

RNA splicing: disease and therapy

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Abstract

The majority of human genes that encode proteins undergo alternative pre-mRNA splicing and mutations that affect splicing are more prevalent than previously thought. The mechanism of pre-mRNA splicing is highly complex, requiring multiple interactions between pre-mRNA, small nuclear ribonucleoproteins and splicing factor proteins. Regulation of this process is even more complicated, relying on loosely defined *cis*-acting regulatory sequence elements, *trans*-acting protein factors and cellular responses to varying environmental conditions. Many different human diseases can be caused by errors in RNA splicing or its regulation. Targeting aberrant RNA provides an opportunity to correct faulty splicing and potentially treat numerous genetic disorders. Antisense oligonucleotide therapies show particular promise in this area and, if coupled with improved delivery strategies, could open the door to a multitude of novel personalized therapies.

Keywords: RNA splicing; alternative splicing; splicing mutations; antisense oligonucleotides; splice correction; DMD

INTRODUCTION

Among the diverse repertoire of mechanisms by which an organism can achieve gene regulation, differential pre-mRNA splicing stands out as a particularly powerful yet subtle mediator. RNA also presents an attractive target for therapeutic interventions. On an *in vivo* cellular basis mRNA is more accessible than DNA and the presence within the cell of multiple different RNA processing pathways (e.g. splicing, nonsense-mediated decay, RNA interference, etc.), means that there is much scope for influencing its control at different levels. Targeting and manipulating RNA avoids many of the risks and concerns associated with DNA-based gene therapy such as random gene insertion. The dynamic nature of RNA turnover also means that therapeutic interventions can be time limited, dose titrated and modified according to response, adding further levels of control.

This review sets out to explain some of the ways in which the complex process of pre-mRNA splicing can lead to disease. It will also discuss a number of the different approaches currently in development that hope to rectify splicing where it goes wrong,

with the ultimate goal of therapeutic clinical applications.

PRE-mRNA SPLICING

When a protein-coding gene is transcribed, the initial transcript (pre-mRNA) must undergo a series of post-transcriptional processing events prior to its translation. Aside from 5' capping and polyadenylation, the most significant modification is that of intron removal and exon ligation through splicing. The major effector of the splicing reaction is the spliceosome, a complex of hundreds of interacting proteins and small nuclear RNAs (snRNAs) including the five small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5 and U6 [1]. In order to perform accurate splicing, the spliceosome must recognize exon/intron boundaries. At a basic level, this occurs through the presence of consensus sequence elements at the 5'- and 3'-splice sites of introns and through the presence of a branch point sequence near to the 3'-end of an intron (Figure 1).

The splicing reaction itself is mediated via a sequence of carefully controlled interactions between

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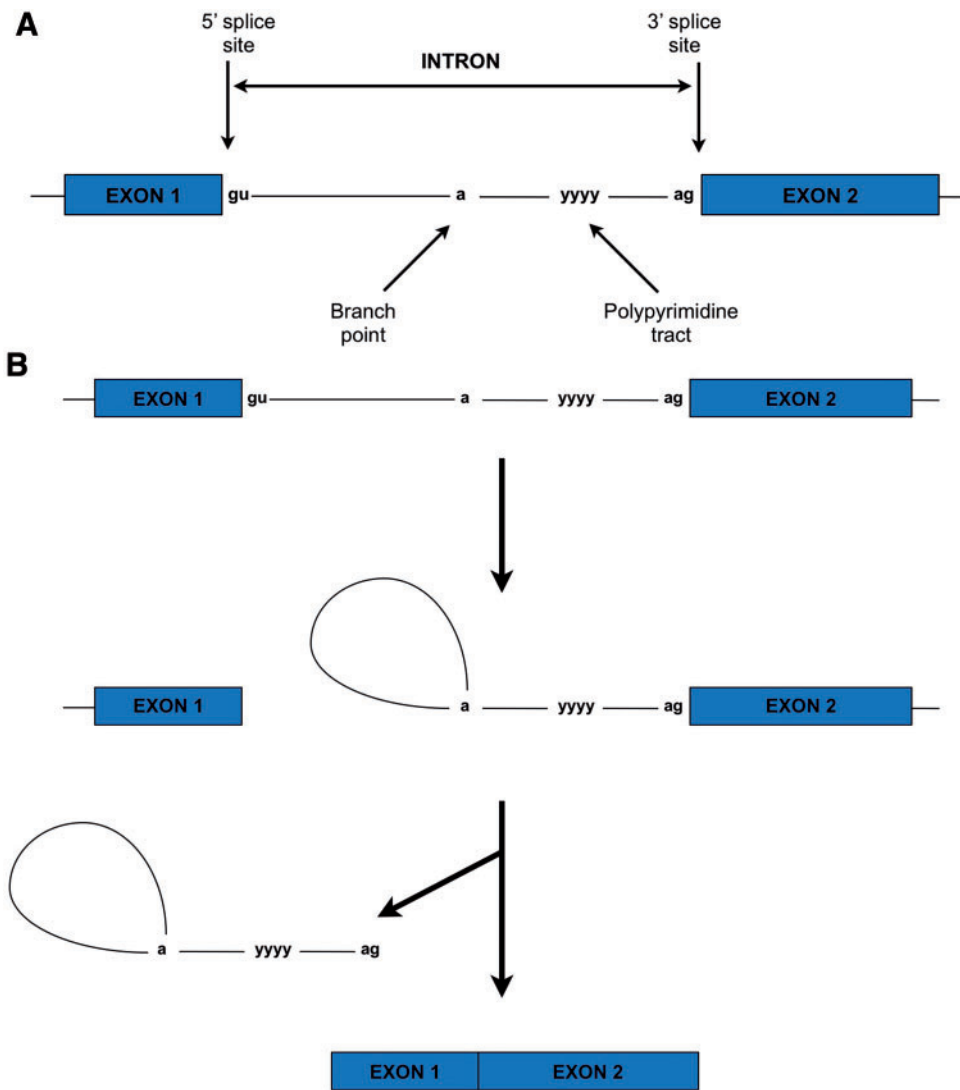


Figure 1: The basic splicing process. (A) Exons are represented by boxes and introns by lines. The invariant GU and AG nucleotide sequences of the 5' and 3'-splice sites are shown. Also shown is the branch point (A) and the nearby polypyrimidine tract (YYYY). (B) The first transesterification reaction creates a lariat structure joined at the branch point. The second transesterification reaction releases the lariat intron and ligates the exons together.

snRNPs, proteins and the pre-mRNA transcript [2, 3]. U1 first binds via complementary base pairing to the 5'-splice site, while U2 binds the intron branch point. A 'triple' snRNP complex consisting of U4, U5 and U6 then moves in to associate with the assembling spliceosome. U4 leaves the complex allowing U6 to replace U1 at the 5'-splice site. U6 then interacts with U2 to bring the branch point into close proximity with the 5'-splice site. At this point a transesterification reaction cleaves the 5'-end of the intron from the upstream exon and attaches it to the branch point, forming a loop-like lariat structure. Further interactions mediated by U5 then bring the 3'-end of the upstream exon and the 5'-end of the

downstream exon into close proximity with each other. This allows a second transesterification reaction to cleave the remaining 3'-end of the intron and join the two exons together.

The splice site sequences that allow this reaction to take place are sufficient to maintain the accuracy of exon-exon junctions. However, splice sites are only loose consensus sequences and on their own they cannot provide the degree of control needed for correct exon selection, particularly where alternative splicing is involved. In order to allow this, exon recognition requires interactions between *trans*-acting factors (proteins and ribonucleoproteins) and *cis*-acting elements (pre-mRNA sequences).

ALTERNATIVE SPLICING

In alternative splicing, the cell can ‘choose’ different combinations of exons to use in the final mRNA transcript of a gene. This creates different splicing isoforms of a single gene despite the original DNA sequence being the same in each case. Most (75%) of the exons that are alternatively spliced have been shown to be protein coding [4]. In addition, the majority of known alternative exons map to the surface regions of protein structures, making them more likely to affect protein function [5]. The process of alternative splicing thus creates different protein isoforms which differ in their functional capacities.

Another variant of this is that different splice sites may be selected by the spliceosome, resulting in longer or shorter exons. Entire introns can also be retained in this way through ‘exonization’. These ‘choices’ are made depending on the relative ‘strength’ of competing splice sites. How well a splice site matches the consensus sequence will determine how well spliceosome components can bind to it and this influences whether or not it is used. Splice site strength also depends on the presence of nearby sequence elements known as splicing enhancers and silencers. These *cis*-acting elements can be located both in exons and/or in introns and exert their effects by facilitating the binding of various splicing factors, which in turn positively or negatively regulate inclusion of a particular exon (Figure 2). Positive factors bind to enhancers and include a family of proteins rich in serine and arginine (SR proteins). Negative factors bind to silencers

and include the family of heterogeneous nuclear ribonucleoproteins (hnRNPs). This, however, is an oversimplification. In some instances SR proteins are known to repress splicing. In adenovirus infection, the SR protein SF2/ASF binds an intronic repressor element near the branchpoint of adenovirus pre-mRNA [6]. This prevents U2 snRNP recruitment and prevents use of the 3′-splice site. Likewise, hnRNPs can also act to stimulate rather than suppress splicing [7]. SR proteins and hnRNPs possess protein- and RNA binding domains and through these they bind with low specificity to regulatory sequences and to each other. The unique arrangement of protein interactions a particular pre-mRNA makes forms part of the so-called ‘splicing-code’ [8].

Enhancer and silencer sequences are much more variable than splice site sequences and much remains unknown about how changes to these sequences affect splicing factor binding. Splicing factors are examples of *trans*-acting factors and their up- or down-regulation within a cell provides a clear opportunity for splicing regulation to be influenced by independent pathways and external factors. Indeed the variability of these sequences is indicative of the fact that the individual RNA–protein interactions involved in splicing factor binding are weak and of only low affinity. While this makes enhancer and silencer characterization more difficult, it is precisely this property of low affinity binding of multiple interacting factors that allows for fine regulation and control [3].

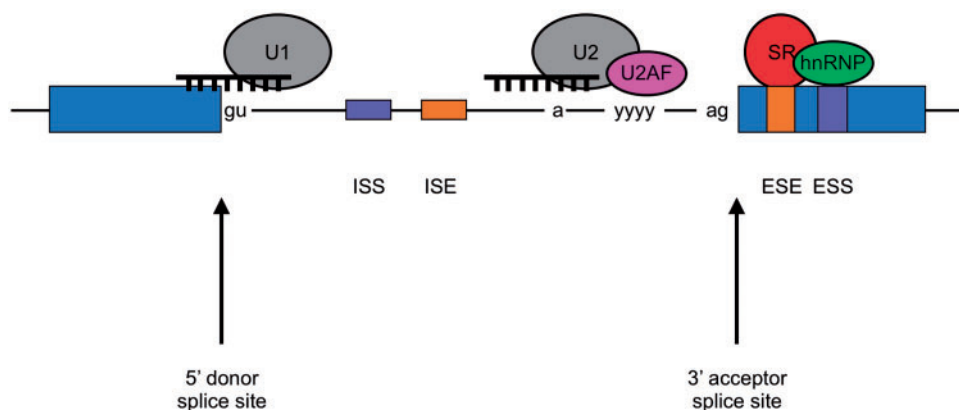


Figure 2: Control elements regulating splicing. U1 and U2 snRNPs bind via complementary base pairing to loose consensus sequences at the 5′ splice site and branch point respectively. U2AF (U2 auxiliary factor) recognizes and binds to the polypyrimidine tract and facilitates correct U2 binding. SR proteins bind to ESEs and increase splice site use, while hnRNPs bind to ESSs and exert a negative effect on splice site use. Other splicing factor proteins bind to intronic splicing enhancers (ISEs) and silencers (ISSs).

ADDITIONAL FACTORS GOVERNING SPLICING

Other factors including the rate of transcription and epigenetic factors such as chromatin conformation and histone modifications are known to play important roles in regulating splicing [9]. Much work is ongoing to help define the precise mechanisms by which such regulation occurs. It has been known for some time that splicing is coupled to the transcription process. RNA pol II recruits spliceosome components via its C-terminal domain and this allows cotranscriptional initiation, though not necessarily completion, of splicing or at least the commitment to use specific splice sites [10]. The rate of transcript elongation can also affect the splicing process and promoter structure influences the outcome of alternative splicing [11]. Chromatin structure appears important for correct spliceosome assembly and the positioning of nucleosomes within genes has been found to be non-random with particular enrichment at intron–exon junctions, suggesting a role in exon definition [12, 13]. Similarly, histone modifications have been found to be non-randomly enriched at exons, even taking into account relative nucleosome overrepresentation [14].

In addition, pre-mRNA secondary structure can influence selection of splice sites [15]. For example, a stem–loop structure at the 5′-splice site of exon 10 in the gene for tau protein regulates usage of the exon. Another example of this is alternative exon usage in the fibronectin gene, where pre-mRNA secondary structure affects the availability of an enhancer element [16]. In this case, splicing of the EDA exon of fibronectin is dependent upon the presence of an exonic splicing enhancer (ESE) displayed within the exposed part of an RNA stem–loop structure. Disruption of this secondary structure prevents recognition of the exon.

Small nucleolar RNAs (snoRNAs) have also been found to regulate splice site selection. For example, the snoRNA HBII-52 regulates alternative splicing of the serotonin receptor by binding to an alternative exon [17]. Interestingly, this same snoRNA, HBII-52, is not expressed in Prader-Willi syndrome (PWS) and this is thought to contribute to the disease. A child with a microdeletion encompassing HBII-438A, the HBII-85 cluster and a portion of the HBII-52 snoRNA cluster exhibited features of PWS [18]. Although small nucleolar ribonucleoproteins (snoRNPs) exist in the nucleolus and splicing occurs in the nucleoplasm, evidence suggests that

snoRNPs are transported though the nucleoplasm as they are being assembled, allowing an opportunity for them to influence splicing [19]. This may occur through interactions between snoRNP associated proteins and splicing factors such as hPrp31 [20].

Alternative splicing is frequently regulated in response to external stimuli [21]. Signal transduction pathways can lead to phosphorylation of *trans*-acting factors such as SR proteins. Targeted phosphorylation of RS-domains (characteristic arginine/serine rich domains at the C-terminal end of SR proteins) can affect a protein's ability to bind to and interact with its usual protein partners [22]. Splicing factors can also be dephosphorylated by phosphatases and phosphatase modulation affects alternative exon usage [23].

PSEUDOEXONS

The same nucleotide sequence can, under different conditions, be defined as an exon or an intron [24, 25]. Attempts to design exons using current knowledge have yielded unexpected results and have proved the underlying complexity of the spliceosome's functions [26]. *In silico* analysis reveals the abundant presence of sequences lying within the intronic domains of many genes that look like exons and have both 5′ and 3′ consensus splice sites, yet are not used as such [27]. These sequences are known as pseudoexons. The exclusion of these pseudoexons is thought to be mediated through intrinsic sequence defects, splicing silencers and inhibitory RNA secondary structures [28–30]. Looking at the splicing process more globally, rather than on an individual gene basis, will help to clarify what makes an exon an exon and what differentiates pseudoexons, allowing a fuller understanding of how the splicing machinery distinguishes between them.

THE SCOPE OF SPLICING IN DISEASE

Over 90% of human protein-coding genes are alternatively spliced [31]. However, since in fact every intron-containing gene requires splicing, any mutation affecting a canonical splice site in such a gene can lead to gene dysfunction and potentially to disease. Such splice site mutations are a common finding in clinical diagnostic laboratories and it is estimated that they may account for some 10% of all pathogenic mutations [32]. However, this does

not include mutations affecting splicing enhancers, silencers or *trans*-acting factors. Many such mutations will have been overlooked historically, either because they appear to be silent synonymous changes with no effect on amino acid sequence, or else because of their apparently innocent intronic location. Ever increasing numbers of these mutations are now being identified in patients with genetic disease and according to some estimates up to 50% of all pathogenic mutations may affect splicing in some way [33].

Familial dysautonomia—a splice site mutation

Familial dysautonomia (FD) is a rare recessively inherited disorder affecting both the autonomic nervous system and somatic sensory neurones. It is caused by mutations in *IKBKAP*, which encodes a transcription factor component of the elongation complex known as IKAP. In nearly all cases (99.5%) of FD the pathogenic mutation is found to be an intronic T > C substitution at position 6 of intron 20 [34]. This disrupts binding of U1 to the 5'-splice site of exon 20, causing exon skipping and resulting in a frameshift and premature termination codon. *IKBKAP* appears to promote expression of genes involved in oligodendrocyte formation and so this could explain the demyelinating phenotype observed in FD [35].

Spinal muscular atrophy and medium-chain acyl-CoA dehydrogenase deficiency—disrupted regulatory elements

Spinal muscular atrophy (SMA) is the second most common recessive disorder in humans and is the most common inherited cause of infant mortality. It is caused by mutations in the *SMN1* gene which encodes the survival motor neurone (SMN) protein [36]. SMN is required for snRNP synthesis and its loss of function leads to degeneration of motor neurones particularly evident in the spinal cord. In humans there has been a gene duplication event of *SMN1* that has given rise to an almost identical gene called *SMN2*. However, *SMN2* contains a silent C > T substitution in the sixth nucleotide of exon 7. This causes skipping of exon 7 and ineffective protein production, with the result that *SMN2* is unable to compensate for the loss of function of *SMN1* [37]. The *SMN2* mutation both destroys an

ESE by abolishing a binding site for the SR protein SF2/ASF and also creates an exonic splicing silencer (ESS) by allowing a binding site for hnRNPA1 [38, 39].

A very similar mechanism to this occurs in medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. MCAD is required for the degradation of medium chain length fatty acids and MCAD deficiency is the most common defect of mitochondrial β -oxidation. Medium chain acylcarnitines accumulate in the urine and this can be detected diagnostically. One particular missense mutation in exon 5 of *MCAD* (c.362C > T) causes exon skipping and degradation by nonsense-mediated decay [40]. Exon skipping occurs because of disruption of a splicing enhancer that is nearly identical to the enhancer in exon 7 of *SMN2*.

Hutchinson–Gilford progeria syndrome—activation of a cryptic splice site

Hutchinson–Gilford progeria syndrome (HGPS) is a genetic disorder characterized by features of premature ageing. There is postnatal growth retardation, premature atherosclerosis, bone dysplasia and a distinctive facial appearance with micrognathia, alopecia, narrow nasal bridge and pointed nasal tip [41]. HGPS is caused by mutations in the lamin A/C gene (*LMNA*). *LMNA* codes for two proteins, lamin A and C, dependent on alternative splicing of the transcript. Lamins A and C are members of the nuclear lamin family of structural proteins that form intermediate filaments and constitute the nuclear lamina, a meshwork structure which supports the inner nuclear membrane in eukaryotic cells [42]. HGPS is most commonly caused by a recurrent mutation in exon 11 (c.1824C > T) [43]. This point mutation does not alter the coding amino acid sequence (p.Gly608Gly) but instead activates a cryptic splice site 5 nt upstream. The single base change turns the sequence GGTGGGC into GGTGGGT and this altered sequence is recognized as a splice donor site. The effect of the mutation is production of a truncated protein that lacks the last 50 amino acid residues encoded by exon 11. This means the mutant protein, known as 'progerin', is missing an 18 amino acid C-terminal domain needed for a number of post-translational modifications such as farnesylation.

Menkes disease—splicing as a modifier of disease

Disease severity can be influenced by alterations in splicing. One example is Menkes disease, an X-linked disorder of copper metabolism caused by mutations in *ATP7A* [44]. This encodes an ATPase that transports copper across intestinal mucosa into blood. A significant proportion of *ATP7A* mutations involve the conserved dinucleotide sequences at 5' and 3' splice sites. Mutations at these sites severely disrupt normal splicing and the result is the severe phenotype of Menkes disease, which includes severe neurological impairment, kinked brittle hair, dysmorphic features, failure to thrive and death usually occurs before the age of 3 years. However, mutations affecting the less well-conserved more degenerate sequences surrounding the invariant dinucleotides tend only to partially abrogate normal splicing. The result of these 'weaker' mutations is a clinically distinct and milder condition known as occipital horn syndrome [45]. This is a disorder of the extracellular matrix leading to skeletal and cutaneous manifestations.

Altered splice isoform ratios

Disrupting the relative abundance of alternatively spliced RNA isoforms can lead to disease. Frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) arises when mutations occur in the gene *MAPT*. This gene encodes tau protein, which is needed for microtubule assembly and stability. Mutations within regulatory elements of *MAPT* exon 10 that promote its inclusion, increase the ratio of a tau isoform containing four microtubule-binding sites (4R) relative to the three (3R) site isoform. This causes disease by precipitating tau aggregation [46]. Alzheimer's disease also involves tau aggregations in the brain but investigation of 4R to 3R ratios has not shown a consistent pattern related to this disease. However, other splicing factors influencing exon 10 splicing such as *clk2* and *tra2-beta1* have themselves been found to have altered splicing patterns in Alzheimer's, suggesting that disordered splicing may indeed be playing a role in this disease [47].

Myotonic dystrophy—splicing factor sequestration

Myotonic dystrophy (DM) is an autosomal dominant condition characterized by progressive myopathy, delayed relaxation of muscle contractions

(myotonia), cardiac conduction defects, cataracts and a characteristic myopathic facies with frontal balding. Two forms of DM occur, known as type 1 (DM1) and type 2 (DM2). DM1 is due to a CTG expansion in the 3' untranslated region of the *DMPK* gene [48]. DM2 is clinically milder and is caused by a CCTG expansion in intron 1 of the *ZNF9* gene [49]. DM is an example of a disease where microsatellite expansions cause RNA gain of function. When these expansions are transcribed, the RNA contains many CUG or CCUG repeats and these have a high affinity for the splicing factor MBNL1. Depletion of MBNL1 from the nucleoplasm causes a functional loss of this protein [50]. In DM1, another protein called CUGBP1 becomes upregulated because of hyperphosphorylation and stabilization mediated by protein kinase C [51]. This action is induced by RNA containing CUG repeats. MBNL1 depletion and CUGBP1 upregulation together cause widespread disruption of alternative splicing. This directly leads to many of the clinical features seen in DM, including myotonia, where aberrant splicing of the muscle-specific chloride channel gene *CLNC1* causes impaired chloride conductance in muscle [52].

Mutations of the splicing machinery

Mutations in genes encoding fundamental components of the splicing machinery are relatively rare, presumably because the effects are incompatible with life. However, a few such mutations are seen in several diseases. In SMA, the SMN protein is involved in snRNP assembly and a deficiency of functional SMN protein results in multiple splicing defects across many tissues [53]. Motor neurones appear to be particularly affected, giving rise to the classic phenotypic picture of SMA. Autosomal dominant retinitis pigmentosa can also be caused by mutations in splicing factors PRPF31/U4-61k and PRP8 [54–56].

TDP43 (TAR DNA binding protein 43 kDa) is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and contains two RNA binding domains, one of which binds to UG repeats. It has been found to bind to a 12 UG repeat in the *CFTR* gene, causing exon 9 skipping resulting in cystic fibrosis [57]. It has also been implicated in neurodegenerative disorders such as ALS and frontotemporal dementia, where it has been found in ubiquitinated protein aggregates forming cytoplasmic inclusions [58, 59]. Interestingly TDP43 mutations

have been found in both sporadic and familial forms of ALS [60, 61]. Sequestration and depletion of this splicing factor from the nucleus could be contributing to splicing abnormalities and neurodegeneration.

Splicing and cancer

Alternative splice variants, which may be tumour specific, can significantly influence cellular processes in cancer, including proliferation, motility and drug response [62]. However, the degree to which aberrant splicing is involved in carcinogenesis and how much is just a reflection of the generally disordered cell processes present in tumours, remains largely uncertain.

Notwithstanding this, splicing mutations can affect tumour suppressor genes and oncogenes just as they can affect any other type of gene. *KLF6* is one such tumour suppressor gene that inhibits cell growth through various mechanisms including activation of p21, a cyclin-dependent kinase inhibitor [63]. A variant splice isoform of *KLF6* is formed by use of an alternative 5'-splice site in exon 2. This isoform (*KLF6-SV1*) antagonizes *KLF6* and acts in a dominant negative fashion, promoting cell proliferation [64]. A single nucleotide polymorphism (SNP) near the exon 2 intron/exon boundary leads to upregulation of the *KLF6-SV1* isoform because of binding of SRp40, an SR protein [65]. This particular SNP has been associated with prostate cancer and studies have shown that overexpression of *KLF6-SV1* accelerates prostate cancer progression [66].

Another example is *CDKN2A*, a gene that encodes two separate tumour suppressor proteins p14ARF and p16INK4a through the use of alternate reading frames. Loss of these proteins is associated with increased risk of melanoma. A particular mutation in the intron 1 splice acceptor site that leads to skipping of exon 2 in both p14ARF and p16INK4a has been seen in a family with melanomas and neurofibromas [67].

Oncogenes are also subject to mis-splicing. The receptor tyrosine kinase *KIT* is a proto-oncogene that can be activated by gain-of-function mutations resulting in aberrant splicing. Such mutations are found in gastrointestinal stromal tumours (GISTs). Deletions of the 3'-splice site of intron 10 activate a new 3'-splice site within exon 11. The deleted portion is critical to *KIT* inhibition and so the mutant aberrantly spliced *KIT* kinase remains constitutively active [68].

The upregulation of particular splice isoforms in preference to others has been implicated in several cancers. The apoptotic regulator *Bcl-X* is one example where two isoforms have opposing effects on apoptosis [69]. *Bcl-XS* is pro-apoptotic while *Bcl-XL* is anti-apoptotic. This difference in function depends on use of an alternative 5'-splice site in the first coding exon.

In addition to *cis*-acting mutations, specific alterations in *trans*-acting factors such as splicing factor expression have also been found in cancer. SR proteins are, for example, frequently upregulated in tumours. SF2/ASF, an archetypal splicing factor, is known to regulate alternative splicing of the *Ron* oncogene and this modulates cell motility, which is related to metastatic formation [70]. Overexpression of SF2/ASF can generate tumours *in vivo* and in this way it can be thought of as a proto-oncogene [71].

THERAPEUTIC APPROACHES

Small molecule modulators of splicing

Factors governing alternative splicing are modulated in response to various cell signalling pathways. Post-translational modification of splicing factors is one such mechanism. SR protein phosphorylation alters the protein's ability to enhance exon recognition [72]. Inhibition of specific protein kinases could be a means of modulating SR protein-mediated splicing events. Such an inhibitor could take the form of a small molecule [73]. However, targeting such fundamental processes is likely to result in widespread off-target effects. Blanket inhibition of SR protein phosphorylation would probably cause far-reaching global changes in splicing profiles. In addition, intracellular signalling pathways involving kinases and phosphorylases often have multiple and diverse effects, many of which remain unknown. Inhibition of specific enzymes could, therefore, have effects on entirely different cellular mechanisms other than splicing.

DM presents a potential target for small molecule therapy. Since the pathogenesis of this disorder is thought to involve RNA gain of function through sequestration of splicing factors such as MBNL1 and CUGBP1, an agent that antagonizes this process could potentially be used therapeutically. Screening of small molecule libraries has shown that the drug pentamidine is able to block MBNL1 binding the CUG repeats present in DM1 [74].

Antisense oligonucleotides

A more target-specific approach to splicing modulation can be achieved through the use of antisense oligonucleotides (AONs). Short oligonucleotides (ONs) can be synthesized that are complementary to a particular RNA sequence transcribed from a specific gene. The sequence specificity of ONs means that only the RNA sequence of interest will be targeted. By designing AONs that bind to splice sites or to enhancer or silencer elements within the transcript, the splicing mechanism can be manipulated in a precise and reproducible way (Figure 3). Blocking splice sites and/or regulatory sequences prevents snRNPs and splicing factors such as SR proteins and hnRNPs from binding to the RNA transcript. This allows directed exon skipping or inclusion depending on the sequence blocked [75].

The most advanced use of this technology in terms of therapeutic development has been for Duchenne muscular dystrophy (DMD). This is an X-linked disorder of muscle characterized by progressive muscle weakness in childhood, cardiomyopathy and death in early adulthood [76]. The molecular defect is due to out-of-frame mutations affecting the dystrophin gene, leading to absence of functional dystrophin protein. Normal dystrophin consists of two terminal functional domains joined by a central repetitive, non-essential rod domain. The majority of causative mutations occur in the

central rod domain. The functionality of dystrophin can be restored by restoration of the RNA reading frame [77]. This can be achieved by selective exon skipping within the section of RNA transcript encoding the rod domain. Since the beginnings and ends of exons are not defined by reading frame or codon position and since exon lengths do not adhere to being in multiples of three nucleotides, different exon–exon junctions within a given gene can lie at different positions within a codon: i.e. after positions 1, 2 or 3. Thus, by using a targeted AON to inhibit the inclusion of a specific exon during the splicing process, the reading frame of mutated frame-shifted pre-mRNAs can be restored. In the case of dystrophin, although the resulting mRNA is internally shortened, the functionally important terminal domains are retained. Clinical trials using AONs have been carried out in human patients with DMD with promising results confirming restoration of dystrophin expression after local intramuscular injection [78, 79]. The challenge now is to develop an effective method to deliver ONs systemically.

In order to achieve lasting effect, AONs need to be able to resist degradation by endogenous nucleases, particularly RNase H. A number of different ON chemistries have been developed to address this problem. In all cases, this entails making modifications to the molecular structure of the sugar-phosphate backbone found in naturally occurring

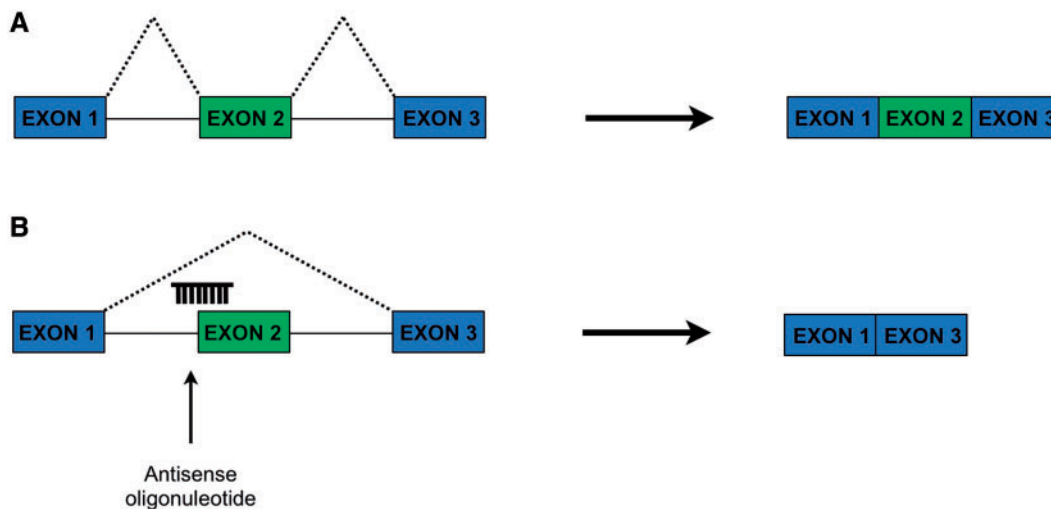


Figure 3: The principle of exon skipping using AONs. **(A)** In the top figure, consecutive exons are spliced together through recognition of consecutive 5'- and 3'-splice sites. **(B)** In the bottom panel, an AON hybridizes to the 3'-splice site of the first intron, preventing its recognition by the splicing machinery. Instead, the next available 3'-splice site (in the following intron) is used, resulting in exclusion of the intervening exon. AONs may be targeted to other regulatory sequences such as ESEs, ESSs, ISEs or ISSs in order to achieve the desired effect.

nucleic acids while maintaining the molecule's ability to perform Watson–Crick base pairing with native RNA. The most common examples currently in use include 2'-O-methyl phosphorothioates, locked nucleic acids (LNAs), peptide nucleic acids (PNAs) and phosphorodiamidate morpholinos (PMOs) (Figure 4).

AONs can be designed to block cryptic splice sites and prevent pseudoexon inclusion. AONs targeting activated cryptic splice sites have been used to restore

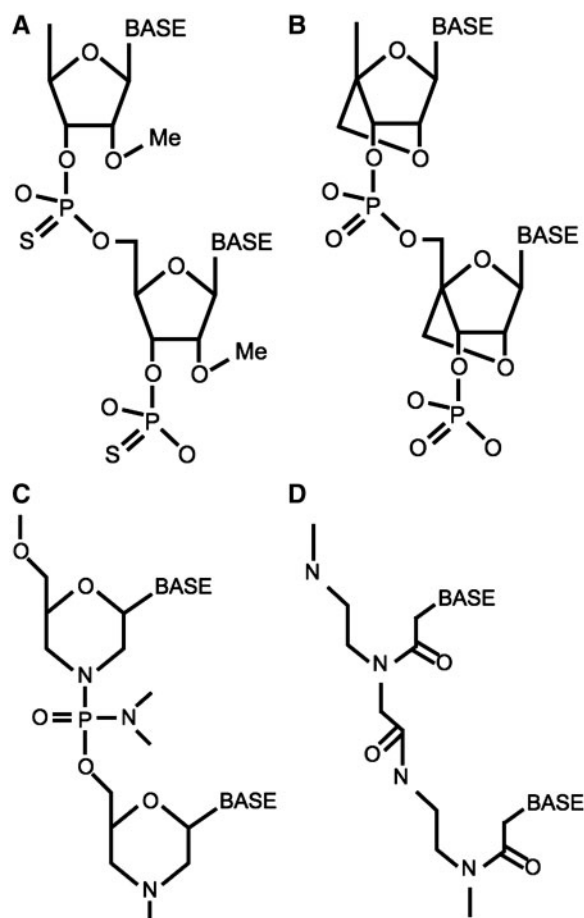


Figure 4: Chemical structures of commonly used AONs. **(A)** 2'-O-methyl phosphorothioates. This chemistry resembles RNA but has a methyl group at the 2'-O position and has a phosphorothioate rather than a phosphate group linking the ribose molecules. **(B)** LNAs. This closely resembles RNA but incorporates an extra carbon linker between the 2'-O and the 4' carbon. **(C)** PMOs. The ribose molecules are replaced by morpholine ring moieties and the phosphodiester bonds are replaced by phosphorodiamidate linkers. **(D)** PNAs. The entire sugar–phosphate backbone structure has been replaced by a repeating aminoethylglycine backbone and acetyl linkers carry the bases.

normal splicing in β -thalassaemia (β -globin) and cystic fibrosis (*CFTR*) [80–82]. In both examples, 2'-O-methyl phosphorothioate AONs were used. Another related AON chemistry, 2'-O-(2-methoxyethyl) phosphorothioate AON, has been used to upregulate exon 7 inclusion in *SMN2* and this rescues the phenotype in a transgenic mouse model of SMA [83]. The same approach has been tested in primates [84]. In β -thalassaemia, splicing defects have also been corrected by engineering U7 snRNA to target aberrant splice sites [85]. Other notable conditions involving pseudoexon inclusion and for which AON therapeutic approaches are being investigated include congenital disorders of glycosylation (*PMM2*) [86] and afibrinogenemia (*FGB*) [87]. AONs are also being developed to treat DM [88]. By designing ONs that bind to CUG repeats in DM1, the expanded region is prevented from binding to and sequestering proteins such as MBNL1. This disrupts the toxic gain-of-function mechanism thought to account for pathogenesis in DM1.

Bifunctional ONs

Bifunctional ONs are a variant on the theme of AONs. They contain an antisense-targeting domain at one end and an effector domain at the other which contains binding sites for known splicing factors [89]. Bifunctional ONs have been used to facilitate the inclusion of *SMN2* exon 7 by acting as an ESE. Chimeric effectors have also been designed which again contain an antisense domain but also have a peptide effector domain such as RS repeats that mimic the effects of SR proteins [90].

Trans-splicing

The majority of naturally occurring splicing occurs between exons of a single pre-mRNA. Occasionally splicing can take place between two separate pre-mRNA transcripts, which may be from different genes. This process is known as *trans*-splicing and offers a potential route for the 'correction' of aberrant RNAs [91]. *Trans*-splicing is mediated by the spliceosome and specific pre-mRNAs can be targeted by designing sequence-specific pre-*trans*-splicing molecules (PTMs). PTMs are ONs that consist of a binding domain complementary to part of the target intronic sequence, a splicing domain incorporating the required splicing sequence elements and a coding domain that carries the exon(s) to be *trans*-spliced (Figure 5). The complementarity between the PTM binding domain and the intronic

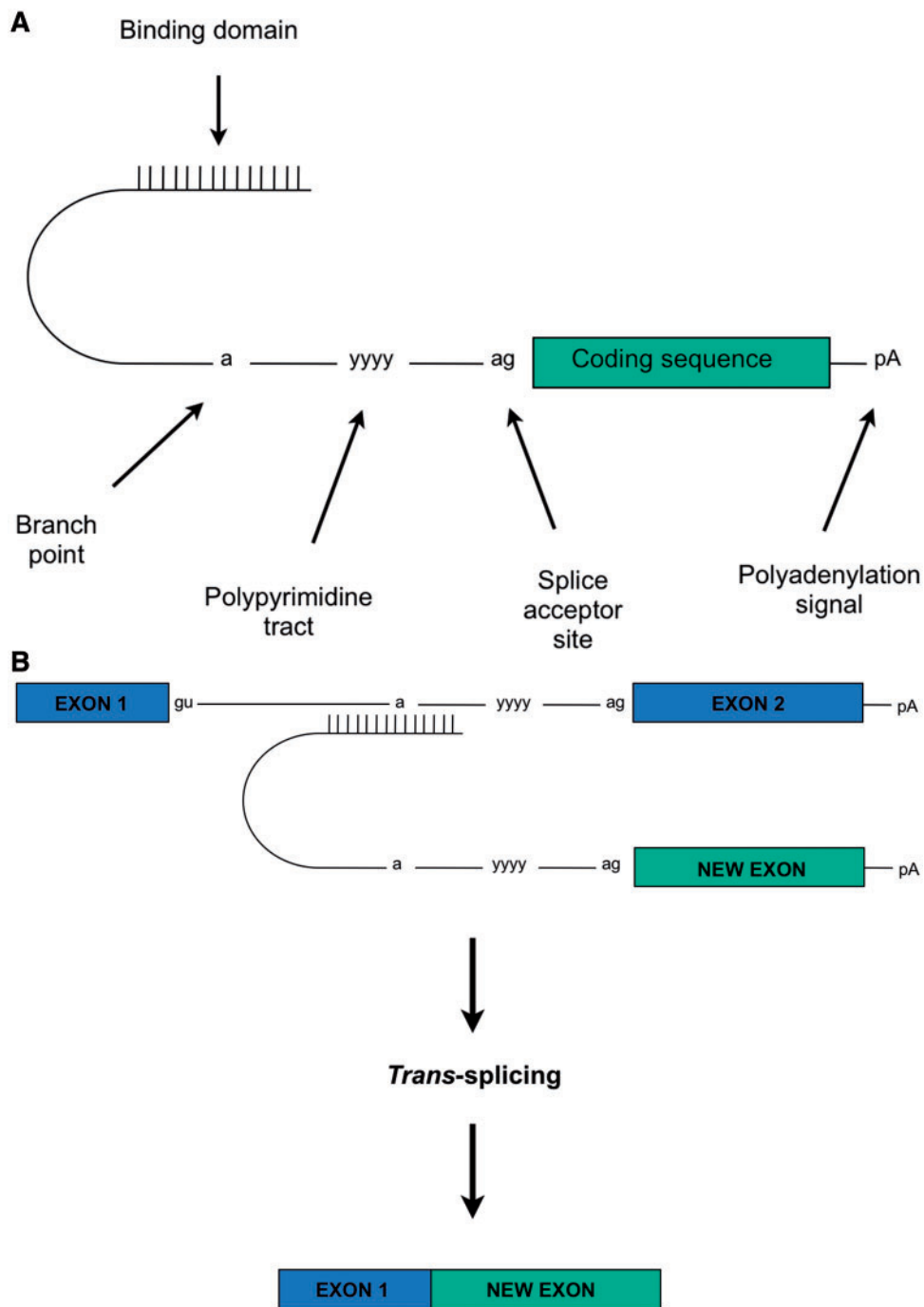


Figure 5: *Trans-splicing.* (A) Example of a pre-*trans-splicing* molecule (PTM). (B) The binding domain of the PTM hybridizes to its target pre-mRNA. Strong splice sites within the PTM encourage preferential *trans-splicing* to generate a 'reprogrammed' mRNA.

sequence of interest enables targeting of specific pre-mRNAs. Typically the binding domain includes the branchpoint region of the native pre-mRNA and this has the effect of preventing the usual splicing reaction from taking place. By designing strong splice sites in the PTM, the spliceosome can be 'tricked' into using the PTM splice site in preference

over that of the endogenous transcript. By using this principle and different conformations of PTM design, it is theoretically possible to effectively reprogramme the 5'- or 3'-ends of an mRNA, or even to selectively replace a single internal exon. Such approaches have been used in models of cystic fibrosis, haemophilia A and SMA [92–94].

CHALLENGES

Delivery

The traditional concept of gene therapy entailed restoring the function of a defective gene by introducing the correct DNA sequence of a particular gene into the relevant cells. With the advent of RNAi and antisense technologies, the emphasis of gene therapy has increasingly moved towards modulation of RNA rather than DNA. However, irrespective of approach, the primary difficulty that still arises is one of delivery.

A number of different delivery vectors, both viral and non-viral, are potentially available as means of transport for ON-based splice-correction therapies [95]. Viral vectors including retroviruses, adenoviruses and adeno-associated viruses have long been used in laboratory settings but their inherent risks and immunogenicity has limited their clinical application [96]. Although modifications can be made to reduce the immunogenicity of viruses, it is perhaps worth considering that evolution of the complex adaptive immune system of higher organisms was likely driven for the most part by the need to prevent viral infection and propagation. It is perhaps therefore no surprise that the therapeutic use of viral vector gene therapy has so far proved elusive and problematic. Another widely studied approach has been the use of liposome vectors. Complexing nucleic acids with cationic lipid particles can facilitate effective cellular uptake *in vitro*. However, efficiency of *in vivo* uptake remains generally poor [97].

One particularly intriguing and promising avenue of research involves the use of cell-penetrating peptides (CPPs) to deliver conjugated ON cargoes. Such peptides include B-peptide and derivatives of Penetratin, a *Drosophila* protein rich in arginine residues. The exact mechanism by which CPPs enter cells is not fully elucidated [98]. However, ongoing studies involving peptide-conjugated AONs for the treatment of DMD are producing extremely promising results. These studies show that conjugation to CPPs dramatically increases ON uptake systemically in both skeletal muscle and heart [99, 100].

Personalized medicine

Mutations found in clinical practice, including those affecting splicing, are largely 'private' mutations, so-called because they are only found in a single individual or in a single kindred. Designing bespoke sequence specific therapies for such situations is personalized medicine in the truest sense of the term.

However, if each new ON sequence designed is classed as a novel therapeutic agent, it will be unfeasible to subject each new agent to all the rigorous drug development tests and trials used in current pharmaceutical practice. When the cohort of treatable patients consists of a single individual, there can be no prospect of a clinical trial. This issue is one of the major challenges facing personalized medicine and it must be resolved if we are to derive the full benefit promised by ON-based therapies.

Predicting splicing

A growing number of *in silico* software programs are available to help predict the effects of mutations on splicing. While these can provide useful information regarding mutations close to canonical splice sites, their accuracy regarding more subtle sequence changes in poorly conserved elements such as splicing enhancers and silencers is much more variable. In the clinical diagnostic setting, such predictions regarding unknown variants are generally not yet reliable enough to allow clinical decisions to be based upon them. In such cases, there is still a reliance on functional RNA studies to help elucidate the presence of aberrant splicing. However, even this approach has limitations, since the studies are almost always done in blood and there can be no guarantee that the pattern of splicing in leucocytes will necessarily reflect that in other tissues.

Predicting the effects that a particular sequence will have on splicing is currently one of the greatest challenges in molecular genetics. As we have seen, the answer is likely to be complex, since variations in *trans*-acting factors can alter the splice isoform pattern and different cell types are likely to splice genes differently in response to both intra- and extracellular conditions. Novel methods of global RNA analysis such as exon-junction microarrays and deep sequencing, together with detailed cataloguing of the targets of RNA binding proteins will lead to a fuller understanding of the complex regulatory networks that govern splicing and shed light on the effects of individual mutations on global patterns of splicing [101].

CONCLUSIONS

The examples cited in this review are far from comprehensive. However, they do serve to illustrate some of the many and varied ways in which splicing contributes to disease. RNA splicing is one of the fundamental processes of cell biology. The more that

is learnt about it, the more can be appreciated about its multilayered complexity and its relevance in terms of health and disease. Furthermore, by unpicking the mechanisms by which cells choose how to splice their RNA, a fuller picture is gradually emerging of how external factors of the cellular environment interact with internal genetic factors. This understanding brings with it too increasing opportunities to manipulate the splicing mechanism and to correct it when it causes disease. By advancement in areas such as ON delivery, splicing prediction and the understanding of splicing in disease pathogenesis, the scientific and medical communities are equipping themselves with much of the knowledge and tools needed for the next rapidly approaching frontier of biomedical science, that of personalized genetic medicine.

Key Points

- Pre-mRNA splicing is a highly complex process regulated by *cis*-acting sequence elements and *trans*-acting splicing factors.
- Aberrant pre-mRNA splicing is a frequent cause of human genetic disease.
- Therapeutic strategies to treat splicing diseases include small molecule modifiers of splicing, *trans*-splicing and AONs.
- Current challenges in this field include effective delivery systems, accurate splicing prediction and the development of personalized mutation-specific therapies.

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