Targeting splicing factors for cancer therapy

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ABSTRACT

Alternative splicing (AS) of mRNAs is an essential regulatory mechanism in eukaryotic gene expression. AS misregulation, caused by either dysregulation or mutation of splicing factors, has been shown to be involved in cancer development and progression, making splicing factors suitable targets for cancer therapy. In recent years, various types of pharmacological modulators, such as small molecules and oligonucleotides, targeting distinct components of the splicing machinery, have been under development to treat multiple disorders. Although these approaches have promise, targeting the core spliceosome components disrupts the early stages of spliceosome assembly and can lead to nonspecific and toxic effects. New research directions have been focused on targeting specific splicing factors for a more precise effect. In this Perspective, we will highlight several approaches for targeting splicing factors and their functions and suggest ways to improve their specificity.

Keywords: splicing; splicing factor; cancer therapy; small molecules; decoy oligonucleotides

INTRODUCTION

Splicing of protein-coding genes is an essential regulatory mechanism in eukaryotic gene expression. Alternative splicing (AS) utilizes exon skipping, intron retention, mutually exclusive exons and differential 5′ or 3′ splice sites to generate mRNA and protein isoforms with distinct properties, originating from the same gene, thereby diversifying the proteome. AS is a highly regulated process, and misregulation of this process contributes significantly to the susceptibility for and development of diseases, including cardiovascular diseases, immune diseases, neurodegeneration, metabolic diseases, and cancer (for review, see Zhang et al. 2021).

Specifically in cancer, AS misregulation was shown to contribute to tumor initiation, progression and invasion by modifying the relative expression of isoforms of various oncogenes and tumor suppressors (for reviews, see Shilo et al. 2015; Kozlovski et al. 2017; Siegfried and Karni 2018; Yoshimi et al. 2021). A comprehensive study of 32 cancer types has demonstrated that the majority of cancers have up to 30% more AS events in tumor samples compared to the corresponding normal tissue (Kahles et al. 2018). Using transcriptomic analyses, cancer-specific splicing patterns, including nonsense-mediated mRNA decay (NMD) events induced by intron retention and intronic cryptic splice-site activation, were discovered in thousands of genes in both hematological malignancies and solid tumors (Graubert et al. 2012; Furney et al. 2013; Brooks et al. 2014; Ferreira et al. 2014; Danan-Gotthold et al. 2015; Jung et al. 2015; Yoshimi et al. 2019; Calabrese et al. 2020). Differentially spliced genes were shown to contribute to cancer initiation and progression (Brown et al. 2011; Ben-Hur et al. 2013; Maimon et al. 2014; Shilo et al. 2014; Climente-González et al. 2017; Mogilevsky et al. 2018; Yoshimi et al. 2019), alter tumor sensitivity to chemotherapy and hormonal treatment (Calabretta et al. 2016; Paschalis et al. 2018; Tripathi et al. 2019), and can be used as diagnostic or prognostic biomarkers (Hofstetter et al. 2010; Zhang et al. 2019). Among the differentially spliced genes linked to the development of neoplasms are genes involved in proliferation and apoptosis (Bechara et al. 2013; Maimon et al. 2014; Pavlyukov et al. 2018), telomere elongation (Wang et al. 2016), cell cycle regulation (Yeo et al. 2016; Baker et al. 2021), tumor metabolism (Christofk et al. 2008; Ben-Hur et al. 2013), and angiogenesis (Pradella et al. 2021).

AS is controlled by both *cis*-acting regulatory elements within the pre-mRNA and *trans*-acting splicing factors (Fig. 1A). Accordingly, cancer-specific misregulation can be induced either by mutations in intronic/exonic *cis*-acting regulatory sequences of the spliced gene, often generating novel splice sites and thereby affecting splicing (Roca

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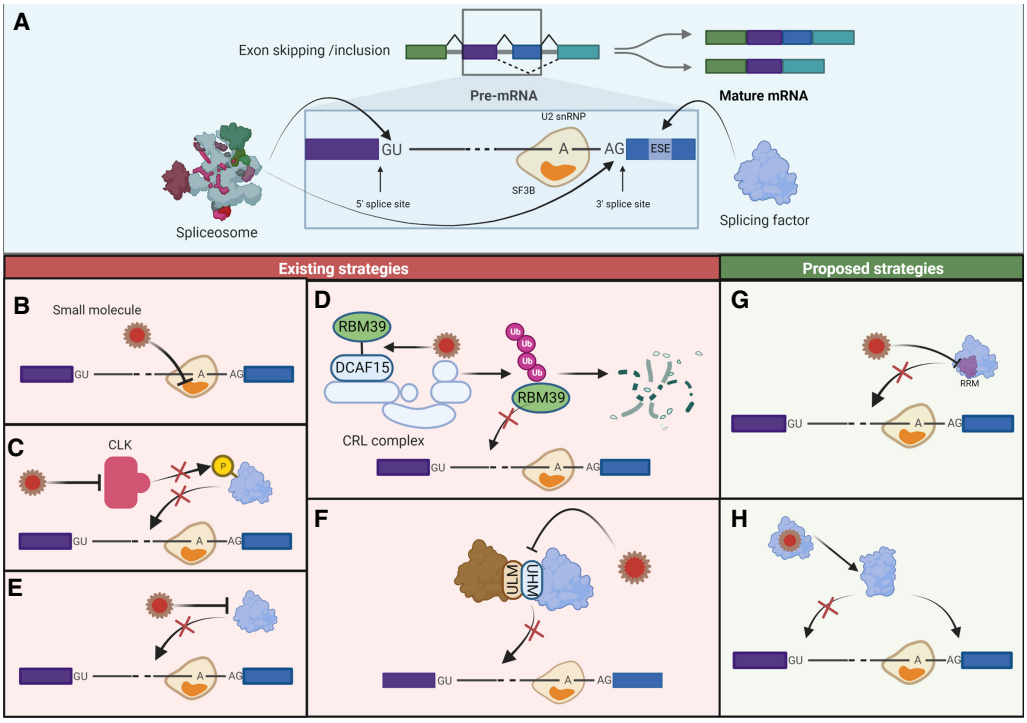

FIGURE 1. Targeting splicing factors by small molecules. (*A*) Scheme showing both *cis* and *trans* components involved in alternative splicing. (*B*) Inhibition of SF3B/U2 snRNP by a small molecule. (*C*) Inhibition of CLK protein by a small molecule. (*D*) Recruitment of RBM39 to the E3 ligase CRL4 substrate receptor DCAF15 by a small molecule, leading to its ubiquitination and degradation. (*E*) Direct inhibition of a splicing factor by a small molecule. (*F*) Inhibition of the UHM–ULM bond between two splicing factors by a small molecule. (*G*) Direct inhibition of a splicing factor by a small molecule targeting specific RRM. (*H*) Direct binding of a small molecule to a splicing factor causing allosteric modulation resulting in either inhibition or activation of the splicing factor.

et al. 2013; Supek et al. 2014; Frampton et al. 2015; Jung et al. 2015), or by misregulation of *trans*-acting splicing factors (e.g., over- or underexpression, copy-number variation or mutations) that may affect their function (for review, see Lee and Abdel-Wahab 2016; Urbanski et al. 2018). Thus, targeting AS has become a desirable goal in cancer therapy, and multiple therapeutic strategies targeting AS misregulation in cancer are in different stages of development. In this Perspective, we will discuss recent advances in AS modulation, focusing on methods to specifically target splicing factor activity. In addition, we will suggest ways to develop these approaches to be safer and more efficient.

SPLICING FACTORS AND CANCER

Trans-acting splicing factors are the most prominent mediators of splice-site recognition, selection and alternative splicing regulation. In general, the splicing machinery can be divided into two groups of components. The first group is represented by core spliceosomal components that bind and assemble around the 5′ splice site (the U1 complex) and the 3′ splice site and branch site (U2 complex) and consist of small nuclear ribonucleoproteins (snRNPs) and proteins (for reviews, see Patel and Steitz 2003; Wahl et al. 2009). The second group is comprised of splicing factors that interact with *cis*-elements within exons or introns and enhance or suppress spliceosome assembly leading to splicing activation or inhibition, respectively (for reviews, see Cartegni et al. 2002; Black 2003). Two important families of splicing regulators are serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). Some of the other splicing factors relevant to cancer biology are the RBFOX1/2/3 proteins, CELF proteins, MBNL1, NOVA proteins, and STAR (signal transduction and activation of RNA metabolism) proteins, including SAM68 (Chen and Manley 2009). SR proteins mediate splicing, in part, by recognizing exonic and intronic splicing enhancers (ESEs and ISEs, respectively), stabilizing the interactions of the splicing machinery on the splice sites. hnRNPs, on the other hand, recognize exonic and intronic splicing silencers (ESSs and ISSs, respectively), compete with SR protein binding and, in most cases, inhibit splicing by various mechanisms, that are only partially understood (Black 2003; Busch and Hertel 2012). The orchestrated binding and competition of multiple splicing factors, sometimes with opposing functions, on a target mRNA, results in the tight regulation of AS. This fine-tuning is disrupted in cancer, where certain SR proteins and hnRNPs have been shown to be dysregulated, acting as either oncoproteins

or tumor suppressors (Karni et al. 2007; Golan-Gerstl et al. 2011; Anczuków et al. 2012; Cohen-Eliav et al. 2013; Jbara et al. 2021).

The activity of both SR proteins and hnRNPs is regulated at the transcriptional level (e.g., activation of SRSF1 transcription by c-Myc) (Das et al. 2012) and post-transcriptionally by multiple modifications such as phosphorylation, methylation and ubiquitination (de Kesel et al. 2022), and by mechanisms such as NMD (Lareau et al. 2007; Sun et al. 2010). Phosphorylation is known to affect splicing factors' protein–protein interactions, binding to target transcripts and intracellular localization (Naro and Sette 2013). An extreme example is SRSF10 (SRp38), which functions as a specific splicing activator when phosphorylated, but upon dephosphorylation becomes a splicing repressor (Feng et al. 2008). SR protein phosphorylation is mediated by serine–arginine protein kinases (SRPKs) and CDC-like kinases (CLKs), as well as proteins involved in cellular signal transduction pathways, such as MAPK, PI3K, and AKT, which also mediate phosphorylation of hnRNPs (Naro et al. 2021; de Kesel et al. 2022). These modifications are potential targets for modulating the activity of splicing factors.

Mutation of a single splicing factor can alter the RNA processing of thousands of genes and therefore can have a significant impact on the cell's transcriptome. One possible outcome is tumor initiation and progression (Dvinge et al. 2016). Since the first reports identifying somatic mutations in genes encoding core spliceosomal proteins in hematological malignancies (Papaemmanuil et al. 2011; Wang et al. 2011; Yoshida et al. 2011; Graubert et al. 2012; Quesada et al. 2012), many other studies have followed. Whole-exome sequencing data analysis from 33 tumor types in The Cancer Genome Atlas (TCGA) revealed that somatic mutations in *SF3B1*, *U2AF1*, *SRSF2*, and *RBM10* are common in various types of cancers (Seiler et al. 2018a). Splicing factor mutations are usually heterozygous and mutually exclusive (Yoshida et al. 2011). It has been demonstrated that cancer cells harboring mutated splicing factors, such as U2AF1, SF3B1, and SRSF2, are more dependent on wild-type spliceosomal activity for viability than wild-type cells, and therefore are more sensitive to pharmacological perturbation of the spliceosome (Lee et al. 2016; Shirai et al. 2017; Obeng et al. 2022). These studies support the development of spliceosomal-targeting drugs as potential therapy for cancers with mutations in splicing factors.

TARGETING SPLICING FACTORS FOR CANCER THERAPY

Several splicing factors have been shown to act as potent driver oncogenes in specific cancers (Karni et al. 2007; Golan-Gerstl et al. 2011; Anczuków et al. 2012; Cohen-Eliav et al. 2013). Over the past few years, several therapeutic strategies targeting aberrant splicing factors activity have been under development, with some of them already in clinical trials for cancer therapy.

Small molecules

Small molecules can be used to inhibit or enhance splicing by targeting distinct aspects of splicing; by interfering with proteins participating at any stage of spliceosome assembly, by inhibiting accessory splicing factor protein kinases, or by directly targeting the accessory splicing factors themselves. Here we will highlight several examples of small molecules that have been developed and tested to interfere with splicing factor activity.

Some of the first small molecules targeting splicing were developed from natural compounds and their derivatives. These molecules, which were discovered as anticancer drugs, were later characterized by their binding to and inhibition of the SF3b subcomplex in U2 small nuclear ribonucleoprotein (snRNP), which recognizes the branchpoint sequence in the pre-mRNA and is part of the catalytic core of the splicing reaction (Fig. 1B). Among the SF3b inhibitors are R901464, and its methylated derivative spliceostatin A, which were shown to inhibit splicing in vitro and promote pre-mRNA accumulation by binding to SF3b in the spliceosome (Kaida et al. 2007). Pladienolide B is another natural compound that showed antitumor activity and binds directly to the SF3b subunit SAP130, causing its inhibition (Kotake et al. 2007). Preclinical studies demonstrated that E7107, a semisynthetic derivative of Pladienolide B, and H3B-8800, a small, orally bioavailable molecule with a pladienolide-based scaffold, inhibit activity of the spliceosome by blocking an ATP-dependent remodeling event that exposes the branch point-binding region (BBR) of U2 snRNA (Folco et al. 2011; Seiler et al. 2016, 2018b). E7107 was the first splicing modulator to enter clinical trials. Phase I trials of E7107 in solid tumors resulted in the expected splicing modulation; however, little to no clinical benefit was achieved. Moreover, an ocular toxicity was observed that further prevented the continuation of these trials (Eskens et al. 2013; Hong et al. 2014). Similar outcomes were seen in the preclinical studies of H3B-8800, and further in phase 1 trials in myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients, which showed splicing modulation but no clinical response (Steensma et al. 2021). The authors suggest that the amount of splicing inhibition necessary for cell death may be higher than what was achieved in human subjects, or that abnormal splicing, although it plays a role in disease development, is not sufficient to sustain the survival of tumor cells. Alternatively, it may be that prolonged inhibition is required for clinical activity. Another possibility may be that the cancer cells have acquired additional mutations and

are no longer dependent on the specific splicing modulation.

High-throughput screening of small molecule libraries using cell-free in vitro splicing assays, as well as cell-based assays, have been used to identify direct spliceosome inhibitors. Using these methods researchers have been able to screen small molecule libraries ranging from ~2000–70,000 compounds (Effenberger et al. 2017). In one such assay, using reverse transcription followed by quantitative PCR as a readout, three small molecules that inhibit splicing were identified: (i) Tetrocarcin A (NSC333856), a known antibiotic and antitumor compound that inhibits the antiapoptotic gene *BCL2*, interferes with the stability of complex A, an early spliceosomal complex, or with the transition to the next assembly stage; (ii) an indole derivative (NSC635326) with no known biological activities, inhibits all stages of spliceosome assembly; and (iii) a Naphthazarin derivative (NSC659999), which was previously shown to suppress tumor growth, is assumed to inhibit late-stage spliceosome assembly. However, these compounds showed low potency, with IC_{50} for in vitro splicing in the micromolar range. Therefore, further improvement of the activity was tested using structure activity relationship (SAR) approaches but showed limited success (Effenberger et al. 2013, 2015).

An alternative approach to target splicing factors is to target the kinases that phosphorylate them (Fig. 1C). There are several examples of molecules that inhibit phosphorylation of splicing factors with the potential to treat various diseases such as Duchenne muscular dystrophy (DMD), Down syndrome, angiogenic diseases and lung cancer (for review, see Ohe and Hagiwara 2015). For example, a glycosylated indolocarbazole derivative (NB-506) was shown to affect SRSF1-mediated splicing targets, possibly by inhibiting topoisomerase I's ability to phosphorylate SRSF1 (Pilch et al. 2001; Soret et al. 2005). Chlorhexidine, a compound widely used in the clinic as a disinfectant and topical anti-infective agent, was identified by a high-throughput cell-based assay to be a specific inhibitor of the CDC2-like kinase (CLK) family of SR protein kinases. In vitro, chlorhexidine had a selective effect on members of the CLK family, with Clk4 and Clk3 being the most sensitive to treatment with IC_{50} values of 10 and 15 μM, respectively. Clk2 and Clk1 were less sensitive to chlorhexidine, with IC_{50} values of 25 and >50 μM, respectively (Younis et al. 2010). Through an extensive screening of 100,000 chemical compounds in an in vitro phosphorylation assay, it was found that TG003, a benzothiazole compound, inhibits the activity of Clk1/Sty and Clk4. TG003 inhibits Clk1/Sty SRSF1 phosphorylation activity, resulting in splicing alterations in vitro and in vivo (Muraki et al. 2004). In a later study, TG003 was also found to promote the skipping of exon 31 of dystrophin when it harbors a nonsense mutation, leaving the wild-type exon 31-bearing dystrophin intact, thus increasing the production of the dystrophin protein in a dystrophinopathy patient's cells ex vivo (Nishida et al. 2011). The exact mechanism by which TG003 acts on the dystrophin gene is yet to be determined. Additionally, its instability hinders its clinical application, therefore a better solution for increasing dystrophin protein level in patients is needed. In 2017, an orally available inhibitor of Clk1, named TG693, was demonstrated to promote the skipping of the mutated exon 31 in DMD patient-derived cells. TG693 increased the production of a functional protein through inhibition of Clk1 phosphorylation activity, particularly of SRSF4 and SRSF6. These results were recapitulated also in vivo (Sako et al. 2017). It should be noted that since one kinase phosphorylates multiple targets, this approach is expected to affect several splicing factors, and therefore might lead to nonspecific effects (Ohe and Hagiwara 2015). Nevertheless, in some cases, this inhibition affects specific substrates, as in the case of SRSF6 (Ajiro et al. 2021).

Anticancer sulfonamides were the focus of drug-discovery for many years due to their anticancer activity, although their targets and mechanisms of action were not established. However, in 2017, Han et al. identified the mechanism of action of indisulam, a sulfonamide previously tested in patients with solid tumors (Han et al. 2017). They found that indusulam, and other related sulfonamides, killed cells by causing degradation of an accessory RNA splicing factor, RNA-binding protein 39 (RBM39). RBM39 participates in transcriptional regulation, alternative splicing, and protein translation and is up-regulated in many types of cancer (Xu et al. 2021). Inhibition of RBM39 activity results in RNA splicing alterations and was shown to be lethal to cancer cells (Xu et al. 2021). Aryl sulfonamides recruit RBM39 to the E3 ligase CRL4 substrate receptor DCAF15 (which is part of the CRL complex), leading to its ubiquitination and degradation (Fig. 1D; Han et al. 2017; Ting et al. 2019). Proteins that mediate protein degradation are often referred to as "molecular glues" (Dong et al. 2021). This class of molecules consists of RBM39 degrading aryl sulfonamides, including indisulam (E7070), tasisulam, E7820, and chloroquinoxaline sulfonamide.

A phase 2 study of indisulam in combination with idarubicin and cytarabine, the standard-of-care therapy for AML, showed good safety properties and yielded a 35% response rate in patients with relapsed/refractory AML (Assi et al. 2018). After these encouraging results, the authors of this study proposed that the combination of drugs should be studied in a more homogeneous group of patients with AML or high-risk MDS whose leukemic cells express mutant splicing factors.

A more specific method for splicing inhibition is direct inhibition of specific splicing factors, rather than targeting the spliceosome (Fig. 1E). Quercetin, a phytochemical compound targeting splicing, is a naturally occurring polyphenolic flavonoid shown to have efficacy in multiple cancers, including lung, breast, prostate and pancreatic

cancers. In addition to other activities, quercetin triggers cell cycle arrest, promotes apoptosis, and inhibits angiogenesis (for review, see Vargas and Burd 2010). hnRNPA1 was identified as the target of quercetin, and the anticancer effects of quercetin are mediated, in part, by impairing functions of hnRNPA1 (Ko et al. 2014). Quercetin acts by binding directly to the C-terminal region of hnRNPA1, interfering with its ability to move freely between the nucleus and the cytoplasm, which results in its cytoplasmic retention (Ko et al. 2014). The authors report that the K_d value of quercetin for binding to full-length hnRNPA1 was approximately 8.9 μM, and that the K_d for binding to the carboxy-terminal region of hnRNPA1 was approximately 1.7 μM, leading them to conclude that the carboxy-terminal region of hnRNPA1 is required for interaction with quercetin. Further studies with quercetin have shown that targeting hnRNPA1 by quercetin can overcome enzalutamide resistance in prostate cancer cells (Tummala et al. 2017). Therefore, quercetin constitutes a potential therapy in cancers that overexpress hnRNPA1, such as gastric cancer and lung cancer (Chen et al. 2018; Ryu et al. 2021).

Protein–protein interactions play an important part in spliceosomal assembly (Corsini et al. 2007; Hegele et al. 2012; Loerch and Kielkopf 2016). U2AF homology motifs (UHMs) and U2AF ligand motifs (ULM) are common among splicing factors and are crucial for early spliceosome assembly (Kielkopf et al. 2004). Recent studies have identified phenothiazines as inhibitors of UHM–ULM interactions, which act by targeting the tryptophan binding pocket of UHM domains, and thus disrupting the activity of all UHM domain-containing proteins, such as U2AF2, RBM39, SPF45, and PUF60 (Fig. 1F; Jagtap et al. 2020). The affinities of these inhibitors were compared for three different UHM domains of SPF45, PUF60, and U2AF65. Three of these compounds (Cmp7–9) showed similar affinity for the three different UHMs (IC_{50} range 6.6–12.1 μM) confirming that the inhibitors are mainly recognized by the conserved tryptophan binding pocket of the UHM domains and no other significant specific contacts are made. UHM–ULM interactions were also identified in other splicing factors such as U2AF1 and SF3b1, which are frequently mutated in myelodysplastic syndromes (Loerch and Kielkopf 2016). Thus, UHM–ULM interaction inhibitors are another class of promising molecules for splicing inhibition.

General splicing inhibitors are expected to be nonspecific and thus more toxic. Ideally, inhibitors of specific splicing factors would target their RNA recognition motif (RRM) and interfere with their RNA binding activity (Fig. 1G). In this way, other functions of the splicing factor will not be affected. Alternatively, small molecules can be tailored to function as allosteric modulators of splicing factors. Some progress has been made using this approach to target transcription factors. The p53 tumor suppressor protein, encoded by the *TP53* gene, is mutated in many cancers. *TP53* mutations in cancer mostly lead to loss of tumor suppressor function. One category of such mutations is conformational or structural mutants, causing extensive misfolding of p53. Small molecules are being developed that can either protect p53 from its negative regulators or restore the function of mutant p53 proteins. In addition, there is a focus on drugs tailored to specific p53 mutations that are more prevalent in the population (Hassin and Oren 2022). A similar therapeutic approach of conformational change/stabilization by allosteric modulators can be applied toward either inhibition or activation of splicing factors (Fig. 1H). The ability to activate splicing factors is relevant in certain cancers, where down-regulation of splicing factors occurs, such as RBFOX1/2 down-regulation in ovarian cancer and glioblastoma multiform (Venables et al. 2009; Hu et al. 2013).

Oligonucleotide-based molecules

mRNA-targeting oligonucleotides

Some cancer-specific AS events can be potential targets for cancer therapy. Splicing factor mRNA can be inhibited by various methods: small-interference RNAs (siRNAs), antisense oligonucleotides (ASOs) (including GAPmers) and CRISPR/Cas9 editing. Oligonucleotides are short single-stranded nucleic acid sequences that bind to the target mRNA through base-pairing. Unlike small molecules, these methods target the splicing factor mRNA rather than the splicing factor protein. Since this Perspective focuses on splicing factor inhibition, we will only briefly mention splice-switching oligonucleotides (SSO). As reviewed in Havens and Hastings (2016), SSOs are RNA oligonucleotides that sterically block access of spliceosome or specific splicing factors to their exonic/intronic specific regulatory sites on the pre-mRNA molecule. Hence, SSOs cause AS changes, affecting the balance between distinct isoforms originating from the same pre-mRNA. In this way, SSOs can up-regulate a specific gene product, for example by producing productive isoforms over non-productive isoforms, or by stabilizing isoforms destined for NMD (Kole et al. 2012). Since 2016, the FDA has approved several SSOs for clinical treatment of several diseases, such as neurodevelopmental disorders, with many more candidates in clinical development. Currently there are numerous clinical trials evaluating oligonucleotides for cancer therapy (Desterro et al. 2020; Quemener et al. 2020). However, so far, no oligonucleotide-based drug has been approved by the FDA for cancer treatment.

Protein-targeting oligonucleotides

RNA binding proteins such as splicing factors bind to a specific motif sequence within pre-mRNA molecules of the target gene, and either recruit the spliceosome to nearby splice sites or interfere with spliceosome binding (Cartegni et al.

2002). A study by Denichenko et al. proposed a new class of specific splicing modulators named decoy oligonucleotides, which bind directly to splicing factors, rather than to their target pre-mRNAs. These decoy oligonucleotides are single stranded RNA molecules between 21 to 24 nt, with a 2′-O-methyl modification on the ribose of each nucleotide, which increases the stability of the molecule. The first and last three nucleotides are modified with 2′-O-methoxyethyl. Oligonucleotides were also modified with a phosphorothioate backbone. These oligonucleotides contain three to four repeats of an RNA binding motif of a specific splicing factor. Increased affinity of binding was observed with increased number of RNA binding motif repeats (Denichenko et al. 2019). The decoy competes with the endogenous pre-mRNA targets for the binding of a specific splicing factor, leading to inhibition of binding to its target RNA and splicing activity. In this study, three alternative splicing factors were targeted: RBFOX1/2, PTBP1, and SRSF1, all of which are known to be involved in various types of cancers, where their expression is often altered (Karni et al. 2007; Venables et al. 2009; Hu et al. 2013; Georgilis et al. 2018). Decoys designed for these three splicing factors were shown to specifically bind their target splicing factor, alter AS of known targets and have biological effects in line with inhibition of their respective splicing activities in vitro and in vivo. In contrast to gene silencing methods, an important advantage of the decoy oligonucleotides is that decoys bind to the splicing factor's RNA binding domain, and therefore inhibit only the splicing activity of the factor, without interfering with other activities such as protein–protein interactions (Denichenko et al. 2019). In vivo, in mouse models, decoy oligos were injected along with tumor cells and were not examined using systemic delivery. This approach has not yet been applied in clinical settings. Further work in vivo and improved delivery systems are necessary for decoys to become a feasible approach to target splicing factors.

One exciting future direction for decoy oligonucleotides is to design decoy oligonucleotides that are specific to mutated splicing factors (Fig. 2B). Mutations in the splicing factors, SF3B1, SRSF2, U2AF1 have been identified in certain cancers (Papaemmanuil et al. 2011; Wang et al. 2011, 2016; Graubert et al. 2012; Quesada et al. 2012; Furney et al. 2013; Harbour et al. 2013; Brooks et al. 2014; Shirai et al. 2017; Seiler et al. 2016, 2018a). Decoy oligonucleotides targeting cancer-specific mutant splicing factors could improve treatment specificity and avoid unwanted side effects. An alternative approach is to design decoy oligonucleotides based on the secondary structure of the target mRNA (Fig. 2C). DNA and RNA endogenous molecules can fold into G-quadruplex three-dimensional (3D) structures, consisting of four guanines that are held together by Hoogsteen hydrogen bonds (Biffi et al. 2013). G-quadruplex motifs were found enriched within certain regions of the genome, and their formation can trigger genome instability and increase mutation rates in different cancers, making G-quadruplex 3D structures a target for cancer therapy (Kosiol et al. 2021). Protein pull-down experiments demonstrated that G-quadruplex structures bind to different proteins such as hnRNPs, ribosomal proteins and splicing factors such as SRSF1 (Brázda et al. 2014). These findings suggest the possibility of designing splicing factor decoy oligonucleotides based on the 3D structures of the target mRNAs, as a new type of splicing factor-specific therapeutic agent.

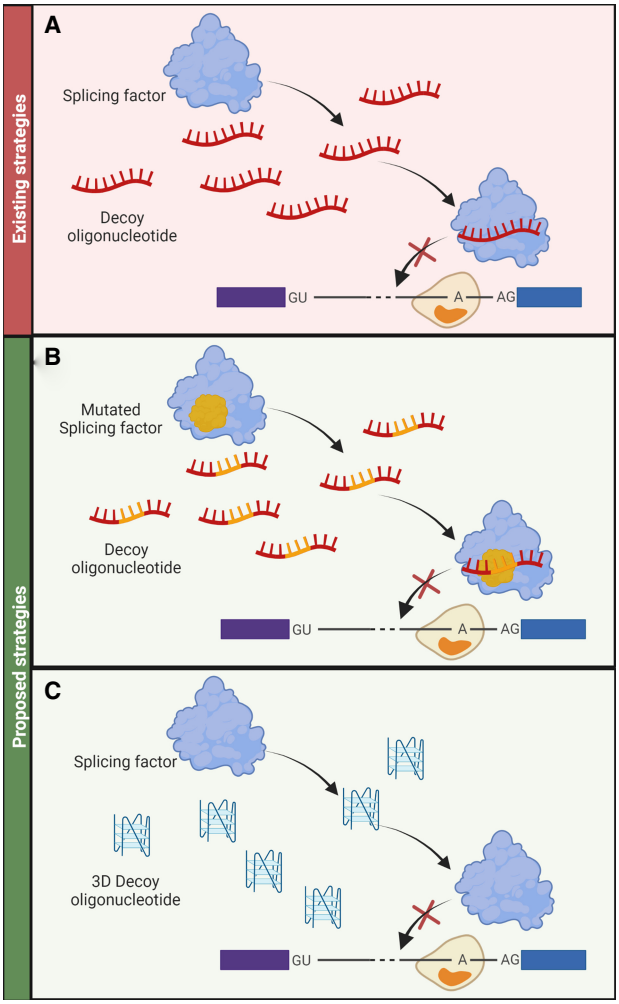

FIGURE 2. Targeting splicing factors by decoy oligonucleotides. (*A*) Inhibition of a splicing factor by direct binding to decoy oligonucleotides. (*B*) Decoy oligonucleotides designed to specifically target mutated splicing factors. (*C*) Decoy oligonucleotides designed to target specifically the binding of splicing factors to 3D RNA structures.

CONCLUDING REMARKS

Intensive research in the past two decades has shown that AS misregulation, which can be caused by splicing factor dysregulation or mutation, is causally involved in cancer development and progression. Thus, the notion that

specific splicing factors and the splicing machinery can be targeted for cancer therapy has gained attention. In recent years, multiple classes of pharmacological modulators of splicing have been under development, including small molecules and oligonucleotides, targeting either the mRNA or proteins involved in spliceosome assembly or alternative splicing. Targeting the core spliceosome components disrupts early stages of spliceosome assembly and can lead to various nonspecific and toxic effects. Thus, research focus has shifted toward targeting a specific spliceosome component for more controlled splicing inhibition. Further specificity can be achieved if specific alternative splicing factors that modulate a narrower set of targets can be targeted. Although there is still a long way to the clinic, we expect to see many new splicing factor inhibitors in preclinical and possibly clinical stages of development for cancer treatment in the coming years.

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