

Since the discovery that synthetic siRNA can trigger specific and potent gene silencing in mammalian cells (49), genes that are linked to diseases ranging from neurodegenerative disorders to various forms of cancer have been targeted to evaluate the therapeutic potential of RNAi (43,61,62). Therapeutic potential of RNAi is based on the fact that mammalian cells, unlike *C. elegans*, appear to lack transitive RNAi, a phenomenon by which many secondary siRNAs can target sequences 5' of the target region and can silence significant numbers of unintended genes within the genome (63). Although RNAi holds enormous potential for the treatment of human diseases, its success depends upon the ability to design siRNAs that can selectively silence a disease-causing mRNA without affecting the levels of the wild-type allele or isoform that has an essential function. In addition, while it is relatively easy to design an exon-specific siRNA (64), to target an exon-exon junction generated due to the skipping or inclusion of a particular exon, could be challenging due to partial sequence similarity (65). Here I review studies in which RNAi has been used to target splicing events that are linked to human diseases. Given the rapidly advancing nature of this field, only well-characterized examples of aberrant splicing have been reviewed.

Figure 1. Networks of RNA-RNA, RNA-protein, and protein-protein interactions regulate alternative splice site choice. The GU and AG at intron-exon boundaries represent the 5' and 3' splice sites, respectively. The binding of U1 and U2 small nuclear ribonucleoprotein (snRNPs) to the 5' splice site and the branchpoint sequence (BPS), and U2AF to the 3' splice site region play an important role in spliceosome assembly. Embedded within the BPS is the adenosine (underlined A) whose 2'-hydroxyl group acts as a nucleophile to initiate the first catalytic step of the splicing. The conserved splice site signals and several exonic (ESS and ESE) and intronic (ISS and ISE) *cis* acting elements contribute to splice site pairing mediated by the spliceosome. The competition between splicing activators and repressors for binding to their respective *cis* acting elements and their ability to communicate with the component(s) of the basal splicing machinery determines whether or not a particular exon will be excluded or included. Y, pyrimidine; N, any base; R, purine; ESS, exonic splicing silencer; ESE, exonic splicing enhancer; ISS, intronic splicing silencer; ISE, intronic splicing enhancer.

RNAi-MEDIATED KNOCKDOWN OF DISEASE-LINKED SPLICING ISOFORMS

Targeting Splicing Isoforms that Inhibit Apoptosis

The expansion of genetically altered, infected, or damaged cells is thought to result in the development of cancer. To eliminate these cells, metazoan organisms have evolved the cell suicide mechanism termed apoptosis, also known as programmed cell death (66). A number of genes that regulate apoptosis use alternative splicing as a mechanism to modulate their expression (13,67). The splice variants of these regulatory genes often display antagonistic roles. For example, *Bcl-x*, a member of *Bcl-2* gene family, undergoes alternative splicing to generate two major isoforms, Bcl-xL and Bcl-xS. While Bcl-xL is antiapoptotic (68), forced overexpression of Bcl-xS (69,70) or decreased levels of Bcl-xL sensitize cells to a variety of antineoplastic agents and radiation (71–73). This raises the possibility that RNAi-mediated knockdown of the Bcl-xL isoform may prove to be an important therapeutic tool in the fight against cancer. Indeed, an siRNA designed to specifically destroy the Bcl-xL isoform has been shown to inhibit the proliferation of 5-fluorouracil-resistant human colon cancer cells (74) (Figure 2). Furthermore, siRNA-mediated down-regulation of Bcl-xL isoform in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant cells has been shown to inhibit cell proliferation and sensitize TRAIL-induced apoptosis in human cancer cells with both acquired and intrinsic TRAIL resistance (75).

Fas (APO-1/CD95), a 45-kDa membrane protein that regulates apoptosis in many lymphoid cell types, also uses alternative splicing as a mechanism to regulate apoptosis (76). Published studies indicate that although full-length Fas promotes apoptosis by Fas-Fas ligand interactions, Fas mRNA isoforms lacking exon 6 encode soluble forms of the Fas receptor that prevent programmed cell death by multiple mechanisms (76–78). This finding suggests that like Bcl-xL,

Figure 2. RNA interference (RNAi)-mediated down-regulation of a splicing isoform. Bcl-xL-specific small interfering RNA (siRNA) down-regulates Bcl-xL protein and inhibit the proliferation of 5-fluorouracil and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant cells. D, distal 5' splice site; P, proximal 5' splice site.

siRNA-mediated knockdown of the Fas mRNA isoform that lacks exon 6 may rescue the blockade of Fas-signaling and promote apoptosis.

Targeting Splice Variants of Cancer-Linked Genes

Genes that regulate apoptosis can be categorized as the “general guardians” of the cell. However, a number of other genes regulate cell growth and differentiation in a tissue-specific manner and use alternative splicing as a mechanism to control their activity. For instance, *KLF6*, a key tumor suppressor gene, is mutated and/or lost in prostate cancer tumors and cell lines (79,80). Moreover, decreased levels of *KLF6* expression has been associated with recurrent prostate cancer (79,80). A common *KLF6* germ line mutation containing a single nucleotide polymorphism (IVSΔA allele), which is linked to an increased relative risk of prostate cancer and increased production of three alternative spliced dominant-negative *KLF6* isoforms, was recently characterized (81). Although the wild-type *KLF6* acts as a tumor suppressor and undergoes alternative splicing to generate three isoforms (KLF6 SV1, SV2, and SV3), the IVSΔA allele increases the amount of KLF6 SV1 isoform over the wild-

type, and this increase is predominant in tumor cells (81). In contrast to tumor suppressor function of wild-type KLF6 protein, the KLF6 SV1 isoform displays a markedly opposite effect on cell proliferation, colony formation, and invasion (81), suggesting that a critical balance between KLF6 splice variants and the wild-type *KLF6* is necessary to maintain normal cell function. Therefore, targeting KLF6 SV1 isoform could be an attractive therapeutic strategy for the treatment of prostate cancer. Martignetti and coworkers (82) showed that siRNAs directed against KLF6 SV1, but not KLF6 SV2 reduced tumor growth in nude mice by approximately 50% and decreased the expression of a number of growth and angiogenesis related proteins. Although the efficacy and specificity of the RNAi therapy in humans remain to be tested, the results in preclinical models are significant in terms of target validation.

The *HER2/neu* gene, a member of epidermal growth factor receptor family (83), is overexpressed in approximately 30% of breast and ovarian cancer cases and often indicates a poor prognosis (84,85). Silencing of *HER2/neu* gene by siRNA inhibits proliferation and induces apoptosis in breast cancer cells that overexpress *HER2/neu* (86). In addition, a novel alternative splicing form of

HER2, Δ *HER2*, has been identified in human cell lines and has been suggested to elicit stronger transformation activity than wild-type *HER2* (87). Since this novel splice variant appears to play a regulatory role in *HER2* transformation activity, siRNA that can target Δ *HER2* selectively may turn out to be a more effective therapeutic strategy for treating breast cancer.

Splicing Isoforms Linked to Growth Hormone Deficiency

The gene that encodes human growth hormone (*HGH*) contains five exons and four introns. Removal of all four introns generates mRNA that encodes the full-length 22-kDa HGH protein (Figure 3). However, the mutation of the splice sites or ESE in the *HGH* gene leads to increased levels of an mRNA isoform that lacks exon 3 (88). In addition, two single base pair substitutions (exon 3 +5 A→G, ESEm1) and a 17-nt deletion in intron 3 (IVS3 Δ 28–45, ISEm2) result in a similar phenotype. The exon 3-skipped mRNA encodes a 17.5-kDa protein that is linked to isolated HGH deficiency (IGHD) type II, an autosomal dominant form of HGH deficiency (GHD) (Figure 3) (89,90). Because exon 3-skipped GH1 transcript encodes a protein that adversely affects normal growth hormone function and secretion (88–90), therapies that specifically target the 17.5-kDa isoform would be useful in patients with IGHD II. Patton and colleagues demonstrated that siRNA designed to

target the exon 2-exon 4 junction of GH1 mRNA, the sequence exclusively present in the 17.5-kDa isoform, specifically degraded transcripts encoding the dominant-negative 17.5-kDa isoform in cultured cells (65). Undoubtedly, RNAi has the potential to be an effective alternative to the current HGH replacement therapy, which has many side effects such as insulin resistance and benign intracranial hypertension edema (91); however, additional in vivo experiments with model organisms will be required to determine the efficacy of this approach.

Splicing Isoforms Linked to Neurological Disorders

Mutations in the human *tau* gene causes a dominant inherited neurodegenerative disease, also known as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (92). In addition, several other neurodegenerative disorders, such as progressive supranuclear palsy and cortical-basal ganglionic degeneration, have been associated with the mutations in the *tau* gene (93). While some *tau* mutations promote aberrant splicing, others lead to the expression of mutant proteins (94–96). A V337M mutation, located in exon 12 of tau mRNA has been linked to frontotemporal dementia. Identifying a siRNA that silences a specific mutant allele that differs from the normal allele by only a single base can be a challenging task. By using a series of chemically synthesized and in vitro transcribed

siRNAs, Paulson and colleagues were able to selectively knockdown the *tau* allele (with V337M mutation) without altering the expression of normal allele (Figure 4) (97,98). To demonstrate the generality of this approach, these authors also targeted the transcript encoding amyloid precursor protein (APP), the precursor of neurotoxic β -amyloid ($A\beta$) that plays a central role Alzheimer's disease (AD) (97,98). To make allele specific knockdown even more selective, Kubodera et al. (99) designed a new strategy to target spinocerebellar ataxia type 6 (SCA6). In this approach, the expression of normal and mutant alleles was inhibited by a single but most effective siRNA. Next, a modified mRNA that is resistant to the siRNA was used to restore the expression of wild-type protein. These studies suggest that carefully designed, double-stranded siRNAs may permit specific silencing of a dominant mutant allele that differs from the wild-type allele by a single nucleotide.

TARGETING GENES THAT AFFECT THE FUNCTION OF SPLICING MACHINERY

Given the central role of splicing in numerous aspects of cell growth and development, it is expected that mutations in genes that encode splicing factors for basic splicing machinery could be involved in diseases. Spinal muscular atrophy (SMA) and retinitis pigmentosa are two known diseases linked to the genetic mutations that affect assembly of the spliceosome.

Spinal Muscular Atrophy

SMA, the second most common autosomal recessive disorder, is caused by homozygous deletions/mutations in the survival motor neuron 1 (*SMN1*) gene (100,101). The *SMN1* gene encodes an essential protein, SMN, that is ubiquitously expressed in all metazoan cells and is part of a stable multiprotein complex present in the cytoplasm and in nuclear gems (102,103). Although a nearly identical copy of *SMN1*, *SMN2* is present in the human genome as an inverted duplication on 5q13; a critical C→T substitution at position 6 of exon

Figure 3. RNA interference (RNAi)-mediated suppression of exon 3 skipped human growth hormone (HGH) isoform. A small interfering RNA (siRNA) designed to target the exon2-exon 4 junction specifically degrade exon 3-skipped transcripts (65). mRNA, messenger RNA.

7 in *SMN2* results in alternative splicing with exclusion of exon 7 as the major spliced product (101,104). Thus, *SMN2* generates limited amounts of full-length protein and appears to complement *SMN1* loss in most cell types, but not in motor neurons. Two different models have been proposed to account for the differences between the splicing of *SMN1* and *SMN2*. The first model proposes that the presence of a splicing factor 2/alternative splicing factor (SF2/ASF)-specific splicing enhancer within exon 7 of *SMN1* helps its inclusion in the mRNA, and a C→T transition in *SMN2* disrupts SF1/ASF binding, thereby preventing the inclusion of exon 7 (105). According to the second model, rather than disrupting an ESE, the C→T transition in *SMN2* creates an ESS whose binding by heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) protein represses exon 7 inclusion (106). Whether it is the abrogation of an enhancer element associated with SF2/ASF or the gain of a silencer element associated with hnRNP A1, forced exon 7 inclusion in *SMN2* may prove to be a potential therapy for the treatment for SMA. Among various approaches that have been tested to improve exon 7 inclusion (107–109), siRNA-mediated knockdown of hnRNP A1 appears to hold promise for therapeutic intervention (106). Manley and colleagues showed that transfection of living cells with siRNAs designed to specifically knockdown hnRNP A1 and the related protein hnRNP A2 promotes efficient *SMN2* exon 7 splicing (106).

Retinitis Pigmentosa

Mutations in the systemically expressed pre-mRNA splicing-factor genes *PRPF31*, *HPRP3*, and *PRPC8* are linked to the autosomal-dominant retinitis pigmentosa (adRP), a genetic

eye disease affecting the retina. These genes are the human orthologs of the yeast genes *PRP31*, *PRP3*, and *PRP8*, respectively (110–112). In yeast, these genes are thought to be involved in the functional assembly of the U4/U6. U5 tri-snRNP, and their human orthologs, were found to be associated with the functional spliceosome (113). It appears that mutations of the above mentioned splicing factors could interfere with the assembly of the functional spliceosome, which most likely results in a tissue specific dominant-negative effect. Recently, using human retinal pigment epithelium (ARPE19) cells, Hu and colleagues (114) demonstrated that RNAi-mediated knockdown of endogenous *PRPF3*, which encodes the human splicing factor Hprp3 and is also linked to adRP, led to cell death. Importantly, the authors showed that this phenotype could be rescued by exogenous *HPRP3*. To avoid the RNAi-mediated knockdown of exogenously expressed *HPRP3*, shRNAs were designed to target only the untranslated regions (UTRs; 5' and 3' UTRs) of the endogenous *HPRP3*, which are absent in the exogenous gene. Thus, RNAi-mediated specific knockdown of a defective splicing factor coupled with the ability to exogenously express the wild-type copy may provide therapy for RP and may have broad therapeutic implications in other diseases.

RNAi FOR DISSECTING THE ROLE OF SPLICING REGULATORS

That 35%–70% of human genes encode alternatively spliced transcripts (12) suggests that a large number of proteins must be involved in regulating alternative splicing. However, only a limited number of splicing regulators

have been fully characterized (3). This could be due in part to the fact that assigning gene function in mammalian cells is a difficult task. The advent of RNAi has presented a unique opportunity to identify cellular factors that may be involved in alternative splicing. To identify proteins involved in alternative splicing and to determine the alternative splicing events controlled by a known splicing factor, the Rio and Gravelly labs have performed RNAi screens in cultured *Drosophila melanogaster* cells (115,116). The reasons for choosing *D. melanogaster* cells over mammalian cells are simple. First, the components of the splicing machinery are highly conserved between human and the fruit fly (117). Second, it is much easier to perform both genetic and biochemical experiments in *Drosophila*. Lastly, RNAi experiments in *Drosophila* are cost-effective. To identify genes that are specifically regulated by a splicing factor, Rio and colleagues (118) selected four known splicing regulators (dASF/SF2, B52/SRp55, hrp48, and PSI) and knocked down their expression by employing RNAi. To simplify data interpretation, they developed a high-density microarray that can assay all annotated alternative-splicing events in *D. melanogaster*. Interestingly, the number of splicing events controlled by each of these factors was found to be highly variable. For example, dASF/SF2 strongly affected the splicing of more than 300 genes, whereas PSI affected the splicing of only 43 genes. Park et al. (115) conducted a large RNAi screen consisting of 250 potential splicing regulators, roughly 70% of the *Drosophila* RNA binding proteins. This study led to the identification of 47 splicing regulators that are involved in regulating the splicing of 19 alternative exons from three different pre-mRNAs (115).

CONCLUSIONS AND FUTURE DIRECTION

The last few years have witnessed the emergence of several novel strategies aimed at regulating alternative splicing for therapeutic benefits. RNAi, which harnesses the power of an innate cellular system for the targeted down-regulation of gene expression appears highly promising and therefore

Figure 4. Allele-specific small interfering RNA (siRNA) for suppression of a missense *tau* mutation. The siRNA designed to target the mutant allele forms a perfect duplex, but contain mismatches with the normal allele. This leads to the preferential degradation of the mutant transcript (97,98). WT, wild-type; mRNA, messenger RNA.

continues to be the subject of intense research. The relative ease with which an siRNA can be designed and synthesized, its specificity and potency, and, most importantly, the ability to preferentially suppress the expression of mutant alleles makes this approach highly appealing. Moreover, RNAi not only has the potential to be an effective therapeutic tool, but also enables the identification of genes that regulate alternative splicing. However, RNAi is faced with limitations when compared with antisense oligonucleotides, which unlike RNAi, can modulate mRNA isoform levels (42). Also, whereas the spliceosome (119) and ribozymes (120) can be exploited to repair mutant mRNA, in its present form RNAi has been used to destroy mRNA. Finally, to fully grasp the therapeutic potential of RNAi, several important challenges, such as the development of safe and efficacious delivery systems, off-target effects, and degradation by cellular nucleases, must be addressed.

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COMPETING INTERESTS STATEMENT

The author declares no competing interests.

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