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Review Article

Splicing in oncogenesis and tumor suppression

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Post-transcriptional modifications, such as 5′ end capping, 3′ end polyadenylation and splicing, are necessary for the precise regulation of gene expression and transcriptome integrity. Therefore, it is not surprising that abnormalities of these post-transcriptional modifications prompt numerous diseases, including cancer. In fact, many studies revealed that misregulation of mRNA processing, especially splicing, are observed in a variety of cancer cells. In this review we describe how changes within RNA splicing regulatory elements or mutations in the processing factors alter the expression of tumor suppressors or oncogenes with pathological consequences. In addition, we show how several small molecules that bind to spliceosomal components and splicing regulators inhibit or modulate splicing activity. These compounds have anticancer activity and further development of small molecule modulators has potential in next generation cancer therapy. (*Cancer Sci* 2012; 103: 1611–1616)

mRNA Splicing

In eukaryotes, nascent transcripts (precursor messenger RNA [pre-mRNA]) are subjected to post-transcriptional modifications, including capping of the 5′ end, intron removal by splicing, as well as cleavage and polyadenylation at the 3′ end, to become mature mRNA that are templates for translation.[1] These post-transcriptional modifications are important for efficient gene expression and for the integrity of the transcriptome, hence aberrations in these modifications might perturb gene expression and interfere with vital cellular functions, enabling pathogenesis including carcinogenesis or tumor progression.[2]

Pre-mRNA consist of protein coding regions, exons and intervening sequences, introns.[3] The splicing process joins the exon sequences while removing the introns. These reactions are coordinated and catalyzed by the spliceosome, a large, multi-component ribonuclear complex, consisting of five subcomponent small nuclear ribonucleoprotein particles (snRNPs), named U1, U2, U4, U5 and U6 (Fig. 1). The splicing reactions start with recognition of the intron's 5′ end, the 5′ splice site (5′ ss), by U1 snRNP, followed by SF1 binding to the branch point sequence and interaction of U2AF with the 3′ end of the intron, the 3′ splice site (3′ ss), to form complex E. Complex E turns over to complex A when U2 snRNP replaces SF1. Complex B results from U4/U6•U5 tri-snRNP binding to complex A. In the last step, conformational changes enable two transesterification reactions by which the intron sequence is excised and the adjacent exons joined.

This process requires high precision and fidelity as gene expression depends on the integrity of the transcript. A change by only one nucleotide would introduce a frame-shift, which would not only alter the amino acid sequence of the protein but likely introduce a premature termination codon (PTC). Similarly, intron retention by failure to splice will even more likely yield mRNA with a PTC, as intron sequences appear enriched in stop codons. Such mRNA with a PTC are degraded by nonsense mediated decay (NMD) or translated into truncated proteins.[4,5] Consequently, frequent failure in recognition of the splice site would completely disable transcriptional regulation and meaningful gene expression.

Alternative Splicing

Alternative splicing is a mechanism to produce multiple isoforms from a single gene through the use of alternative splice sites. Alternative splicing not only increases protein variety, but the resultant gene products might have considerably different functions. In some cases, alternative splice isoforms might even oppose each other's effect. Production of splice isoforms depends on the tissue at hand, as well as the organism's developmental stage.[6,7] Multicellular eukaryotes, like mammals, take advantage of this mechanism to expand protein diversity from a limited number of genes. In fact, more than 90% of genes are subjected to alternative splicing in humans.[8,9] As noted, alternative splicing greatly contributes to biological complexity, while failure to properly express the correct isoform can have deleterious consequences and aid tumor formation (see below).

Alternative splicing is regulated by cis elements within the RNA and trans RNA binding factors. Well-studied trans factors are serine/arginine-rich (SR) proteins and heterogeneous nuclear RNPs (hnRNPs)[10] (Fig. 1). SR proteins, binding to exonic splicing enhancers (ESE) and intronic splicing enhancers (ISE), recruit spliceosomal components and promote splicing and exon inclusion.[11] In contrast, hnRNPs mainly bind to exonic and intronic splicing silencer sequences (ESS and ISS) and inhibit splicing or promote exon skipping by inhibiting the positive splicing factors.[12] Therefore, mutations in splicing enhancer/silencer elements and RNA binding trans factors might result in the expression of undesirable isoforms, which in turn can induce or aid carcinogenesis. We describe some examples in which a change of splicing pattern leads to cancer development below.

Splicing Factors Implicated in Cancer Development

Serine/arginine-rich splicing factor 1 (SRSF1) is a well-known SR protein regulating both constitutive and alternative splicing.[11] Recently, it was reported that SRSF1 is upregulated in various human cancers and overexpression of SRSF1 transforms immortal cells, suggesting that SRSF1 has oncogenic activity.[13] Actually, SRSF1 regulates splicing patterns of several important oncogenes and tumor suppressors, resulting

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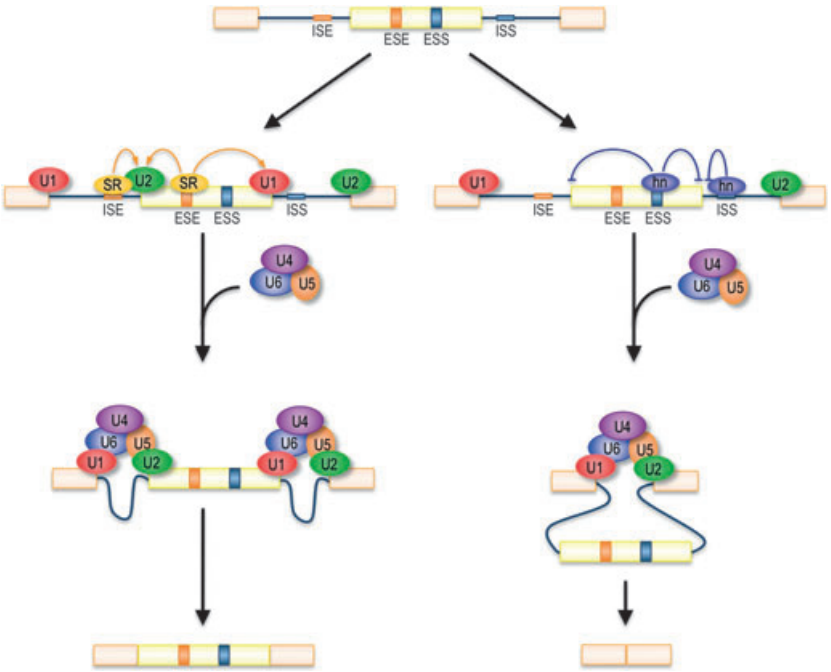

Fig. 1. Schematic representation of mRNA splicing. U1 small nuclear ribonucleoprotein particle (snRNP) and U2 snRNP recognize 5′ splice site (5′ ss) and branch point sequence, respectively. U4/U6•U5 snRNP joining is followed by conformational changes to become the catalytically active spliceosome. Binding of SR proteins to exonic or intronic splicing enhancers (ESE and ISE, respectively) stimulates splicing efficiency, while binding of hnRNP to splicing silencers (ESS and ISS) suppresses splicing and causes exon skipping.

in inhibition of apoptosis and increase of the rate of cell growth.

Tumor suppressor BIN1 interacts with the product of the *MYC* proto-oncogene and suppresses oncogenic activity of MYC.[14] Tumor-suppressing activity of BIN1 is controlled by alternative splicing. Inclusion of exon 12A diminishes BIN1 binding to MYC and hence also decreases its antitumor activity (Fig. 2). Overexpression of this isoform has been observed in melanoma cells.[15] Inclusion of exon 12A is increased by SRSF1 overexpression and decreased by knockdown of SRSF1, suggesting that SRSF1 contributes to cancer progression by changing the splicing pattern of the *BIN1* mRNA.[13]

RPS6KB1 is another SRSF1 regulated gene. It encodes a kinase of the small subunit ribosomal protein S6 and regulates cell growth and apoptosis.[16] RPS6KB1 has two isoforms (isoform-1 and isoform-2) and expression of isoform-2 is stimulated by SRSF1 overexpression.[13] Overexpression of isoform-2 transforms NIH3T3 cells and the SRSF1 expression level is correlated with the isoform-2/isoform-1 ratio in human lung cancer cells, suggesting that a splicing pattern change in *RPS6KB1* by SRSF1 also promotes cancer development.

RON is a tyrosine kinase receptor for the macrophage stimulating protein (MSP) and is involved in cell dissociation, mobility and invasion.[17,18] An alternative spliced isoform of RON without exon 11, named ΔRON, is constitutively active even in the absence of its ligand[19] (Fig. 2). ΔRON is highly expressed in several cancers, suggesting that the splicing pattern change of *RON* regulates tumor progression.[20] The splicing pattern of *RON* is regulated by SRSF1, which directly binds to an ESE of *RON* to stimulate exon 11 skipping and enhances cell motility, consistent with upregulation of SRSF1 in cancer cells.[13,20]

The hnRNP protein family, which functions as a splicing silencer, is also implicated in cancer development. One of its family members, hnRNP H, regulates alternative splicing of the *IG20/MADD* gene encoding several alternative splicing isoforms including IG20 that triggers apoptosis, and another isoform MADD, the MAP kinase activating death domain protein, that is expressed at high levels in cancer cells, contributing to their survival by inactivating caspase-8[21–23] (Fig. 2). HnRNP H binds to exon 16 of *IG20/MADD* and induces exon 16 skipping to produce the MADD isoform.[21] Conversely, knockdown of hnRNP H results in exon 16 inclusion and IG20 isoform expression, leading to reduction of cell survival in both U373 glioma and HeLa cells. Interestingly, hnRNP H also controls alternative splicing of *RON*. The *RON* gene has several binding motifs for hnRNP H in exon 11 and binding of hnRNP H promotes exon 11 skipping and production of ΔRON that induces invasion and migration.[21] HnRNP H is actually overexpressed in glioma cells and might function as an oncogene by modulating splicing of both *IG20/MADD* and *RON*.

Another hnRNP family member, hnRNP A2/B1, is overexpressed in several tumors, such as glioblastoma, lung cancer and breast cancer.[24–26] Its expression level is correlated with poor prognosis. Knockdown of hnRNP A2/B1 induces apoptosis only in cancer cells, but not in normal cells.[27] HnRNP A2/B1 modulates the splicing patterns of numerous genes including pyruvate kinase M (*PKM*), which controls glucose metabolism.[28] PKM has two isoforms, PKM1 and PKM2, expressed in adult tissues and in embryonic cells, respectively (Fig. 2). The embryonic isoform PKM2, which is re-expressed in cancer cells, promotes aerobic glycolysis and tumor growth, whereas PMK1 activates oxidative phosphorylation and reduces tumorigenicity.[29] HnRNP A2/B1 binds to the flanking region of exon 9 of the *PKM* gene and promotes exon 9 skipping and exon 10 inclusion, leading to PKM2 production.[28] These results suggest that hnRNP A2/B1 regulates tumorigenesis through the alternative splicing of *PKM1/2*. However, since Bluemlein *et al.*[30] reported most recently that they did not observe a transition from PKM1 to PKM2 during tumorigenesis, further study is required to elucidate whether hnRNP A2/B1-dependent alternative splicing of *PKM* does indeed enhance carcinogenicity.

Recently, Yoshida *et al.* reported that mutations in core components of the splicing machinery, namely SF3b1 and U2AF35, are found in patients with myelodysplasia, a myeloid neoplasm characterized by deregulated dysplastic blood cell production and a predisposition for acute myeloid leukemia.[31,32] Furthermore, a mutation in U2AF35, which is observed in patient samples, causes a reduction in the rate of

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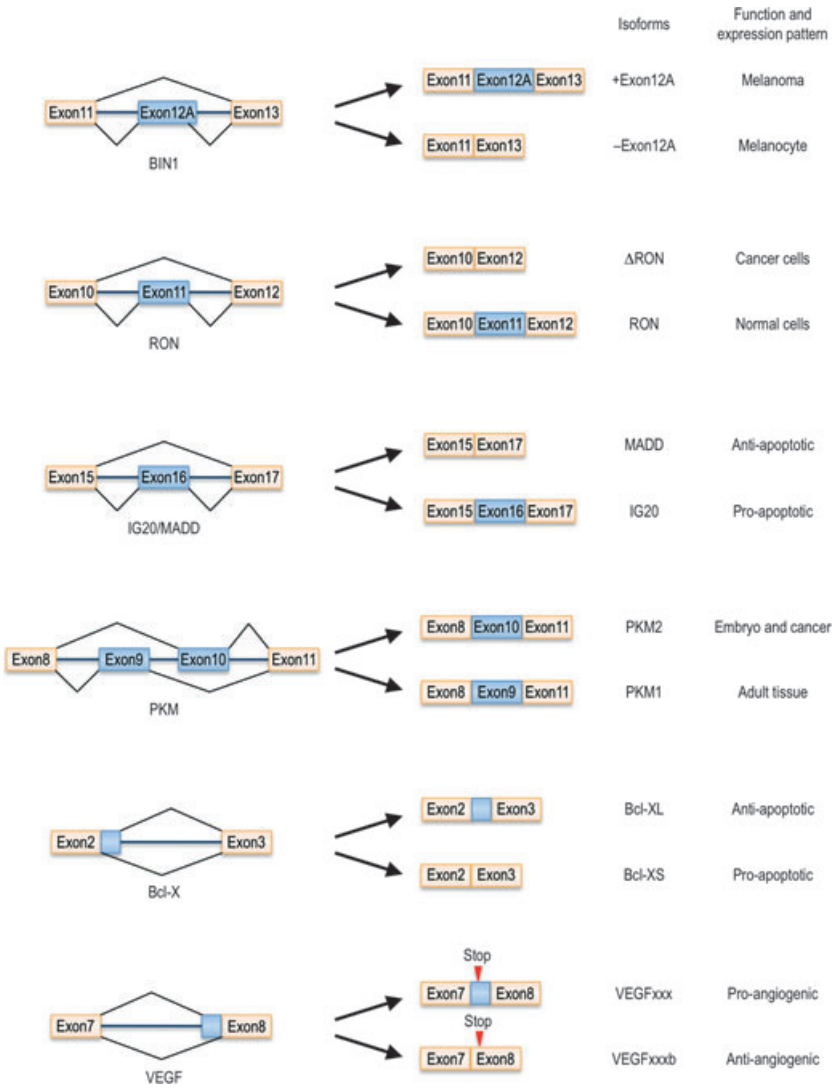

Fig. 2. Alternative splicing isoforms discussed in this review. Example of proteins whose alternative splicing patterns greatly influence their activity and carcinogenic potential. Each isoform's function and expression pattern is noted on the right side of each diagram.

cell growth and seems to induce apoptosis, which are features of myelodysplasia. While it is possible that abnormal splicing causes this hematological disorder, it currently remains uncertain how it could be involved in disease progression and tumorigenesis.

Splicing Pattern Changes by Alternative Splicing Affect Cancer Development

Beyond IG20/MADD or PKM1/2, the function of several other genes is regulated by alternative splicing, with each splice isoform fulfilling distinct or even opposing functions.[6] Further examples include the apoptosis regulator Bcl-X and the vascular endothelial growth factor (VEGF).

Bcl-X. Bcl-X is a well-known regulator of apoptosis.[33] *Bcl-X* pre-mRNA has two alternative 5′ ss in intron 2 (Fig. 2). If the upstream 5′ ss is used, the pro-apoptotic isoform Bcl-X_S is produced. However, splicing at the downstream 5′ ss results in generation of the anti-apoptotic isoform Bcl-X_L.[34–36] Bcl-X_L is overexpressed in a wide variety of cancer cells,[37,38] whereas Bcl-X_S is usually downregulated.[39] The splicing pattern of Bcl-X is controlled by many splicing regulators.[6,40] One such factor is RBM25, also referred to as hRED120. It acts as a RNA binding protein, which interacts with RNA processing factors both physically and genetically.[41] Co-suppression of RBM25 with splicing and 3′ end processing factors results in aberrant growth or developmental defects in *Caenorhabditis elegans*. RBM25 binds to the *Bcl-X* pre-mRNA and recruits U1 snRNP to the upstream 5′ ss of intron 2 of the *Bcl-X* gene through interaction with hLuc7A, a U1 snRNP binding protein, resulting in production of the short Bcl-X_S isoform.[42,43]

Another RNA binding protein playing a role in alternative splicing is hnRNP K, which also influences the splicing pattern of the *Bcl-X* gene. HnRNP K binds to a splicing silencer element located in the vicinity of the upstream 5′ ss of the *Bcl-X* gene and represses the production of the Bcl-X_S isoform.[40]

Sam68, which was identified as a target of the oncogenic protein kinase Src-family, is yet another splicing regulator of Bcl-X.[44–46] Inactivation of Sam68 causes neoplasmic transformation, while overexpression of Sam68 leads to cell cycle arrest and apoptosis. This suggests that Sam68 is a potential tumor suppressor.[47,48] Sam68 binds to *Bcl-X* mRNA and promotes splicing at the upstream 5′ ss to increase the relative amount of isoform Bcl-X_S. This effect is reversed by tyrosine phosporylation of Sam68 through Src-like kinase Fyn, indicating that dephosphorylated Sam68 induces apoptosis through alternative 5′ ss choice of *Bcl-X*,[46] although Sam68 also contributes to tumor progression in other contexts.[49]

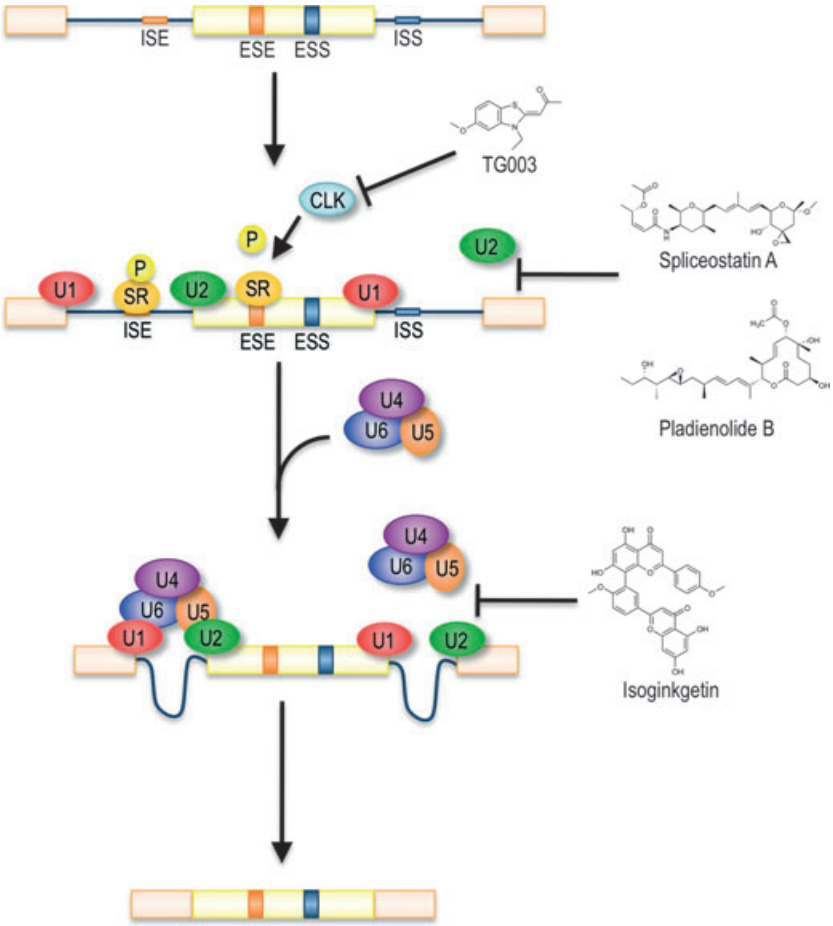

Fig. 3. Small molecules inhibiting splicing. Spliceostatin A and pladienolide B inhibit stable binding of the U2 small nuclear ribonucleoprotein particle (snRNP) to precursor messenger RNA (pre-mRNA). Isoginkgetin interferes with U4/U6•U5 binding. TG003 decreases the phosphorylation level of SR proteins by inhibiting the SR protein kinase CLK. ESE, exonic splicing enhancer; ESS, exonic splicing silencer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer.

Vascular endothelial growth factor. Vascular endothelial growth factor is a key regulator of angiogenesis, a prominent feature of cancer.[50,51] The VEGF family consists of several members, with each isoform designated as VEGFxxx, with xxx indicating the number of amino acids in the mature protein. Their lengths depend on whether alternative forms of exons 6 and 7 become incorporated into the mature mRNA. For a long time, it was not sufficiently appreciated that the *VEGF* gene gives rise to an entire family of growth regulators, some displaying rather different properties. VEGF isoforms fall into two main categories, VEGFxxx and VEGFxxxb.[52,53] The *VEGF* gene contains two possible splice sites in exon 8[53] (Fig. 2). If the proximal splice site is selected, the product will belong to the angiogenic VEGFxxx family. If the distal site is chosen, the sequence of the six most C-terminal amino acids will be changed. In this case, the product belongs to the VEGFxxxb family and will have anti-angiogenic properties, exactly the opposite of VEGFxxx. This splice site selection is controlled by SR proteins.[54] Overexpression of SRSF1 and SRp40 causes VEGFxxx isoform production. In contrast, SRp55 enhances distal splice site selection, resulting in production of anti-angiogenic VEGFxxxb isoforms.

Expression of VEGFxxxb isoforms is downregulated in renal cell carcinoma, prostate cancer and malignant melanoma.[53,55,56] Not surprisingly, VEGFxxx is overexpressed in many cancer cells.[57] VEGFxxx binding to VEGF receptor 2 (VEGFR2) induces dimerization of the receptor and activates several signal transduction cascades.[58] It appears that the different C-terminus of VEGFxxxb is unable to bind the neurophilin 1 co-receptor required for the full activation of VEGF-induced signaling.[59] This difference of binding mode can explain opposing functions of each VEGF isoform.

Modulating Splicing by Small Molecules for Cancer Therapy

Thus far we have outlined the importance of accurate splicing in eukaryotes and described some of the pathologies arising from aberrant splicing or from loss of control over its regulation. With the discovery of small molecules that interfere with splicing, the possibility of using splice inhibition for therapeutic purposes arose.

Spliceostatin A. Spliceostatin A (SSA) is a methyl-ketal derivative of FR901464, a metabolite from the bacterium *Pseudomonas* sp. No. 2663.[5,60] FR901464, which was originally isolated as a transcriptional activator, has potent cytotoxic activity against a number of different human cancer cell lines and the ability to prolong the life of tumor-bearing mice.[60,61] The other distinguishing feature of FR901464 is that it causes cell cycle arrest at the G1 and G2/M phases. Further study revealed that FR901464 treatment causes production of a C-terminal truncated form of the cyclin-dependent kinase (CDK) inhibitor and tumor suppressor, p27, designated p27*. It became clear that under SSA treatment p27 pre-mRNA accumulated and became translated, resulting in production of a shortened protein. As p27* was constitutively active and more stable than the original p27 protein, it acted as a negative regulator of cell growth. This finding suggests that splicing inhibition could allow targeted therapy and restore cell cycle control in transformed cells.

Spliceostatin A binds to the SF3b complex, which is a subcomponent of the U2 snRNP that recognizes the branch point sequence[5,62] and inhibits splicing both *in vivo* and *in vitro*. Recent studies revealed that SSA destabilizes interaction between pre-mRNA and SF3b1, the largest subunit of the

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SF3b complex, resulting in reduced fidelity of branch point recognition[63] (Fig. 3). In addition to production of p27*, SSA decreases VEGF in both mRNA and protein levels and inhibits tumor angiogenesis *in vivo*,[64] suggesting that downregulation of VEGF is another reason why SSA displays antitumor activity.

Pladienolide. Pladienolide is another potent antitumor natural compound, isolated from *Streptomyces platensis*.[65] Pladienolide shares many common features with SSA. It decreases gene expression controlled by the VEGF promoter, arrests the cell cycle at G1 and G2/M phases and exerts potent antitumor activity in several human cancer xenograft models in mice.[65,66] Like SSA, the molecular target of pladienolide is SF3b1 and consequently pladienolide inhibits splicing in a similar manner.[67,68] In pladienolide-treated cells, conformational changes and ATP-dependent remodeling events of the U2 snRNP are inhibited and this inhibition weakens the binding between U2 snRNP and pre-mRNA[69] (Fig. 3). A synthetic derivative of pladienolide, E7107, has entered phase I clinical trials against thyroid cancer and has led to stable disease or delayed disease progression in a subset of patients.[70]

Other compounds. Spliceostatin A, pladienolide and GEX1A directly bind to the spliceosome and exert their antitumor activity through direct inhibition of splicing.[5,67,71] Isoginkgetin is another general splicing inhibitor with antitumor activity.[72,73] It prevents recruitment of U4/U6•U5 tri-snRNP and inhibits splicing, suggesting that the spliceosome is a novel anticancer drug target (Fig. 3). TG003 is not a general splicing inhibitor but modulates alternative splicing patterns through dephosphorylation of SR proteins, including SRSF1, by inhibiting SR protein kinases CLK1 and CLK4[74] (Fig. 3). Because SRSF1 is a proto-oncogene (see above), this compound also has potential as an anticancer drug.

Perspective

As shown here, missplicing of cancer-related genes can drive tumor development. Modulation of splicing patterns and restoring the balance between two alternative splicing forms using splicing modulators/inhibitors might be an effective method for cancer therapy. Several small molecules binding to splicing factors are in phase I clinical trials and the results will be available soon. Although it is challenging to control specific splicing events, the difficulty can be overcome by screening for novel small molecules and identifying subsets of tumors that might be susceptible to splice-inhibition therapy. Further studies will surely uncover the detailed mode of action of further splicing modulators. Because the discovery of splicing inhibitors is still recent, the development of chemical modulation of pre-mRNA processing has just begun.

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Disclosure Statement

The authors have no conflict of interest.

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