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Alternative Splicing and Human Disease

Stefan Stamm, *University of Kentucky, Lexington, Kentucky, USA*

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Advanced article

Article Contents

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26950 (p0001) **Almost all human protein coding genes undergo alternative splicing, and an increasing number of diseases is associated with the selection of 'wrong' splice sites. Such missplicing can be caused by mutations in DNA (deoxyribonucleic acid), mutations in splicing factors and changes in the concentration of splicing factors. The effect of these mutations can be predicted using bioinformatic tools, but any prediction needs to be validated experimentally. Advances in understanding the molecular mechanisms regulating splice site selection resulted in the development of treatment options using RNA (ribonucleic acid) oligonucleotides as well as small molecules that inhibit or alter alternative splicing. Oligonucleotides for treatment of spinal muscular atrophy and Duchenne muscular dystrophy have advanced into clinical trials and serve as a paradigm for the treatment of other diseases caused by missplicing.**

Introduction

26950 (p0002) All polymerase II transcripts undergo pre-mRNA (messenger ribonucleic acid) processing, and almost all transcripts are subject to pre-mRNA splicing where parts of the pre-mRNA are removed as introns and the remaining sequences are joined together as exons and exported into the cytosol. This process can be alternative, where parts of the nuclear pre-mRNA are either included or excluded in the mature mRNA. This mRNA is subsequently exported into the cytosol and typically encodes a protein. Almost all (95–100%) multiexon genes undergo alternative splicing, where an exon is either included or skipped in the final pre-mRNA (Barbosa-Morais *et al*., 2012; Merkin *et al*., 2012). On average, a human gene contains a median of 26 exons per gene and generates 3.4 isoforms, indicating that alternative splicing increases the coding capacity of the human genome more than threefold. Alternative splicing is more abundant in humans than in other species, for example mice generate only 2.4 isoforms per gene and *Caenorhabditis* elegans 1.2 isoforms (Lee and Rio, 2015). The overall function of alternative splicing is to increase the diversity of the mRNA expressed from the genome (Kelemen *et al*., 2013). A genome-wide analysis showed a large influence of alternative splice variants on protein interaction networks. Within an interaction network, most alternative splicing variants behave more like distinct proteins than like minor variants (Yang *et al*., 2016), suggesting that alternative splicing generates functional distinct proteins from a single gene. Frequently, alternative splicing generates stop codons or frameshifts that often destine the resulting mRNA isoforms to nonsense-mediated decay. Examples described in the literature show that alternative splicing regulates the binding properties, intracellular localisation, enzymatic activity, protein stability and posttranslational modifications of a large number of proteins (reviewed in Kelemen *et al*., 2013). Defects in alternative splice site selection results in an increasingly recognised number of human diseases (Chabot and Shkreta, 2016; Wang *et al*., 2012).

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Splicing Mechanism

26950 (p0003) Alternative splicing is regulated by combinatorial control through a large network of protein:protein, protein:RNA and RNA:RNA interactions. In humans, splice sites do not contain enough information to identify alternative or constitutive exons. Additional information is provided by sequence elements that bind to nuclear proteins, which in turn interact with elements in the spliceosome to identify exons. This arrangement of RNA elements is called

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the 'splicing code', which is currently being deciphered using genome-wide approaches (Barash *et al*., 2010).

Exons are surrounded by three characteristic sequence elements: the 5′ and 3′ splice sites locates at the exon–intron border and the branch point located 18–50 nt upstream of the 3′ splice site. There are two splicing systems in humans, a major one where the introns are marked by GU–AG bases and a minor one accounting for less than 0.5% of introns, marked by AU–AC dinucleotides (Turunen *et al*., 2013). The splice sites are recognised through interaction with five small nuclear RNAs, U1, U2, U4, U5 and U6 snRNAs. Through base complementarity, the U1 snRNA recognises the 5′ splice site and the U2 snRNA binds to the branch point sequence. U4/U5 and U6 form a complex (U4/U5/U6 trisnurp) involved in the catalysis of the reaction.

Alternative Exon Recognition

26950 (p0005) Characteristic for human splice sites is their poor adherence to a consensus sequence, and only the GU–AG dinucleotides are always conserved in the major spliceosome. Owing to the poor conservation of splicing signals in humans, additional RNA elements are needed to regulate alternative splicing. These elements can act as splicing enhancers that promote exon inclusion or splicing silencers that block inclusion of exons in the mRNA (**Figure 1a**). Bioinformatic analyses indicate that up to three quarters of the nucleotides in an exon contribute to splicing regulation (Chasin, 2007). The splicing regulatory sequences act by binding to proteins that in turn interact with the spliceosome or – depending on the protein bound – block this interaction. The activity and concentration of splicing regulatory proteins is influenced by posttranslational modifications, such as phosphorylation, as well as sequestration by other proteins or RNAs (Zhou and Fu, 2013; Dey *et al*., 2014). In addition to proteins, noncoding RNAs can bind to splicing regulatory motifs (Falaleeva *et al*., 2016) and regulate splice site selection.

26950 (p0006) The RNA binding proteins interact with low specificity to accessible, mostly single-stranded parts of the pre-mRNA. As it can be often predicted what proteins bind to a given sequence, it is possible to generally predict whether a sequence enhances or silences exon usage or has no effect. However, the action of splicing regulatory proteins depends on neighboring RNA elements and secondary RNA structures. Owing to this dependency on the sequence context, the same short RNA sequence can act as a silencer or enhancer. Any functional prediction of a splicing regulatory element therefore needs to be tested experimentally.

26950 (p0007) By forming a transient protein complex on the pre-mRNA, the pre-mRNA processing machinery can overcome the highly degenerate nature of splicing signals. The degeneracy of the splicing signals is biologically necessary, as it allows to overcome the problem that pre-mRNA needs to contain the information for both the protein coding and splicing regulation. The importance of splicing sequences became apparent when synonymous mutations within the coding regions were shown to cause human diseases by altering exon usage (Cooper and Mattox, 1997). The comparison of tumour genomes showed that oncogenes contain an excess of synonymous mutations, likely leading to their splicing deregulation in cancer (Supek *et al*., 2014).

Circular RNAs

26950 (p0008) 26950 (p0004) In almost all protein-coding mRNAs described so far, the alignment of the exons in mRNAs corresponds to the corresponding sequence in the DNA (deoxyribonucleic acid). However, more than 25 years ago, mRNAs with 'scrambled exons' have been reported, that is rather than having exon 1-2-3, mRNAs with exons 1-3-2 were described (Nigro *et al*., 1991). This result was not followed up, as the generation of these exons was not clear. Advances in RNAseq technology then showed that these 'scrambled exons' were part of circular RNAs (circRNAs). circRNAs are generated from splicing in large lariats or are generated by intramolecular splicing of RNAs that are constrained through base pairing, for example owing to strong secondary structures caused by inverted repeats (Jeck *et al*., 2013). The formation of circRNAs due to strong secondary structures might be important for humans, as 11% of the human genome consists of Alu elements (Hasler *et al*., 2007). Owing to their origin from structural 7SL RNA, Alu elements can dimerise through the Alu domains that show self-complementarity. As Alu elements are primate specific, they could generate primate specific circRNAs.

26950 (p0009) Most circRNAs are less than 1% of the linear mRNAs made from the same locus, but in some cases, the circRNA is 10-fold more abundant than the linear mRNA from the locus (Jeck *et al*., 2013; Lasda and Parker, 2014; Salzman *et al*., 2013). circRNAs are mainly cytosolic and can be translated into protein (Abe *et al*., 2015; Jeck *et al*., 2013). In general, circRNAs are much more stable than linear mRNAs, as they will not be degraded by the exosome, which acts like an exonuclease. In addition, most circRNAs do not contain 3′UTR regions that promote deadenylation and exosomal degradation. Owing to their circular structure that recapitulates the binding of the poly(A) tail to the mRNA cap in linear RNAs, circRNAs are potentially excellent ribosomal substrates, allowing for efficient translation in vitro and in vivo (Abe *et al*., 2015). Thus, in addition to alternative splicing, the generation of circRNAs, performed by the spliceosome, largely increases the coding capacity of the human genome. circRNAs change their expression stronger than mRNAs upon cell transformation (Bachmayr-Heyda *et al*., 2015), and could thus contribute to the deregulation in cancer.

26950 (p0010) In summary, alternative pre-mRNA splicing greatly increases the number of proteins encoded by the DNA. A multitude of sequence signals determine whether a piece of the pre-mRNA is spliced into the mature mRNA. The balance of these signals can be disturbed by mutations on the RNA, which interfere with the recognition of a part of the pre-mRNA as an exon. In addition, mutations in splicing factors deregulate splice site selection, which contributes to disease (**Figure 1b**).

Mechanisms and Examples for Diseases Caused by Missplicing

26950 (p0011) Changes in alternative splicing can be caused by mutations in the pre-mRNA, mutations in trans-acting factors or changes in the concentration or localisation of trans-acting factors. As a result,

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the inclusion of alternative exons is altered, ranging from complete exon skipping to constitutive exon inclusion. In addition, new exons can be created by the generation of new splice sites when these splice sites are located next to an authentic or cryptic splice site.

DNA mutations

26950 (p0012) Over 2000 splicing mutations involving 300 genes and resulting in 370 diseases have been described in the literature and were compiled in a database (Wang *et al*., 2012). The best understood mutations that can also be predicted with a high accuracy by bioinformatic means are within the 5′ and 3′ splice site.

Mutations in splice sites

26950 (p0013) About 10% of roughly 80 000 mutations reported in the human gene mutation database affect splice sites (Cooper *et al*., 2006). As the exon–intron structure of human genes is known, these mutations can be easily identified and their outcome can be predicted with a high degree of success by computer programs. Well-studied diseases caused by changes in splice site selection include thalassemias (Birgens and Ljung, 2007) and familial dysautonomia (FD) (Ibrahim el *et al*., 2007) (**Figure 2b**, (**ii**)).

Exonic mutations

26950 (p0014) Exonic mutations can either change the protein encoded by the exon or be translationally silent. In both the cases, they can affect splice site selection (**Figure 2b**, (**i**)). The effect of synonymous exonic mutations that do not change the predicted reading frame could not be explained until their role in splice site selection became apparent (Cooper and Mattox, 1997). Exonic mutations have been very informative for the analysis of the splicing process (Daguenet *et al*., 2015) but are less frequent than intronic mutations. Well-studied examples include mutations causing frontotemporal dementia with parkinsonism-17 (*FTDP-17*) located in the tau gene (Jiang *et al*., 2003), mutations in medium-chain acyl-CoA dehydrogenase (MCAD) leading to MCAD deficiency (Nielsen *et al*., 2007), mutations leading to
AU:3 ic fibrosis (CFTR) (Haque *et al*., 2010) and mutations in lamin A resulting in Hutchinson–Gilford progeria (Lopez-Mejia *et al*., 2011) and a mutation in the survival-of-motoneuron 2 gene that results in spinal muscular atrophy (SMA) when the survival-of-motoneuron 1 gene is lost (Burghes and Beattie, 2009), see the following discussion. In each of these cases, a mutation disrupts a splicing regulatory element, changing the use of a specific alternative exon, which leads to a disease. Studies in cancer showed that about 20% of point mutations in cancer cells are synonymous (Supek *et al*., 2014). Up to 8% of these somatic mutations are selected in cancer cells, suggesting that they are functional. Examples include BRCA2/breast cancer (Raponi *et al*., 2011), APC/familial adomentous polyposis (Pecina-Slaus *et al*., 2008, 2010) and TP53/multiple tumours (Supek *et al*., 2014).

Intronic mutations

26950 (p0015) The sequencing of 25 000 genomes from 50 different cancer types showed that most of the sequence variations occurs outside protein-coding exons (Diederichs *et al*., 2016) and many of them could affect splice site selection. However, as these mutations are located hundreds of nucleotides away from known exons, their effect on flanking exons can currently not be estimated by bioinformatic means but needs to be addressed experimentally. Examples include types of neurofibromatosis type 1 (NF1) (Raponi *et al*., 2006) and hyperinsulinism (Flanagan *et al*., 2013) (**Figure 2b**, (**iii**)).

Mutations in splicing factors

26950 (p0016) Underlining its central role of the evolutionary highly conserved spliceosome in gene expression, germ line mutations in core splicesomal proteins are rare and include PRP3, PRP6, PRP8, Brr2 and PRP31 causing dominant nonsyndromic retinitis pigmentosa (Boon *et al*., 2007; Chakarova *et al*., 2002; Vithana *et al*., 2001; Liu *et al*., 2012). Mutations in the minor spliceosome small nuclear RNA U4atac cause microcephalic osteodysplastic primordial dwarfism (MOPD) type I (He *et al*., 2011).

26950 (p0017) In contrast to the rare germ line mutations, numerous somatic mutation of core spliceosomal and pre-mRNA associated proteins have been detected in cancer cells. Interestingly, they cluster in components that recognise the 3′ splice site, namely, U2AF35 (U2AF1), ZRSR2, a binding partner for U2AF (Shen *et al*., 2010) and the U2 snRNP component SF3B1 (Martin *et al*., 2013; Quesada *et al*., 2012). More than 50% of all patients with myelodysplastic syndrome show mutations in these proteins.

26950 (p0018) Similarly, mutations in several, but not all proteins binding to splicing regulatory elements on the pre-mRNA, have been detected. They include SRSF1 (SF2/ASF); SRSF2 (SC35) (Hong *et al*., 2015). These proteins generally promote exon recognition. In addition, SRSF1 plays a role in genome stability (Wang *et al*., 1996).

Change in concentration of splicing factors

26950 (p0019) The best mechanistically understood changes in splicing factors leading to diseases are caused through sequestration of proteins by trans-acting RNAs (**Figure 1b**, (**iv**)). Microsatelites are frequent, short (2–5 nt) repetitions in DNA that are also frequently found in pre-mRNAs. Expansion of microsatellites can create stable RNA structures that sequester splicing factors. The best understood example is myotonic dystrophy, where expanded CUG repeats in the DMPK gene lead to myotonic dystrophy type I (DM1) and CCUG expansion in the ZNF9 gene leads to myotonic dystrophy type II (DM2). CUG repeats sequester the splicing factor MBNL1 and cause a phosphorylation of the splicing factor CUG-bp, which changes numerous alternative exons that contribute to the phenotype (Fardaei *et al*., 2002; Kuyumcu-Martinez *et al*., 2007; Tiscornia and Mahadevan, 2000).

26950 (p0020) Other repeat extensions cause spinocerebellar ataxia 8 and fragile X-associated tremor ataxia syndrome (FXTAS) (Ranum and Cooper, 2006) and have a similar influence on pre-mRNA splicing.

26950 (p0021) A cytoplasmic agreggation of the RNA binding protein TDP-43 is a hallmark of amyotrophic lateral sclerosis and likely decreases

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the nuclear concentration of TDP-43 which changes alternative splicing of numerous exons (Scotter *et al*., 2015).

Numerous splicing factors show changes in expression in cancer cells. They include SR proteins (SRSF1, SRSF2 and SRSF6), hnRNPA1 and hnRNPI and other RNA binding proteins such as RBM5 and RBM10 (Singh and Cooper, 2012). In general, SR proteins and hnRNPs shuttle between the cytosol and nucleus, which can be affected by hypoxia inside tumours that promotes a cytoplasmic accumulation, resulting in changes in alternative exon usage (Hirschfeld *et al*., 2009, 2013). 26950 (p0027)

26950 (p0023) Thus, changes in the nuclear, active concentration of splicing factors affects numerous exons and the combination of these changes can cause a disease.

Analysis of Diseases Caused by Missplicing

Bioinformatic tools

26950 (p0024) Using experimental and bioinformatic approaches, splicing regulatory RNA sequences and their trans-acting factors have been individually studied in great detail. The result of these studies led to the development of programs that predict splice sites, splicing regulatory sequences and their binding partners, both predicted and experimentally validated through CLIP experiments. **Table 1** lists some of these computational tools implemented with easy-to use web servers. It should be emphasised that due to the complexity of splice site selection the programs are currently fairly inaccurate. However, the usage of several programs allows the generation of hypotheses that can be experimentally tested.

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Experimental testing

26950 (p0025) The most common technique to detect a change in alternative splicing is RT-PCR (reverse transcription polymerase chain reaction) from an affected tissue. A change in splicing that correlates with a mutation is usually a good indication that the mutation causes the disease. As alternative splicing is regulated by numerous other factors, it is necessary to corroborate these findings with other experimental tests. The most common method to analyse exon usage and especially the effect of a mutation on splice site selection is reporter minigene analysis. In this method, an exon of interest, as well as its flanking exons are cloned into an expression vector and analysed after transfecting this construct into eukaryotic cells. The comparison of two minigenes that were mutated to reflect naturally occurring alleles allows one to determine how a change in sequence influences alternative splicing (Gaildrat *et al*., 2010; Stoss *et al*., 1999).

26950 (p0026) The system can be expanded for the analysis of trans-acting factors by cotransfecting an increasing amount of factor-expressing cDNA constructs with the reporter minigene. To facilitate cloning of splicing reporter constructs, a recombination-based system that allows rapid generation of minigenes from PCR products containing the alternative exon has been developed (Kishore *et al*., 2008).

Therapeutic Approaches

26950 (p0022) Owing to the importance of alternative splicing for human diseases, numerous therapeutic approaches have been devised. Oligonucleotides that selectively change a single splicing event, substances that inhibit the spliceosome, and small molecules that promote a missing gene product are the main approaches (**Figure 2**).

Oligonucleotide-based therapies

Spinal muscular atrophy

26950 (p0028) SMA is a neurodegenerative disease that is currently the leading genetic cause of death in children (Lunn and Wang, 2008). Owing to its high prevalence with a carrier frequency of 1:40, SMN is a well-studied example of a fatal disease that could be cured if a single splicing pattern could be changed. The research resulted in splice-site changing oligonucleotides that are now in clinical trials (Rigo *et al*., 2014).

26950 (p0029) The cause of SMA is the loss of the SMN (survival-of-motoneuron) protein that is involved in generating snRNPs that are the core components of the spliceosome. Owing to a recent gene duplication, humans possess two related SMN genes, SMN1 and SMN2. The genes are almost identical and arranged in tandem, which makes them prone to recombination. The loss of the SMN1 gene that encodes the SMN protein causes the death of neurons by an unknown mechanism, presumably involving numerous small changes in alternative splicing of pre-mRNAs and/or a defect in the neuronal transport system (Burghes and Beattie, 2009). Owing to the gene duplication event, humans also possess the SMN2 gene that is almost identical to SMN1. One crucial difference, however, is a silent C->T mutation in exon 7 that causes predominant exon 7 skipping in the SMN2 pre-mRNA. The failure to include this exon leads to a truncated, unstable protein. One possible therapeutic approach for SMA would be to alter the splicing pattern of the SMN2 pre-mRNA in favour of exon 7 inclusion. Exon 7 inclusion is regulated by several enhancer and silencer elements located in exon 7 and its vicinity (Singh, 2007). To identify oligonucleotides that promote exon inclusion, the exon and its flanking regions were tested with overlapping 18 mers in an oligowalk, which identifed an oligonucleotide binding to an intronic silencer (Hua *et al*., 2007; Singh *et al*., 2004) (**Figure 2a**). Optimising the chemistry using 2′-*O*-methylation, 2′-*O*-methoxyethyl, -phosphothioates prevented degradation of the oligonucletide and its targets by RNAseH and improved cellular uptake. This oligonucleotide can be delivered to the spinal cord through intrathecal injections, where it is taken up by neurons and show effects 6 months post injection in mice (Rigo *et al*., 2014). In phase I clinical trials, the oligo showed little adverse effects and significantly improved the clinical features of the patients (Chiriboga *et al*., 2016).

Duchenne muscular dystrophy

26950 (p0030) While exon inclusion is the therapeutic principle for SMA, exon skipping is the principle for Duchenne muscular dystrophy

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(DMD). DMD affects 1:5000–1:10 000 male births and is lethal, in contrast to the milder Becker muscular dystrophy. The most frequent cause for DMD are deletion of exons encoding the muscle structural protein dystrophin. The deletion of exons causes a frameshift as downstream exons are in a different reading frame, which creates truncated dystrophin proteins, resulting in muscle weakness and death in the second decade of life. The dystrophin gene spanns 2.5 Mb and contain at least 79 exons. Thus, it is possible to restore the reading frame by forcing skipping of an exon downstream of the deletion, which can be achieved using oligonucleotides (**Figure 2b**). The splicing change creates a larger, but still truncated dystrophin protein, characteristic for the milder Becker muscular dystrophy phenotype. Currently, oligonucleotides causing skipping of exons 44, 45 and 53 are in clinical trials. Although the oligonucleotides restored a larger but still truncated dystrophin protein, the effect on patients were not statistically significant and so far the drugs have not been approved (Jacobson and Feldman, 2016; Havens and Hastings, 2016).

26950 (p0031) Similarly, antisense oligonucleotides have been tested for other diseases, among them beta thalassemias (Lacerra *et al*., 2000), cystic fibrosis (Friedman *et al*., 1999), Hutchinson Gilford Progeria Syndrome (HGPS) (Scaffidi and Misteli, 2005), Pompe disease (Clayton *et al*., 2014), Usher syndrome (Lentz *et al*., 2013) and hyperphagia (Zhang *et al*., 2016). A recent updated list is given by Havens and Hastings (2016).

Spliceosome Inhibitors

26950 (p0032) Two structurally dissimilar bacterial natural products, FR901464 and pladienolide, were discovered in 2007 and shown to inhibit splicing in vitro by binding to the U2 snRNA component SF3B1. Importantly, the compounds caused selective death of cancer cells (Kaida *et al*., 2007). Two natural compounds, herboxidiene (GEX1A) (Hasegawa *et al*., 2011) and thailanstatins (Liu *et al*., 2013), are structurally related and showed also anti-cancer activity. On the basis of these natural compounds, synthetic sudemycins were developed (Fan *et al*., 2011; Lagisetti *et al*., 2013), which showed strong activity against cancer cells but low toxcicity in primary cells. In contrast to blocking splicing in vitro, the compounds promote skipping of hundreds of exons in vivo, likely by preventing the correct positioning of U2 snRNP at weak 3′ splice sites (Corrionero *et al*., 2011). Sudemycins show long lasting cytotoxic effects following drug 'washout' in vitro after a 30-min incubation (Convertini *et al*., 2014; Fan *et al*., 2011) suggesting additional modes of action. Reflecting the interaction between U2 snRNPs and histones, it was found that sudemycin treatment causes chromatin modifications as well as changes in gene expression (Convertini *et al*., 2014). A clinical trial in cancer patients with the semisynthetic pladienolide analogue E7107 has been suspended due to occular toxicity (Eskens *et al*., 2013), but the improved derivatives are predicted to enter clinical trials.

Low Molecular Weight Substances

26950 (p0033) The use of special screening systems (Stoilov *et al*., 2008) and candidate compound testing identified numerous substances that change alternative splicing including HDAC inhibitors, kinase and phosphatase inhibitors, as well as cAMP (cyclic adenosine monophosphate) antagonist and agonists that change splicing (Ohe and Hagiwara, 2015; Sumanasekera *et al*., 2008). The example of FD shows that small drug screenings can give new insights into disease mechanisms and could be therapeutically beneficial without directly changing splicing. FD is a neuropathy characterised by a progressive degeneration of the sensory and autonomic nervous system. It is caused by a splice site mutation leading to exon skipping in the inhibitor of kappa light polypeptide gene enhancer in B cells kinase complex-associated protein (*IKBKAP*) gene. Usage of a splicing reporter identified a compound that is selective for the crucial IKBKAP exon and 51 other exons in different genes. Treating cells with the compound showed also changes in tRNA modifications, suggesting a novel disease mechanism (Yoshida *et al*., 2015). A different screening approach identified phosphatidylserine as a compound that induces IKBKAP expression, likely through the MAPK/ERK signalling pathway. As the increased expression could compensate the loss through abarrant splicing, the compound holds therapeutic promise (Donyo *et al*., 2016).

Outlook

26950 (p0034) Tremendous progress has been made in understanding the regulation of alternative splicing and its impact on human disease. On the basis of a detailed molecular understanding, clinical trials using oligonucleotides are now conducted for SMA and Duchenne muscular dystropy, which serve as a blueprint for other splicing diseases. The experimental treatment of cancer cells with splicing inhibitors led to the emergence of spliceosome medicinal chemistry as a new field providing anticancer drugs. It can be expected that splicing-changing drugs will become part of medical practice.

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Further Reading

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Key Concepts

- Mechanism of alternative splice site selection.
- Changes in alternative splicing caused by mutations that result in disease.
- Mutations leading to a change in alternative splicing.
- Analysis of mutations that change splice site selection.
- Splicing-changing oligonucleotides to treat diseases.
- Small molecules as splicing inhibitors or modulators to treat diseases.

Glossary

Cryptic exon# Exon that is used only after a mutation occurred.
Exon# pre-mRNA sequence that appears in the mRNA.
Intron# Intervening sequence that is excised from the pre-mRNA.
Splicing-changing oligonucleotide# Oligonucleotide that changes splice site selection.
Synonymous mutation# Mutation that does not change the reading frame

Author(s) and Affiliation(s):

Stefan Stamm, *University of Kentucky, Lexington, Kentucky, USA*

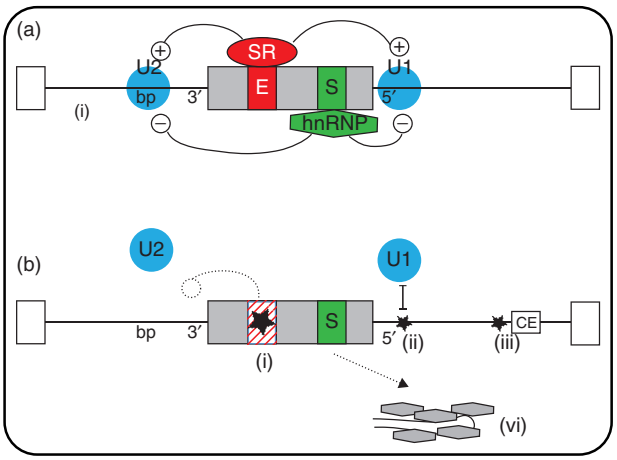

Figure 1 **Schematic overview over disease mechanisms**. (a) *Overview of exon recognition*. A cassette exon flanked by two constitutive exon is shown. Exons are indicated as boxes and introns as lines. The exon contains enhancers (E) and silencers (S) that bind to proteins, SR proteins and hnRNPs. In general, SR proteins promote exon inclusion by stabilising the binding of U2 and U1 snRNPs to the branch point (bp) and 5′ splice site (5′), respectively, 3′: 3′ splice site. hnRNPs have in general the opposite effect. (b) *General disease mechanism*. Mutations on the DNA are indicated with a star. (i) Mutations in exon enhancers prevent SR proteins from binding, which inhibits exon usage; (ii) mutations in 5′ splice sites prevent U1 snRNP binding; (iii) intronic mutations generate cryptic exons; (iv) stable RNAs generated through repeat extensions sequester splicing proteins.

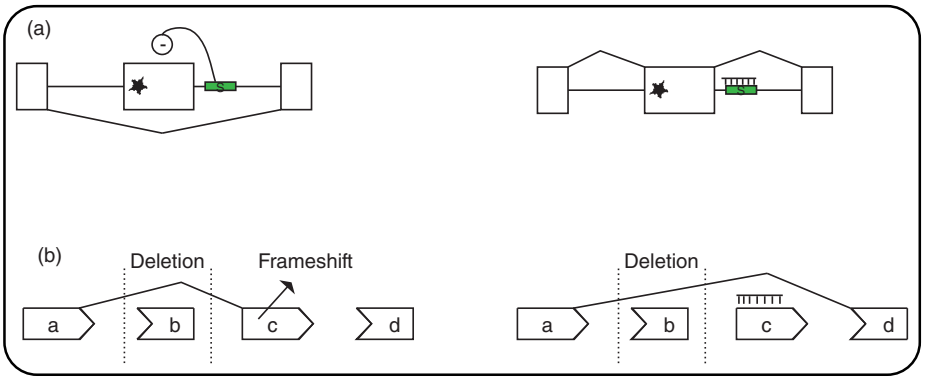

Figure 2 **Therapeutic approaches**. (a) *Spinal muscular atrophy*. A point mutation (star) in the SMN exon 7 generates a splicing silencer, leading to skipping, owing to the presence of other silencers, including an intronic silencer. An oligonucleotide binding to the silencer neutralises its action, leading to exon inclusion. (b) *Duchenne muscular dystrophy*. Exons a–d of the dystrophin gene are schematically indicated. The reading frame of the exons is indicated by the fitting shapes. Thus, a genomic deletion of exon b results in a reading frame change and truncated protein when a is spliced to c. An antisense oligonucleotide causing the skipping of exon c restores the reading frame between a and d.

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Table 1 Bioinformatic resources to detect regulatory features of alternative exons. At the current state, the predictions are highly inaccurate and need to be experimentally verified

Tools	URL	Features	Reference
ESEfinder	http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi	Analysis of sequence to find ESE motifs	Smith *et al.* (2006)
HSP (Human Splicing Finder)	http://www.umd.be/HSF3/	Exonic and intronic motifs identification	Desmet *et al.* (2009)
RegRNA (Regulatory RNA motifs and Elements finder)	http://regrna.mbc.nctu.edu.tw/php/browse.php?ShowType=4	Prediction of the regulatory RNA motifs and elements	Huang *et al.* (2006)
SpliceDisease database AU:10	http://cmbi.bjmu.edu.cn/sdisease	Database of mutations that result in splicing defects	Wang *et al.* (2012)
SpliceAid AU:11	http://193.206.120.249/splicing_tissue.htm	Database of human splicing factors expression data and RNA target motifs	Giulietti *et al.* (2013)
CLIPdb	http://clipdb.ncrnalab.org	CLIP-seq database for protein–RNA interactions	Yang *et al.* (2015)

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