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REVIEW

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The nexus of long noncoding RNAs, splicing factors, alternative splicing and their modulations

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ABSTRACT

The process of alternative splicing (AS) is widely deregulated in a variety of cancers. Splicing is dependent upon splicing factors. Recently, several long noncoding RNAs (lncRNAs) have been shown to regulate AS by directly/indirectly interacting with splicing factors. This review focuses on the regulation of AS by lncRNAs through their interaction with splicing factors. AS mis-regulation caused by either mutation in splicing factors or deregulated expression of splicing factors and lncRNAs has been shown to be involved in cancer development and progression, making aberrant splicing, splicing factors and lncRNA suitable targets for cancer therapy. This review also addresses some of the current approaches used to target AS, splicing factors and lncRNAs. Finally, we discuss research challenges, some of the unanswered questions in the field and provide recommendations to advance understanding of the nexus of lncRNAs, AS and splicing factors in cancer.

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Introduction

According to recent estimates from transcriptome research, more than 90% of the human genome is transcribed, producing thousands of non-coding RNAs (ncRNA) that are not converted into proteins [1]. There are numerous small RNAs in this sizable collection of noncoding transcripts [2]. However, the bulk of ncRNAs are classified as long noncoding RNAs (lncRNAs) because they are longer than 200 bases [2]. LncRNAs regulate nearly every stage of gene expression, including chromatin rearrangement, transcriptional control, splicing regulation, mRNA stability, mRNA translation, miRNA processing and protein stability [3]. Dysregulated lncRNA expression and activity are associated with a number of human diseases, including cancer. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is the first lncRNA with a known function in cancer [4]. The *MALAT1* gene is longer than 6 kb and is very prevalent. Mammals have a largely preserved MALAT1 sequence. The localization of LncRNAs play a crucial role in their function. Many LncRNAs are localized in nucleus and play a direct role in gene expression.

Regulation of gene expression is very important for cellular function. Alternative splicing (AS) is a crucial mechanism that generates diverse proteins from a single gene. It has been established that AS controls signalling networks and is involved in a number of illnesses, including cancer [5–8]. One of the hallmarks of cancer is the accumulation of various alterations in the genome and the epigenome of tumour cells [9]. These alterations affect the expression and function of signalling molecules that regulate cellular processes such as proliferation, differentiation, apoptosis and migration. AS is essential for increasing the diversity and complexity of the cellular functions, but it is extensively deregulated in cancers. Dysregulated alternative splicing can result in the production of cancer-specific splicing isoforms, which are different from the normal splicing isoforms in terms of their presence, absence or abundance [10,11]. Oncogenes and tumour suppressor genes are encoded by many of these transcripts [10,12,13]. The accurate determination of the splice sites and donor sites, as well as the assembly of the spliceosome complex, are essential to the AS procedure. The first is dependent on splicing factors (SFs), a class of tissue- and stage-specific RNA-binding proteins (RBPs) that identify regulatory regions inside exons and introns [14]. It is significant to note that SF activity is further regulated by phosphorylation and dephosphorylation that are activated by cell signalling pathways [15].

Serine- and arginine-rich (SR) proteins and HnRNPs are the two primary families of alternative splicing factors that have been extensively studied [16,17]. Alternative splicing is regulated by both SR proteins and HnRNPs, which can act as enhancers or silencers depending on the context. Contrary to the common view that SR proteins promote alternative splicing and HnRNPs inhibit it, recent studies have shown that both families have dual roles in splicing regulation [11,17,18].

SR proteins are a family of RNA-binding proteins and regulate both general and alternative splicing. SRSF1, a classic example of a SR protein family member, regulates

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the alternative splicing of various oncogenes and tumour suppressor genes important for tumour progression and maintenance [19]. Splicing factors have also been implicated in tumour propagation by directly activating the signalling pathway [6].

HnRNPs, also known as heterogeneous nucleus RNA-binding proteins, are special nucleic acid binding proteins that create complexes with HnRNA [20]. Upon their interaction with pre-mRNAs within the nucleus, these proteins have been found to not only participate in pre-mRNA processing but also contribute to various aspects of mRNA metabolism and transportation [20–22]. Compelling evidence supports the dysregulation of HnRNPs in tumours [23]. For instance, widespread copy number amplification of HNRNPA2B1 has been observed in numerous cancer types [24–26]. HnRNPs have also been implicated in crucial processes such as epithelial-mesenchymal transition (EMT), cell migration and angiogenesis within the context of cancer [23].

Recent studies have demonstrated that lncRNAs have the ability to interact with multiple splicing factors. This interaction has a direct impact on the localization and phosphorylation of splicing factors through the action of the kinase SRPK1. Consequently, these interactions result in alterations to the alternative splicing patterns of the target genes [27]. LncRNAs have also been shown to act as splicing factor hijackers, thereby modulating their concentration at a particular splicing event and regulating splicing [28–31]. LncRNAs act as chromatin remodellers and thereby modulate the binding of the chromatin-splicing adaptor complex by forming a DNA-RNA duplex known as an R-loop, antisense transcript and promoting or inhibiting exon skipping [31]. In addition, they have been shown to facilitate the formation of splicing-related nuclear speckles and paraspeckles around the site of transcription and thereby promote splicing [32]. Long noncoding RNAs form RNA-RNA duplexes with pre-mRNAs, resulting in either recruiting splicing factors at the site or preventing the binding of spliceosomes, thereby regulating splicing [33]. LncRNAs can modulate the expression of splicing factors by acting as miRNA sponges or interacting with transcription factors [33]. Changes in splicing factor levels can impact alternative splicing decisions. Some lncRNAs work in coordination with other regulatory elements, such as enhancers, to influence splicing patterns [33].

Due to the significant roles played by lncRNAs, alternative splicing and splicing factors in cancers, they have been identified as potential targets for cancer therapy. While non-specific inhibition of lncRNA, splicing and splicing factors might result in cellular damage, specific inhibition of them might be beneficial in rectifying abnormal splicing and deregulated expression of lncRNAs and splicing factors in cancer.

This review focuses on interplay between lncRNAs and splicing factors in AS regulation. This study also discusses some of the current strategies employed to target or manipulate lncRNAs, AS and splicing factors. Finally, it addresses the existing research challenges, unresolved questions and provides suggestions to advance our understanding of the intricate relationship between lncRNAs, AS and splicing factors in the context of cancer.

Alternative splicing

Ninety-five per cent of human genes encode several proteins through the phenomenon of alternative splicing (AS) [34]. This process is controlled by a combination of both cis and trans-acting components [14,35]. The trans-acting proteins, known as splicing factors (SFs), detect the cis-elements in the pre-mRNA sequence and attract or repel the spliceosome machinery to catalyse splicing at splice sites [14].

In splicing, introns (noncoding regions) are 'spliced' out of the mRNA precursor (pre-mRNA) in what is now believed to be primarily a co-transcriptional process, and the exons (coding regions) are joined together through two transesterification reactions, which are catalysed by a complex molecular machinery made of small nuclear ribonucleoproteins (snRNPs), known as the spliceosome [14,35] (Figure 1A).

The accurate determination of splice sites and donor sites, as well as the assembly of the spliceosome complex, are essential to the AS process [35]. The first is dependent on SFs, a class of tissue- and stage-specific RNA-binding proteins (RBPs) that identify regulatory regions inside exons and introns [36]. It is significant to note that SF activity is further altered by splicing factor kinases and phosphatases that are activated by cell signalling pathways [15] (Figure 1A).

The splicing process and its regulation are highly relevant for understanding every hallmark of cancer and for the acquisition of resistance to cancer-targeted therapies by cancer cells [37]. Splicing is carried out by the spliceosome. Its assembly starts with the recognition of the 5' splice site by U1 SnRNP through base pairing interactions involving 6–8 nucleotides of U1 SnRNP and the 5' end of the intron [37]. Mutations in the 6–8 nucleotides of U1 SnRNA induce changes in 5' splice site utilization resulting in aberrant alternative splicing [38]. 3' Splice site recognition by spliceosome involves splicing factors (U2AF1, U2AF2 and SF1) [37]. Mutations in splicing factor U2AF1 alters the 3' splice site recognition resulting in splicing changes [38,39].

Mouse models with splicing factor mutant cells have a compromised competitive repopulation capacity compared with that of wild-type cells resulting in altered splicing changes [39,40].

Another step-in spliceosome assembly involves recognition of branch point by U2 SnRNA [37]. Splicing factor SF3B1 is a key component of U2 SnRNP. Mutations in SF3B1 are among the most frequent in a variety of cancers [41]. SF3B1 mutations result in the use of cryptic 3' splice site upstream of the 3' splice site used in wild-type cells resulting in splicing changes [42,43]. Surprisingly, a low level but widespread reduction of intron retention isoforms (that is, enhanced splicing of regulated introns) seems to be the most frequent splicing alteration detected in bone marrow samples of SF3B1-mutated MDS [44].

Recognition of splice sites by U1 and U2 SnRNPs is assisted and modulated by a number of other splicing factors like RNA -binding motif (RBM) proteins, arginine-serine-rich (SR) proteins (including SRSF2) and heterogenous nuclear ribonucleoproteins (hnRNPs) [37]. These factors bound to exonic or intronic regulatory elements and can promote or prevent the recognition of 5' splice

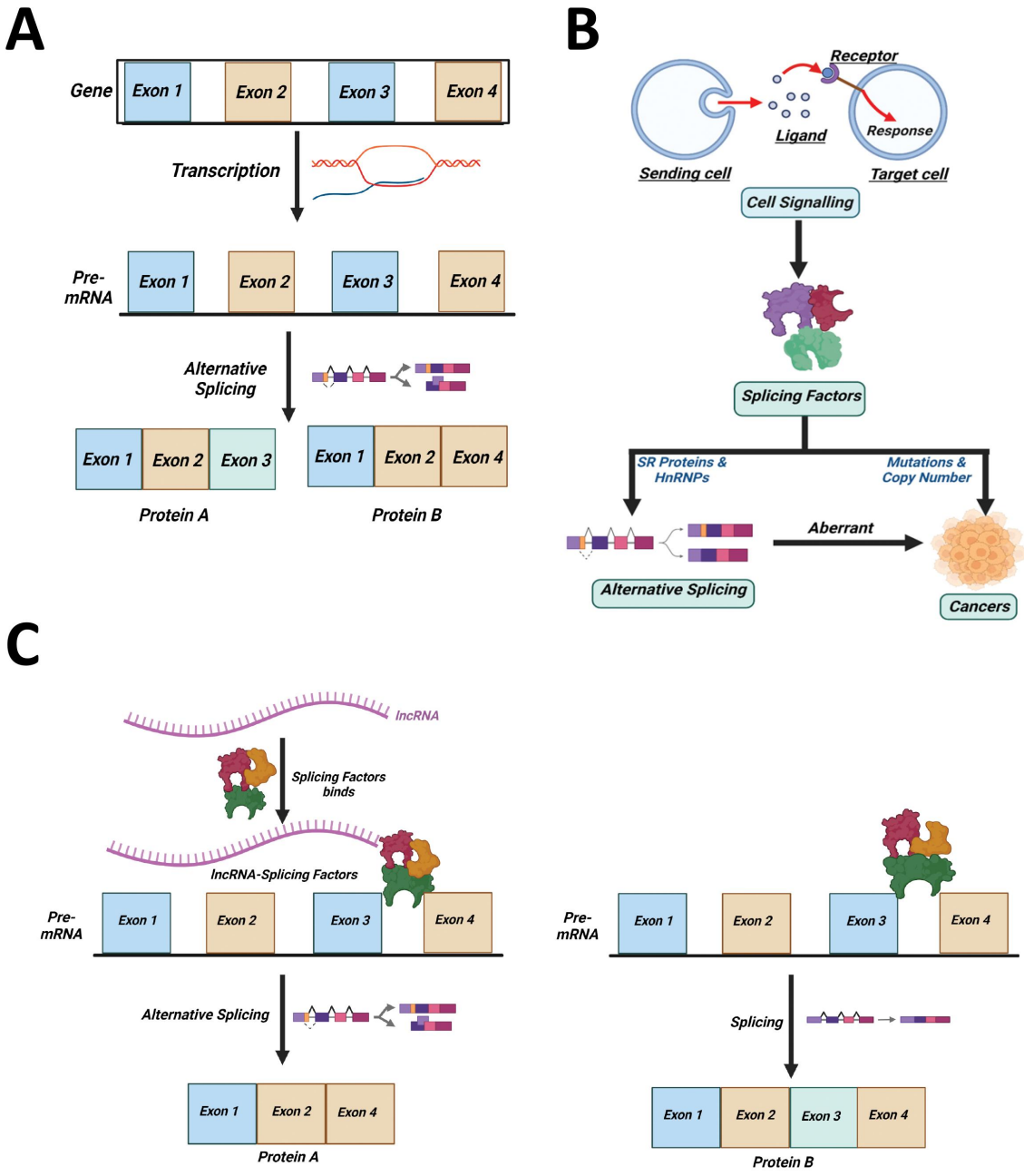

Figure 1. The connection between alternative splicing, splicing factors and long noncoding RNAs. (A) Schematic representation of the process of alternative splicing: pre-mRNA produced from DNA after transcription can produce different mature mRNAs, resulting in two different proteins due to different combinations of exons in a process called alternative splicing. (B) Association of cell signalling, splicing factors, alternative splicing and cancers with each other: cell signalling is the ability of a cell to receive, process and transmit signals to its environment. Cell signalling regulates splicing factor activity. Splicing factors (SR proteins and HnRNPs) regulate alternative splicing. Aberrant alternative splicing has been associated with cancer. Mutations or copy number variations in the splicing factors result in cancer. (C) Interaction of Long noncoding RNAs (lncRNAs) with splicing factors (SFs) to modulate alternative splicing: lncRNAs interact with splicing factors, which are crucial for alternative splicing. The interaction of lncRNAs with SFs would modulate their activity or expression levels, thereby altering splicing. SFs-only interactions result in one type of splicing, while interactions of lncRNA with SFs result in another type of splicing.

site by U1 SnRNP or the 3' splice site by SF1, U2AF1, U2AF2 or U2 SnRNA, thus affecting splicing site choices and therefore alternative splicing decision [37].

In addition to the above-mentioned factors, the process of maturation of SnRNPs influences the levels of SnRNPs, and, consequently alternative splicing decisions [37]. Differential selection of intronic and exonic sequences as differential use of alternative promoters and 3'-end formation sites result in the generation of alternative mRNA isoforms [37].

Many malignancies have disordered alternative splicing, and many tumours contain cancer-specific splicing isoforms that are missing or expressed at variable amounts in the equivalent normal tissues [10,45] (Figure 1A). Oncogenes and tumour suppressor genes are encoded by many of these transcripts [37].

One of the most common examples of altered AS in cancer is BIN1. The BIN1 protein interacts with c-MYC and suppresses its oncogenic activity [46]. Inclusion of exon 12A in the BIN1 transcript abolishes its tumour

suppressor activity, while inclusion of exon 13 is required for the tumour suppressor activity of BIN1 [47]. The aberrant splicing of BIN1 was shown to be under the regulation of the SRSF1 splicing factor [19]. Upregulation of splicing factor SRSF1 resulted in increased inclusion of exon 12A in the BIN1.

Likewise, two distinct alternative isoforms of pre-mRNA for the transcription factor TEAD1 are present: one includes exon 5, while the other lacks exon 5. It has been observed that cell with TEAD1 isoforms containing exon 5 exhibit greater proliferative capacity compared to those without exon 5 [19,48]. The cause of the aberrant splicing of TEAD1 is SRSF1 [49]. SRSF1 overexpression promoted the inclusion of exon 5 in the transcription factor gene *TEAD1* [19,50].

In addition, it was shown that the oncogenic properties of the full-length TEAD1 isoform (TEAD1+E6) were also greater than those of the TEAD1 isoform lacking exon 6 (TEAD1-E6) [50]. Splicing Factor RNA-binding Fox-1 homolog 2 (RBFOX2) was shown to regulate the splicing of TEAD1 exon 6 [50]. RBFOX2 was shown to promote the inclusion of TEAD1 exon 6 via binding to the conserved domain in the downstream intron [50].

Another example of cancer specific AS is *S6K1* gene. S6K1 isoforms exist in two different isoforms: short (Iso2) and long (Iso1). The short transcripts (Iso2) contain stop codons, resulting in transcripts that include only approximately half of the original S6K1 coding sequence (Iso1) and lack the conserved kinase domain. The short isoforms (Iso2) can bind and activate mTORC1, leading to increased phosphorylation of 4E-BP1. Conversely, the long variant of S6K1 (Iso1) produces opposite effects [8]. SRSF1 modulates the splicing of S6K1 [19]. It promotes expression of the Iso2 S6K1 isoform [8,19]. Mouse and human cells overexpressing SRSF1 showed elevated levels of the S6K1 Iso2 isoform [8].

Alternative splicing of mTOR mRNA has been observed, resulting in two distinct forms: mTOR mRNA with intron 5 (designated as mTORi5) and wild-type mTOR mRNA. Wild-type mTOR mRNA consist of exon 4, exon 5 and exon 6, whereas mTORi5 lacks exon 6 but contains exon 4, exon 5 and intron 5. mTORi5 isoform expression resulted in reduction in mTOR protein levels ultimately resulting in reduced mTORC1 activity [51]. This reduction of mTORC1 activity is evident from the phosphorylation of S6 at S240/S244 in cells with increased expression of mTORi5. The exon microarray data revealed that Sam68 may directly influence intron 5 inclusion in the mTOR mRNA [51]. Sam68 inactivation increases the retention of Intron 5 of mTOR mRNA [51].

Various alternative splicing isoforms of the pro-apoptotic Bcl-2 family member BIM transcripts exist, such as EL, L, S, ES and γ1 [52]. γ1 isoform of BIM is associated with reduced apoptosis [48,52]. The ES (extra short) isoform of BIM which lacks exon 4, the BH3 domain, has been shown to behave in a similar fashion to that of the γ1 isoform, as an antiapoptotic protein [48,52]. SRSF1 was shown to regulate the splicing of BIM1, and its overexpression promoted the expression of two novel *BIM* isoforms, the γ1 isoform and the ES (extra short) isoform of BIM [19,48]. The RNA recognition motif 1 (RRM1) domain of SRSF1 is required for its splicing activity.

Splicing factors

Numerous studies conducted over the past two decades have discovered altered splicing patterns in malignancies, and various splicing-related variables have been linked to tumour initiation, progression and maintenance [10,37,45,53,54]. Therefore, it is not unexpected that studies using high-throughput genomic and exomic analysis of a range of malignancies have discovered mutations in key splicing machinery and splicing factor components [55,56]

Specifically, mutations were discovered in the genes for U2AF1 ((U2 small nuclear RNA auxiliary factor 1)) (also known as U2AF35) [57], ZRSR2 ((zinc finger RNA-binding motif and serine/arginine rich 2)) [58], SRSF2 ((serine-/arginine-rich splicing factor 2) [59] (also known as SC35), SF3B1 ((splicing factor 3b subunit 1) [60–63] and PRPF40B (pre-mRNA processing factor 40 homolog B) [64]. Splicing factor SRSF6 (lung and colon cancer), HnRNPA2/B1 (glioblastoma) and SRSF1 (lung, colon and breast cancer) genes copy numbers have been found to change [19,24,65]. The function or location of the splicing factor can also be impacted by post-transcriptional changes, such as phosphorylation on itself, suggesting yet another level of regulation [66] (Figure 1B). In general, splicing factor activity can be changed by signalling pathways (Figure 1B). Active splicing factors can alter the splicing patterns generating tumour specific isoforms. In addition, splicing factors can be mutated or have altered copy numbers in cancers (Figure 1B).

Serine- and arginine-rich (SR) proteins and hnRNPs are the two main families of alternative splicing factors that have been intensively studied [16,17]. Although it is usually accepted that SR proteins encourage alternative splicing while hnRNPs inhibit it, there is mounting evidence that both SR proteins and hnRNPs control AS through a combination of positive and negative regulation [17,18,67]. In general, SR proteins function as exon splicing activators by binding to cis elements in pre-mRNA. These cis elements in pre-mRNA where SR proteins bind are known as exonic-splicing enhancer elements (ESEs). The binding of SR proteins to ESEs helps in the assembly, recruitment and stabilization of the core components of the spliceosome, thereby facilitating the process of splicing. SR proteins have been shown to both activate and repress splicing events [68].

In general, HnRNPs have been shown to repress splicing events. Some of the mechanisms by which HnRNPs repress splicing are as follows: (i) by directly preventing the recognition of splice sites in pre-mRNA by splicing enhancers, for example, SR proteins; (ii) by interfering with the binding of proteins bound to enhancers, for example, SR proteins to exonic-splicing enhancer elements [69]. Furthermore, HnRNPs have been shown to impede communication between splicing factors bound to different splice sites in the pre-mRNA [69]. For a detailed description of other splicing factors, see review by David and Manley [11].

SR proteins have a C-terminal RS domain and one or two RNA-recognition motifs (RRMs) [70]. Several SR proteins have recently been found to undergo either mutation or altered copy number variations and contribute to the development of various cancers [19,59,65,71–73]. AS by SR

proteins can result in increased production of pro-oncogenic isoforms and decreased synthesis of tumour suppressive isoforms [19,65]. The splicing factor SRSF1 is upregulated at the gene and protein levels in colon, kidney, small intestinal and lung tumours [19]. Overexpression of SRSF1 was shown to transform immortal rodent fibroblasts, resulting in the formation of sarcomas in animal model [19]. SRSF1 was shown to control the alternative splicing of the tumour suppressor BIN1, resulting in isoforms that lack tumour-suppressor activity [19]. Furthermore, SRSF1 controlled the splicing of Kinase MNK2 towards an isoform of MNK2 that promotes MAP kinase-independent eIF4E phosphorylation, resulting in increased translation-promoting cancers [19]. In addition, SRSF1 also regulated the splicing of S6K1, a downstream target of mTORC1, resulting in increased mTORC1 activity. The transforming capacity of SRSF1 was shown to be dependent on the oncogenic isoform of S6K1 [19]. In the breast cancer model system, SRSF1 was shown to control the splicing of CASC4 towards +Exon 9 inclusion, resulting in increased acinar size and proliferation and decreased apoptosis, partially recapitulating SRSF1's oncogenic effects [71]. SRSF1 DNA copy number gain and overexpression at RNA levels were associated with poor survival in small cell lung cancers (SCLC) [72]. In SCLC, SRSF1 was also shown to control DNA repair and the chemotherapy response [72].

SRSF2 mutations were observed in patients with myelodysplastic syndromes (MDS) [59]. SRSF2 mutations alter SRSF2's normal sequence-specific RNA binding activity, resulting in unregulated recognition of specific ESE motifs that drive recurrent mis-splicing of key haematopoietic regulators [59]. One of the aberrant alternative splicing isoforms produced due to the SRSF2 mutation is EZH2, which triggers nonsense-mediated decay of various haematopoietic regulators, which, in turn, results in impaired haematopoietic differentiation [59].

Another SR protein called SRSF6 was found to be amplified and overexpressed in lung and colon cancers [65]. Overexpression of SRSF6 in immortal lung epithelial cells resulted in enhanced proliferation and formed tumours when injected into mice [65]. Furthermore, SRSF6-overexpressing immortal lung epithelial cells were protected from chemotherapy-induced cell death [65]. Knockdown of SRSF6 in lung and colon cancer cell lines inhibited their oncogenic properties and potential. SRSF6 overexpression or knockdown altered the splicing patterns of several tumour suppressors and oncogenes to switch towards the oncogenic isoforms and reduce the tumour-suppressive isoforms [65]. SRSF6 was also found to be frequently upregulated in colorectal cancer (CRC) samples and associated with a poor prognosis [73]. SRSF6 overexpression promoted increased proliferation and metastasis *in vitro* and *in vivo*. SRSF6 overexpression resulted in a ZO-1 (+Exon 23) aberrant splicing isoform that functions as an oncogene [73]. However, there are also instances in which the oncogenic activity of SR proteins is not influenced by splicing regulation but by the signalling pathway alone. SRSF1 oncogenic activities were shown to be dependent on mTORC1 [6].

The RNA-binding proteins, known as hnRNPs, interact with heterogeneous nuclear RNA (HnRNA) to form complexes [20]. These proteins appear to have an impact on pre-mRNA processing as well as other facets of mRNA metabolism and transport since they are connected to pre-mRNAs in the nucleus [20–22]. By preventing the creation of short secondary structures that depend on base pairing of complementary areas, the attachment of a pre-mRNA molecule with an hnRNP makes the pre-mRNA available for interactions with other proteins and splicing apparatus [21].

Proposed functions for hnRNPs include prevention of apoptosis, promotion of angiogenesis, cell invasion and the epithelial-mesenchymal transition (EMT) [23]. Various cancer types exhibited extensive copy number amplification of the *HNRNPA2B1* gene [24–26]. Thus, it is hypothesized that hnRNP may be a novel and potential therapeutic target, as well as a marker for prognostic evaluation and therapy response.

Long noncoding RNAs

In addition to aberrant alternative splicing and altered splicing factor expression and function in cancer, there is dysregulated expression and activity of long noncoding RNAs (lncRNAs). The majority of lncRNAs and splicing factors reside in the nucleus. Alternative splicing takes place in the nucleus. lncRNAs interact and bind with splicing factors, thereby regulating their splicing activity.

Splicing factors regulate alternative splicing. lncRNAs, splicing factors and alternative splicing are all key players in the regulation of gene expression and the generation of protein diversity in eukaryotic cells.

Traditionally, lncRNAs have been informally defined as noncoding transcripts greater than 200 nucleotides excluding some noncoding RNAs that lie close to 200nt border such as 7SK (~330nt), 7SL (~300nt) and Alu (~280nt) [74]. Given this grey zone of sizes, leading researchers all-round the globe suggested that lncRNA should be named as noncoding transcript of more than 500nt which are mostly generated by RNA Polymerase II [74]. The fact that splicing and polyadenylation have been observed in many lncRNAs has led to the comparison of these molecules to mRNA [74,75]. Other lncRNAs, however, are neither polyadenylated nor 7-methylguanosine-capped [75,76]. With respect to protein-coding genes, lncRNAs can be intergenic (whose sequences do not trespass on nearby protein coding loci), antisense (overlap protein coding loci) or intronic (expressed from enclosed introns) [74]. lncRNAs are also derived from "pseudogenes " [77]. Additionally, lncRNAs also comprise circular RNA with evident functions, generated by back-splicing the coding and non-coding transcripts [78]. Trans-acting regulatory RNAs are also included, which are obtained from sequences that are typically the 3' untranslated regions of mRNA [79].

Numerous attempts have been made to categorize and name lncRNA, primarily based on its genomic location and orientation in relation to protein-coding genes [80–83]. It has been shown that classifying and naming lncRNAs in relation to nearby genes is helpful since it provides context and, on occasion, suggestions regarding how they function [80–83]. A unanimous recommendation on the nomenclature of lncRNAs released by top researchers suggested that lncRNAs

should be termed according to their discernible trait or function unless they are antisense to a protein-coding gene [84].

LncRNAs have a wide range of functions and can act nearby or far away, which makes it challenging to categorize them into separate RNA classes with regulatory or architectural functions [85]. LncRNAs can be present in either or both cytoplasm or nucleus. Cytoplasmic lncRNAs encode small peptides [85]. Protein coding loci also express lncRNA through alternative splicing [86,87], and surprisingly the major transcript produced by 12% of human protein-coding loci is non-coding [88]. In general, lncRNAs are RNA transcript derived from RNA polymerase II primary transcription units, processed from introns or formed by back splicing [84,86,87].

LncRNAs have drawn a lot of attention over the years as significant regulators of cellular processes including alternative splicing. Here, we will highlight recent findings that demonstrate the important roles that lncRNAs play in the control of alternative splicing (Figure 1C) and examine how they can affect the expression of a variety of splice isoforms by influencing regulation and expression of splicing factors. The contribution of lncRNAs to the regulation of alternative splicing is predicted to increase considerably, as more lncRNAs are identified and defined [27].

LncRNAs can influence the regulation of AS in a variety of ways, either by directly interacting with pre-mRNAs or indirectly, by controlling the activity of splicing factors [27]. Due to their shown ability to function as regulatory agents that regulate gene expression at every level, lncRNAs are especially well adapted to these activities [89,90]. These lengthy RNA polymerase transcripts have been demonstrated to participate in a variety of developmental processes and illness in complex organisms, either on their own or in collaboration with partner proteins [74,89,90].

Here, we examine the way through which lncRNAs contribute to the control of AS through splicing factors (Figure 1C), which are frequently altered or mutated in malignancies.

Interactions of LncRNAs and SFs to regulate AS

LncRNAs exert control over alternative splicing (AS) through their interactions with splicing factors (SFs) [91]. By interacting with the sequence components of pre-RNA, splicing factors play a crucial part in controlling AS, whereas lncRNAs frequently work in tandem with or without splicing factors to control AS [27]. In the following section, we will emphasize several noteworthy recent studies in this area.

In human oesophageal cancer cells, TPM1-AS, a lncRNA that was reverse transcribed from TPM1's second intron, was found in the nucleus and shown to interact with the splicing factor RBM4 [92]. The interaction between TPM1-AS and RBM4 prevents RBM4 from binding to TPM1 pre-mRNA and prevents the inclusion of endogenous exon 2a of TPM1 resulting in decreased expression of oncogenic isoforms [92]. Additionally, oesophageal squamous cell carcinoma (ESCC) exhibits increased lncRNA DGCR5 expression and is associated with a poor prognosis [93]. Studies have demonstrated that DGCR5 interacts with SRSF1 and controls Mcl-1's AS event, allowing the anti-apoptotic isoform Mcl-1 L to be produced and so facilitating carcinogenesis [93].

One of the first identified lncRNAs linked to tumours, lncRNA MALAT1, is significantly expressed in a variety of cancer types [91]. Studies have demonstrated that MALAT1, which is found in nuclear speckles, can interact to varying degrees with a number of SR proteins, including SRSF1, SRSF2, SRSF3 and SRSF5, affecting the transport and distribution of SR protein at transcription sites and between nuclear speckles by controlling the ratio of phosphorylation/dephosphorylation of SR proteins to control the AS events [94].

Further research has demonstrated that MALAT1 can enhance a series of cancer-promoting splicing processes that are mediated by SRSF1 [91]. Binding of nuclear SRSF1 to an RNA oligonucleotide containing the consensus Exonic Splicing Enhancers ESE motif taken from the SRSF1 3'UTR region, through direct RNA affinity purification showed binding of MALAT1 to SRSF1 [91] (Figure 2A). MALAT1 regulates the expression of known splicing targets of SRSF1 [91]. Splicing targets of SRSF1 such as proapoptotic Bcl-2 family member BIM, tumour suppressor BIN1, and the transcription factor TEAD-1 were shown to be regulated by MALAT1 [91]. MALAT1 overexpression increased expression of the ES (extra short) isoform of BIM, which lacks exon 4, the BH3 domain [91]. The ES isoform of BIM has been shown to behave in a similar fashion to that of the Y1 isoform, the BIM isoform without exon 3, 4 and 6 as an antiapoptotic protein [52]. Transient MALAT1 knockdown resulted in reduced expression of the ES isoform of BIM [91]. Another SRSF1 splicing target regulated by MALAT1 is BIN1. The BIN1 protein interacts with c-MYC and suppresses its oncogenic activity [46]. Inclusion of exon 12A in the BIN1 transcript abolishes its tumour suppressor activity while inclusion of exon 13 is required for the tumour suppressor activity of BIN1 [47]. SRSF1 overexpression was shown to increase inclusion of exon12A of BIN1 in human, mouse and rat cells [19,48]. In agreement with these findings, MALAT1 overexpression resulted in increased inclusion of exon 12A. Furthermore, transient MALAT1 knockdown induced skipping of exon 12A [91]. In addition, SRSF1 affects the alternative splicing of the transcription factor TEAD1 by promoting the inclusion of exon 5, resulting in more proliferative activity [19,48]. Consistent with this, MALAT1 overexpression resulted in increased inclusion of exon 5 of TEAD1. In contrast, transient MALAT1 knockdown induced skipping of exon 5 of TEAD1 [91] (Figure 2B). Mutant p53 and ID4 proteins have been shown to interact with MALAT1 and SRSF1 to create complexes that control the AS of VEGF-A pre-mRNA, preventing the synthesis of the anti-angiogenic VEGF-A isoform, and thus enhancing angiogenesis [95]. In addition to cooperating with SRSF1, MALAT1 also competes for SFPQ binding, removing it from the SFPQ/PTBP2 splicing factor complex, thereby promoting tumour growth and metastasis in colorectal cancer [96]. In another study, it was shown that lncNA – uc002yug.2 facilitates the binding of SRSF1, MBNL1 and other splicing factors to the RUNX1 pre-mRNA, resulting in decreased expression of RUNX1 isoform and increased expression of RUNX1a isoform, resulting in

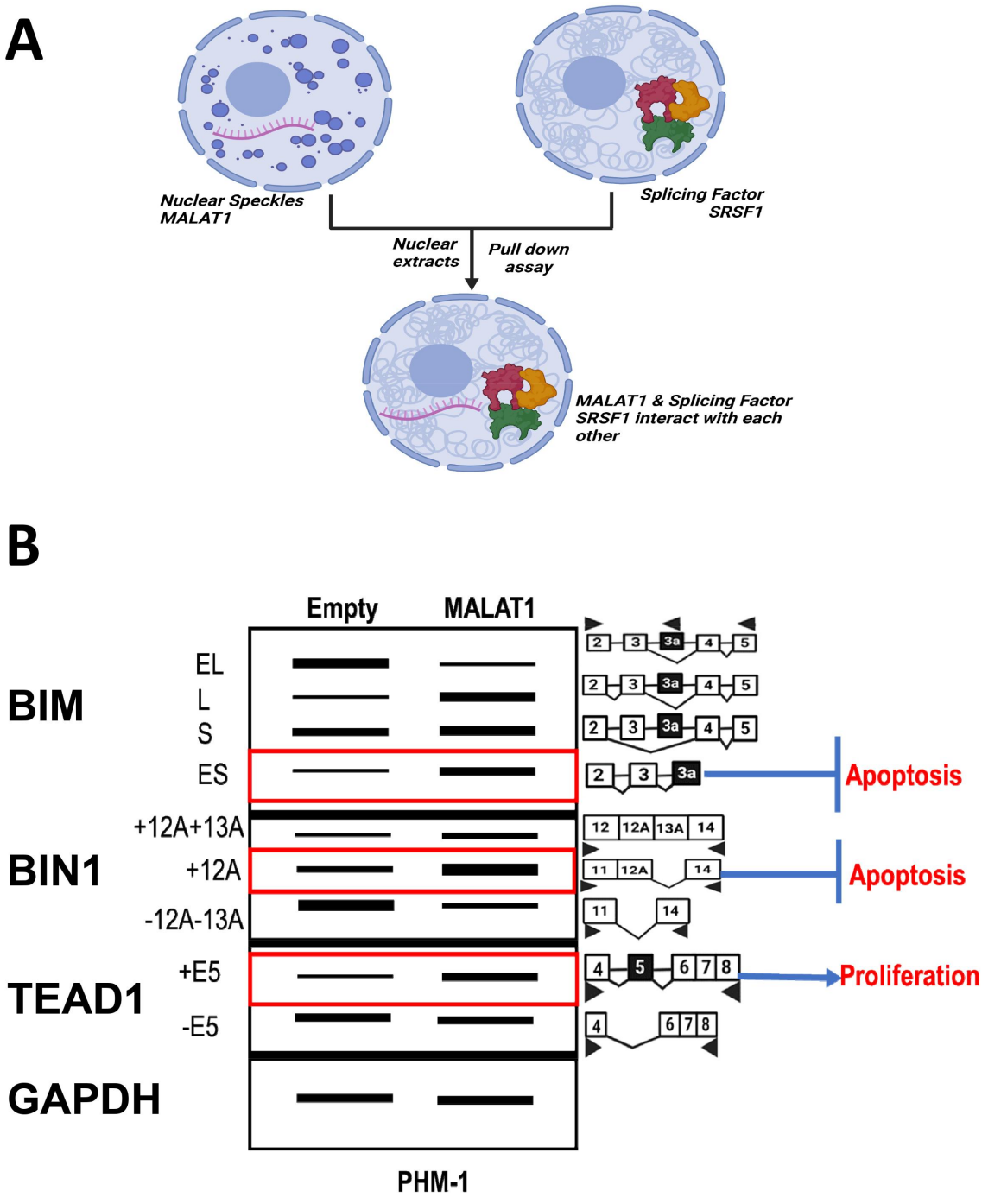

Figure 2. MALAT1 binds with SRSF1 and regulates its splicing targets. (A) Schematic representation of the binding assay used for studying the interaction between SRSF1 and MALAT1. (B) Image showing the regulation of SRSF1 splicing targets by MALAT1.

reduced CCAAT/enhancer-binding protein-α (CEBPα gene expression), and further promote cell proliferation and tumour growth. CEBPα inhibits cancer cell growth. RUNX1 isoform increases CEBPα expression while RUNX1a decreases it [97].

linc01232 has been shown to interact with the splicing factor hnRNPA2/B1 and stabilizes it by preventing ubiquitination and degradation of hnRNPA2/B1. This results in enhanced expression of its splicing target, full-length A-Raf, which has been shown to be involved in pancreatic cancer [98]. These investigations have uncovered a novel mechanism by which lncRNAs regulate cancer progression and maintenance, by alternative splicing modulation through interaction with splicing factors.

A detailed list of lncRNA interacting with splicing factors is provided in Tables 1 and 2.

The observations of deregulated alternative splicing, splicing factors and lncRNAs in cancer have resulted in the need for the development of therapies that would modulate the splicing, expression and function of lncRNAs and splicing factors for therapeutic benefit. In parallel, small molecules that can modulate the RNA processing of individual

Table 1. Serine and arginine-rich (SR) proteins.

Splicing Factors	Binding Experiments	Long Noncoding RNA	Splicing Targets	Effects on Expression	Reference
SRSF1	Oligonucleotide pulldown assay	MALAT1	BIN-1, BIM, RPS6KB1 and TEAD-1	Positively related to expression level	[91]
SRSF1	RNA FISH and immunofluorescence assay	MALAT1	Not done	Not done	[99]
SRSF1	Not done	HOXA11-AS	Not done	Positively related to expression level	[100]
SRSF1	RNA pulldown assay and RIP assay	LINC02580	Not done	Not done	[101]
SRSF1	Not done	MIR205HG	Not done	Negatively related to expression level	[102]
SRSF1	Not done	AGAP2-AS1	Not done	Positively related to expression level	[103]
SRSF1	RIP assay	NEAT1	Not done	Not done	[104]
SRSF1	RNA pulldown assay	OnclncRNA-626	Not done	Not done	[105]
SRSF1	RIP assay	LNC-MIRHG	Not done	Not done	[106]
SRSF1	RNA pulldown assay and RIP assay	LINC01152	Not done	Not done	[107]
SRSF1	RIP assay	MALAT1	VEGF Isoform	Not checked	[95]
SRSF1	HyPR-MS	MALAT1	Not done	Not done	[108]
SRSF1	CHART-MS	MALAT1	Not done	Not done	[109]
SRSF2	Not done	NRON	Not done	Negatively related to expression level	[110]
SRSF2	Not done	MALAT1	Not done	Not done	[111]
SRSF2	HuProt TM protoarray and RIP assay	AC091729.7	Not done	Not done	[112]
SRSF2	Antisense oligonucleotide and RIP assay	MALAT1	Protein kinase C δ	Not done	[113]
SRSF2	RIP assay	PANDAR	Not done	Positively related to expression level	[114]
SRSF2	ChIRP-MS	AFAP1-AS1	Not done	Positively related to expression level	[115]
SRSF3	Pulldown assay and RIP assay	VESTAR	VEGF-C mRNA stability	Not done	[116]
SRSF4	NA	NA	NA	NA	NA
SRSF5	NA	NA	NA	NA	NA
SRSF6	RIP assay	LINC001133	Not done	Not done	[117]
SRSF6	RNA pulldown assay, mass spectrometry and RIP assay	CRNDE	PICALM	Negatively related to expression level	[118]
SRSF6	RIP assay	ZNF561-AS1	Not done	Positively related to expression level	[119]
SRSF7	Not done	MALAT1	Not done	Positively related to expression level	[120]
SRSF8	NA	NA	NA	NA	NA
SRSF9	NA	NA	NA	NA	NA
SRSF10	Mass spectrometry	TRA31P2-AS1	Not done	Not done	[121]

Table 2. Heterogeneous nuclear ribonucleoproteins (HnRNPs).

Splicing Factors	Binding Experiments	Long Noncoding RNA	Splicing Targets	Effects on Expression	Reference
HnRNPA1	Not done	CCAT1	Not done	Positively related to expression levels	[122]
HnRNPA1	ChIRP-MS	SNHG6	PKM	Positively related to expression levels	[123]
HnRNPA2/B1	LC-MS and RIP	H19	RAF-1	Positively related to expression levels	[124]
HnRNPA2/B1	Not done	CACNA1G-AS1	Not done	Positively related to expression level	[125]
HnRNPA2/B1	RNA pulldown and RIP assay	miR503HG	Not done	Negatively related to expression level	[126]
HnRNPA2/B1	RNA pulldown and RIP assay	UC002mbe.2	Not done	Positively related to expression level	[127]
HnRNPA2/B1	RNA pulldown and RIP assay	BC200	BCL-X	Not done	[128]
HnRNP-C	RIP assay	LBX2-AS1	Not done	Positively related to expression level	[129]
HnRNP-C	RAP-MS	Xist	Not done	Not done	[130]
HnRNP-C	RIP assay and CHIRP-MS	DDX11-AS1	Not done	Positively related to expression level	[131]
HnRNP-D	RNA pulldown and RIP assay	LINC01354	Not done	Not done	[132]
HnRNP-F	RNA pulldown and RIP assay	HIF-1α antisense lncRNA	PKM2	Positively related to expression level	[133]
HnRNP-G	Affinity pulldown and mass spectrometry	m6A –MALAT1	NASP	Not done	[134]
HnRNP-H	Affinity pulldown and mass spectrometry	RP11-670E13.6	Not done	Negatively related to expression level	[135]
HnRNP-I	NA	NA	NA	Not done	NA
HnRNP-K	Biotin-labelled RNA pulldown and mass spectrometry	pancEts-1	Not done	Not done	[136]
HnRNP-K	RIP Assay	SINEUP	Not done	Not done	[137]
HnRNP-K	RNA pulldown and RIP assay	CASC11	Not done	Positively related to expression level	[138]
HnRNP-M	RAP-MS	Xist	Not done	Not done	[130]

transcripts and regulate the function and expression of lncRNAs and splicing factors are being pursued for cancer therapy. We will individually look at the modulation of splicing factors, alternative splicing and lncRNAs.

Modulation of splicing factors

In recent years, various types of pharmacological modulators have been used, or are under development, to target AS. The spliceosome machinery has been directly targeted as a potential global anticancer therapy [139]. Amiloride, a drug typically used with other medications to treat high blood pressure, treatment of cells results in changes in alternative splicing [140,141]. Numerous oncogenic genes, including *BCL-X*, *HIPK*3 and *RON/MISTR1*, had altered alternative splicing patterns after treatment of cells with amiloride, most likely as a result of hypophosphorylation of SRSF1 [140,141]. Phosphorylation of SRSF1 is essential for its nuclear speckle accumulation, where it is recruited to the spliceosome machinery (Figure 3A). Hypophosphorylation or dephosphorylation of SRSF1 results in its translocation to the cytoplasm, leading to its proteolytic degradation (Figure 3B).

The SF3b subunit is a part of U2 snRNP, which is an essential component of the splicing apparatus, the spliceosome. Inhibitors that bind to SF3b result in the nuclear export of intron-bearing pre-mRNA, resulting in blocked splicing. Pre-mRNA needs to be in the nucleus for proper splicing (Figure 3B).

Spliceostatin A (SSA) is a small molecule derived from fermentation byproduct of Pseudomonas bacteria that inhibits splicing by binding to splicing factor SF3B [142]. It has been demonstrated that giving tumour-bearing mice SSA therapy increases their lifespan [143]. The SF3b subunit is a part of U2 snRNP, which is an essential component of the splicing apparatus, the spliceosome. Inhibitors that bind to SF3b result in the nuclear export of intron-bearing pre-mRNA, resulting in blocked splicing. Pre-mRNA needs to be in the nucleus for proper splicing. Sudemycin E, an analog of the splicing modulator FR901464 and its derivative spliceostatin A, treatment also resulted in reversible changes in alternative splicing [144] (Figure 3B).

While nonspecific suppression of splicing might result in cellular damage, broad inhibition of splicing by small compounds may be beneficial in rectifying abnormal splicing in cancer [145] (Figure 3B). It is anticipated that general splicing inhibitors would be more harmful and non-specific. Thus, small compounds that only prevent particular instances of alternative splicing are required. The splicing reaction is controlled by multiple phosphorylation and dephosphorylation events [146,147]. Several kinases, including SRPK [148,149], topoisomerase I [150], Clk [151] and DIRK1A [152], have been found to phosphorylate SR proteins. Inhibition of topoisomerase I's kinase activity by glycosylated indolocarbazole derivative (NB-506) results in less phosphorylation of SRSF1/SR proteins resulting no spliceosome assembly and thereby inhibiting splicing [153]. Additionally, it has been discovered that certain small compounds can prevent the phosphorylation of particular SR proteins, and this way regulating specific alternative splicing events [154]. General splicing inhibitor, amiloride, was shown to cause hypophosphorylation of SRSF1 [140,141].

Similar to topoisomerase I inhibition, Clk inhibition has also been demonstrated to interfere with the alternative splicing pattern of multiple target genes by reducing the phosphorylation of SR proteins [155]. In addition, it has been shown that SRSF4 is less phosphorylated when SR kinases, such as SRPK proteins, are inhibited [156]. These findings support the development of a more focused strategy for splicing-based therapies. However, more research must be carried out to determine whether these small compounds can be useful, without harmful side-effects.

Antisense-mediated splicing regulation

While wide-spread suppression of splicing might result in cellular damage, specific inhibition of splicing by small compounds may be beneficial in rectifying abnormal splicing in cancer [16]. Small compounds that only prevent particular instances of alternative splicing are thus required. A more nuanced strategy, targeted antisense oligonucleotide (ASO) splicing modulation, has been developed to target specifically certain splicing events, as opposed to general changes in splicing [157,158].

This method is predicated on the idea that trans-acting factors bind to and recognize particular cis sites on the mRNA in order to carry out splicing. ASOs are synthetic nucleic acids of 15 to 25 bases, complementary to cis regions on the mRNA [157]. ASOs can be made to target splicing enhancers or silencers, to either block or promote splicing, or they can be made to block binding of a splicing factor to a cis element, preventing splicing at that site and leading to enhanced inclusion of a particular exon [157]. With significant effectiveness, this strategy has been utilized to treat neurodegenerative disorders as Duchenne muscular dystrophy [159] and Spinal muscular atrophy [160].

This strategy, which makes use of antisense oligonucleotides (ASOs, tiny bits of chemically altered DNA or RNA), provide a cure for genetic diseases brought on by mutations that interfere with splicing, but it can also be used to address other types of genetic mutations [161]. To increase stability to nucleases and give desirable pharmacokinetic and pharmacodynamic properties for in vivo application, ASOs need to be chemically modified [162]. The negatively charged 2′-O-methyl RNA phosphorothioate chemistry and the charge-neutral phosphorodiamidate morpholino oligomer are the two most often employed ASO chemistries [162].

Another example where antisense-mediated splicing modification has progressed into the clinical trial phase is in the treatment of Hutchinson-Gilford progeria syndrome (HGPS). Lamin A is a nuclear envelope protein encoded by the *LMNA* gene and involved in the control of gene expression, nuclear stability and chromatin structure [163]. Progerin, a shortened version of lamin A, is present in small amounts in normal ageing but accumulates to higher levels in HGPS patients, as a result of a point mutation in this gene that causes a shift in alternative splicing [164]. Accumulation of progerin in these patients results in growth impairment, lipodystrophy, skin and bone abnormalities, cardiovascular changes, hastened ageing and shortened lifespan [164]. This change in the LMNA splicing, that favours

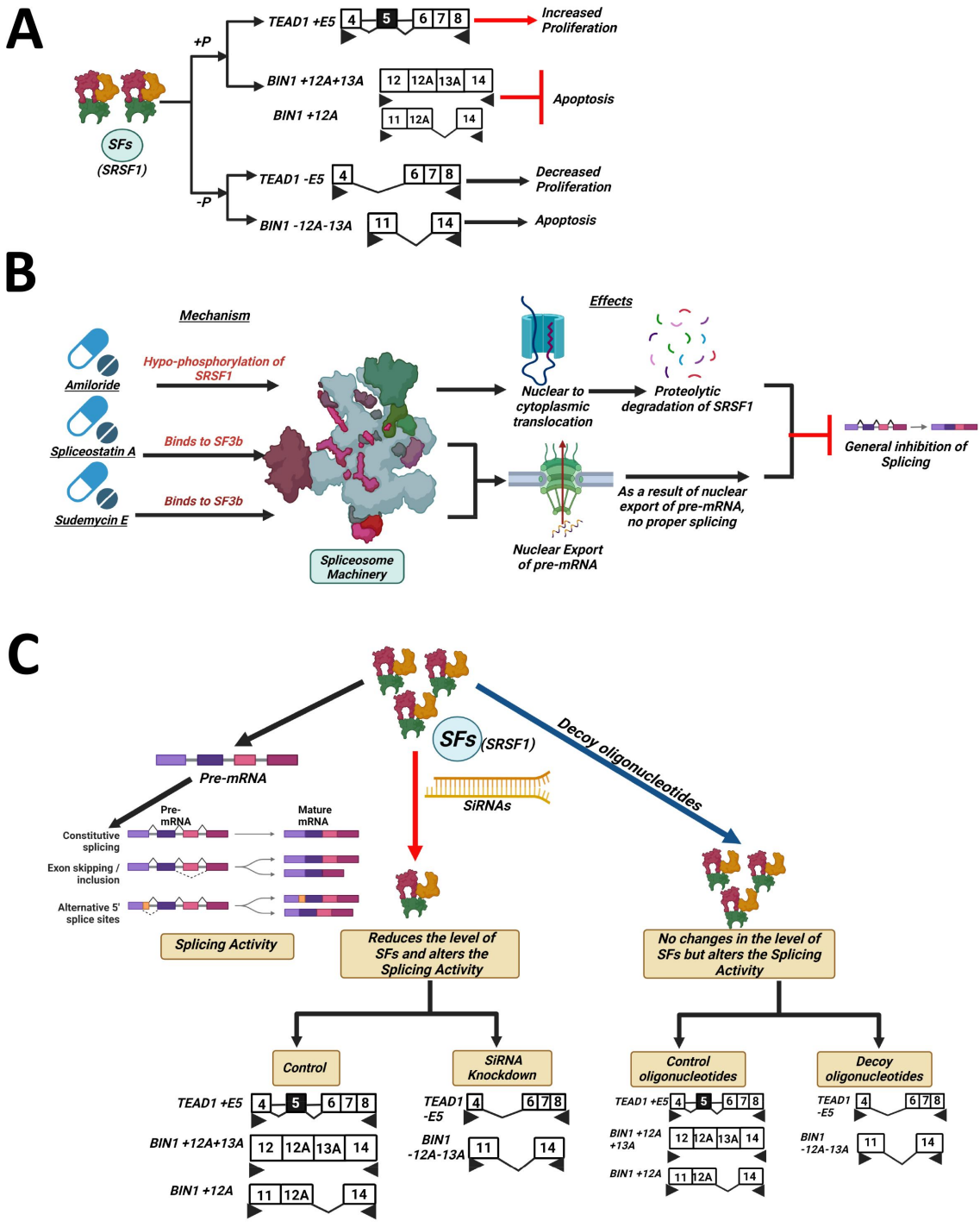

Figure 3. Regulation of splicing factor activity. (A) Splicing factor activity regulation by phosphorylation. Phosphorylation of SR splicing factors (SRSF1) results in increased splicing activity, as shown by the increased inclusion of +Exon 5 in TEAD1 and +Exon 12A and +Exon 13A in BIN1. Hypophosphorylation of the SR splicing factor results in reduced splicing activity, as shown by the decreased inclusion of +Exon 5 in TEAD1 and +Exon 12A and +Exon 13A in BIN1. (B) General inhibition of splicing by targeting splicing factors (SFs). Amiloride induces hypophosphorylation of SRSF1, resulting in nuclear to cytoplasmic translocation of SRSF1, culminating in proteolytic degradation of SRSF1. Degradation of SRSF1 results in general inhibition of splicing. Spliceostatin a and Sudemycin E bind to SF3b of the spliceosome machinery, resulting in the nuclear export of pre-mRNA and leading to general splicing inhibition. (C) Regulation of splicing factor activity and expression by siRNAs and decoy oligonucleotides. Splicing factors act on pre-mRNAs to regulate splicing. SiRNAs against SFs reduce the level of SFs, resulting in reduced splicing activity. Decoy oligonucleotides do not change the level of SFs but reduce splicing activity, similar to the action of siRNAs.

the production of the truncated progerin protein, is an excellent illustration of how normally well-maintained balance of protein isoforms can be upset by the presence of a cryptic splice site [164]. In fibroblast cultures from HGPS patients, a phosphorodiamidate morpholino oligomer ASO blocking the progerin splice site has been utilized to successfully restore normal splicing, rectify nuclear abnormalities and fix additional nuclear envelope protein mislocalization [165]. Following these developments, in vivo-morpholino ASOs were able to restore many of the phenotypical abnormalities identified in a mouse model that replicates the primary clinical symptoms of HGPS [166].

Exon-skipping ASOs can be used to either induce exon skipping and create mRNA isoforms that encode non-functional proteins or trigger nonsense-mediated decay of the mRNA, or they can be used to encode alternative isoforms with desired physiological or therapeutic functions that could modify or antagonize the effect of an undesirable gene [159,167]. In one investigation, ASOs were employed to elucidate the function of pancreatic beta cell insulin receptor (INSR) alternative splicing alterations [5]. It was known that INSR undergoes alternative splicing in pancreatic beta cells, but the role of the different isoforms was not fully understood. In this work, INSR exon 11 skipping was induced by an ASO that competes with the INSR intron 10 3'-splice site, effectively causing skipping of INSR exon 11 when it was introduced into pancreatic beta cell line, MIN6 [5] (Figure 4A). The cells transfected with ASOs resulted in increased INSR exon 11 skipping and decreased proliferation and survival. Cells with more INSR exon 11 skipping were more sensitive the pancreatic beta cells to stress conditions [5]. The study concluded that INSRA produced from exon 11 skipping sensitizes beta cells to stress conditions.

Exon-skipping ASOs have the potential to be therapeutically beneficial, can inhibit the activity of a normal isoform, and/or can mitigate the adverse effects of knockdown of the normal isoform and are preferable to simple knockdown [168]. If a simple knockdown is needed, other techniques such as RNA interference [169], ASOs that cause RNase H-mediated mRNA degradation [170], or translation inhibition [171], are more frequently used. Recently, regulation of alternative splicing by CRISPR technology was shown to alter the mRNA splicing and precisely control alternative splicing events [172].

Thus, exon-skipping technology enables gene expression to be reengineered to create the desired result with definite therapeutic advantages over alternative methods [168].

ASOs have many benefits over directly inhibiting splicing factors [173]. When the expression of a splicing factor is inhibited all the biological processes, such as splicing, transport, or translation, are impacted. Specific inhibition of splicing factor activity by decoy RNA oligonucleotides offers a solution that only inhibits the splicing activity of the splicing factor without affecting the other functions [174] (Figure 3C). The benefit of ASOs is that they only affect the targeted mRNA; all other cell functions are unaffected [16,175]. This strategy does have some drawbacks, though. The ASOs must be absorbed by the target tissue/cells with a very high efficiency [175]. Despite this challenge, ASOs have proven successful in treating neurodegenerative disease [176].

Recently, Gapinske et al. introduced a versatile method called CRISPR-SKIP that promotes exon skipping by mutating target DNA bases within splice acceptor sites [177]. CRISPR-SKIP utilizes single-base editors called cytidine deaminases to induce exon skipping and thereby regulate gene splicing [177]. This technology utilizes a special feature of exon splicing events: nearly every intron ends with a guanosine at the intron–exon junction. This junction that defines exons and introns has conserved sequences that are recognized by the spliceosome machinery, resulting in gene splicing. Mutations that disrupt these conserved sequences at the intron–exon junction of any given exon in genomic DNA would prevent the binding of spliceosome machinery, resulting in exon skipping. Exon skipping means that an exon is not present in the mature transcript. Nearly every intron ends with a guanosine at the intron–exon junction. Gapinske et al. showed that guanosine present at the end of the intron can be mutated by converting the cytidine in the complementary strand to thymidine through the use of cytidine deaminase single base editors through a technique called CRISPR-SKIP, resulting in altered splicing [177] (Figure 4B). Given its precision and simplicity, this technique has huge potential to regulate deregulated alternative splicing events in cancer. Importantly, artificial modulation of specific alternative splicing events, for example, by selectively skipping mutation-containing exons from mature transcripts present in various cancers while keeping normal isoforms intact, would prove to be very useful molecular tools in biomedicine [177].

Given the difficulty of targeting splicing factors (SFs) and aberrant alternative splicing in cancer, it is advisable to target something that acts upstream of them. From the discussion so far, it is clear that lncRNAs, through interaction with SFs or by themselves, regulate alternative splicing; thus, targeting lncRNAs for regulating the aberrant splicing and expression and function of SF would be a better therapeutic approach.

Modulation of lncRNAs

Depletion of lncRNAs

Several techniques have been employed for the depletion of lncRNAs (Figure 5A). RNAi has been widely employed to deplete lncRNAs, building on its success in the suppression of protein-coding genes [91,178–182]. After the transfection of siRNAs or the production of shRNAs, RNAi often causes the direct complementarity of the RNA-induced silencing complex to cause the degradation of target RNA molecules, and for mRNAs, protein translation can also be blocked [182–185]. The location of the target RNA within the cell plays a role in the efficacy of RNAi-mediated knockdown [186,187]. RNAi factors like Argonaute and Dicer have been discovered in cells' nuclei, despite the fact that human RNAi is assumed to occur primarily in the cytoplasm [188], which may explain how RNAi achieves suppression of nucleus enriched lncRNAs like MALAT1 [91,178]

ASOs are an alternative for RNAi in the degradation of lncRNAs. ASOs are single-stranded DNA oligomers that are 15–20 nt in length that are frequently chemically altered to improve knockdown effectiveness and lessen in vivo toxicity [182]. Particularly, it has been demonstrated that the 2'-O-MOE and LNA gapmer modifications improve affinity towards target RNA transcripts and provide nuclease resistance, enabling these modified ASOs to have half-lives between days and several weeks in vivo [170,189–192]. Through complementarity, ASOs bind to and hybridize with target RNA transcripts, leading to the target transcripts' RNaseH-mediated degradation [193,194]. As a result, ASO-mediated knockdown is much more effective in the nucleus than RNAi-based

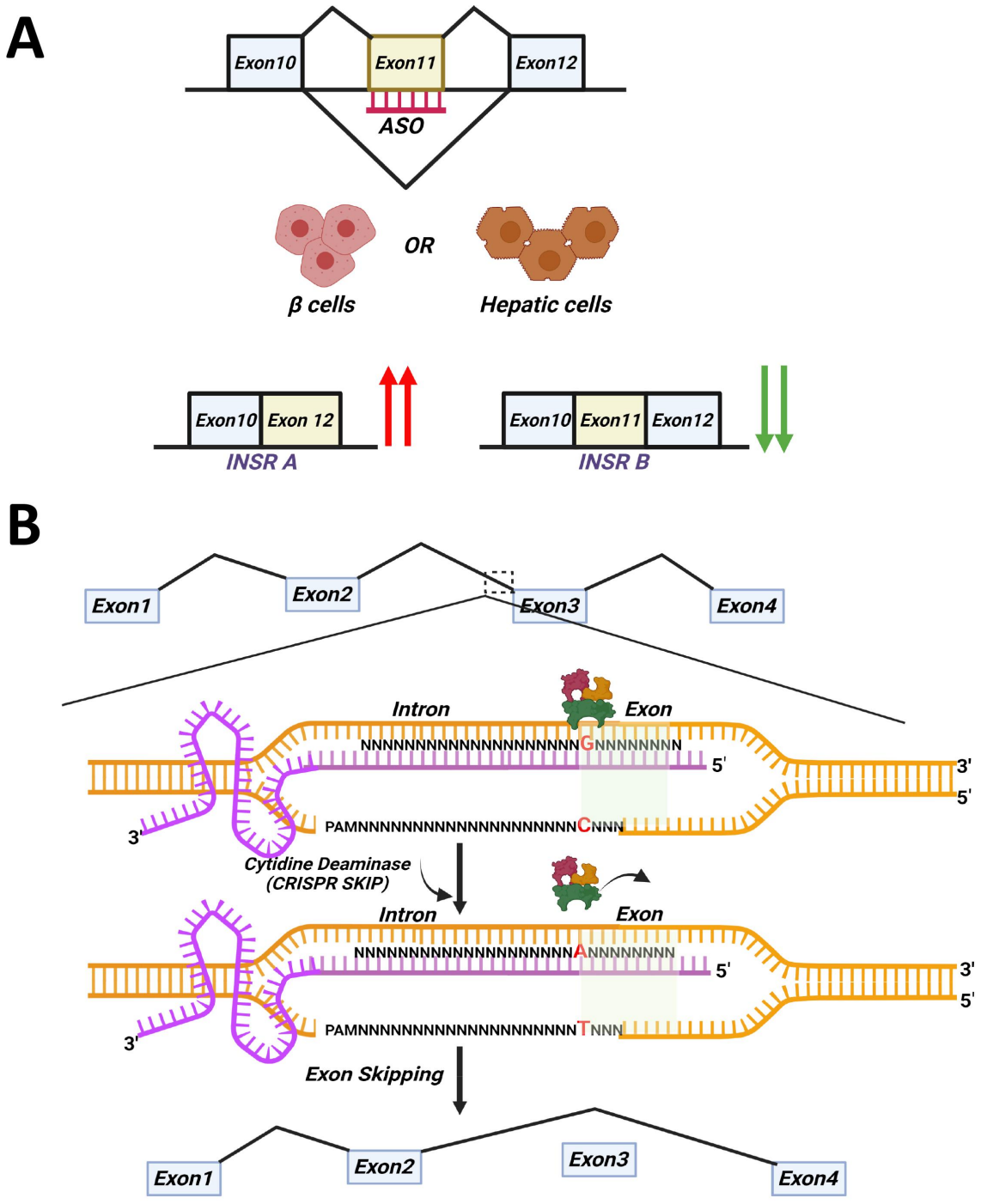

Figure 4. Modulation of alternative splicing through the use of antisense oligonucleotides and CRISPR-SKIP. (A) Schematic representation of INSR exon 11 skipping through the use of antisense oligonucleotides (ASO). use of ASO in β cells or hepatic cells results in increased expression of INSR-A (without exon 11) and decreased expression of INSR-B (with exon 11). (B) Schematic representation of the mechanism of action of exon skipping through the use of CRISPR-SKIP. CRISPR-SKIP utilizes single base editors called cytidine deaminases to induce exon skipping and thereby regulate gene splicing. This technology uses a special feature of exon-splicing events, which is that every intron ends with a guanosine at the intron–exon junction. These conserved sequences are recognized by spliceosome machinery, resulting in exon skipping. CRISPR-SKIP mutates the guanosine present at the end of the intron, resulting in altered splicing.

techniques, making it a good choice for researching the roles of both cis-acting and trans-acting lncRNAs [195]. This has been used in an experimental model system of breast cancer. Intravenous infusion of Malat1 ASOs reduced tumour metastases in a mouse model of breast cancer when compared to scrambled ASO controls [196].

Another method of depleting lncRNAs is Cas13-based direct RNA cleavage of the target lncRNA. The Cas13 family of CRISPR ribonucleases is a recent technique that can directly destroy lncRNAs [197]. CRISPR-Cas13 May effectively cleave target RNA when sgRNAs complementary to the target guide it to the site [197]. Since sgRNAs can be generated stably from viral vectors and Cas13 has been utilized to silence lncRNAs in mammalian cells, Cas13-based approaches may be appropriate for genome-scale screening for lncRNA transcript function [198,199].

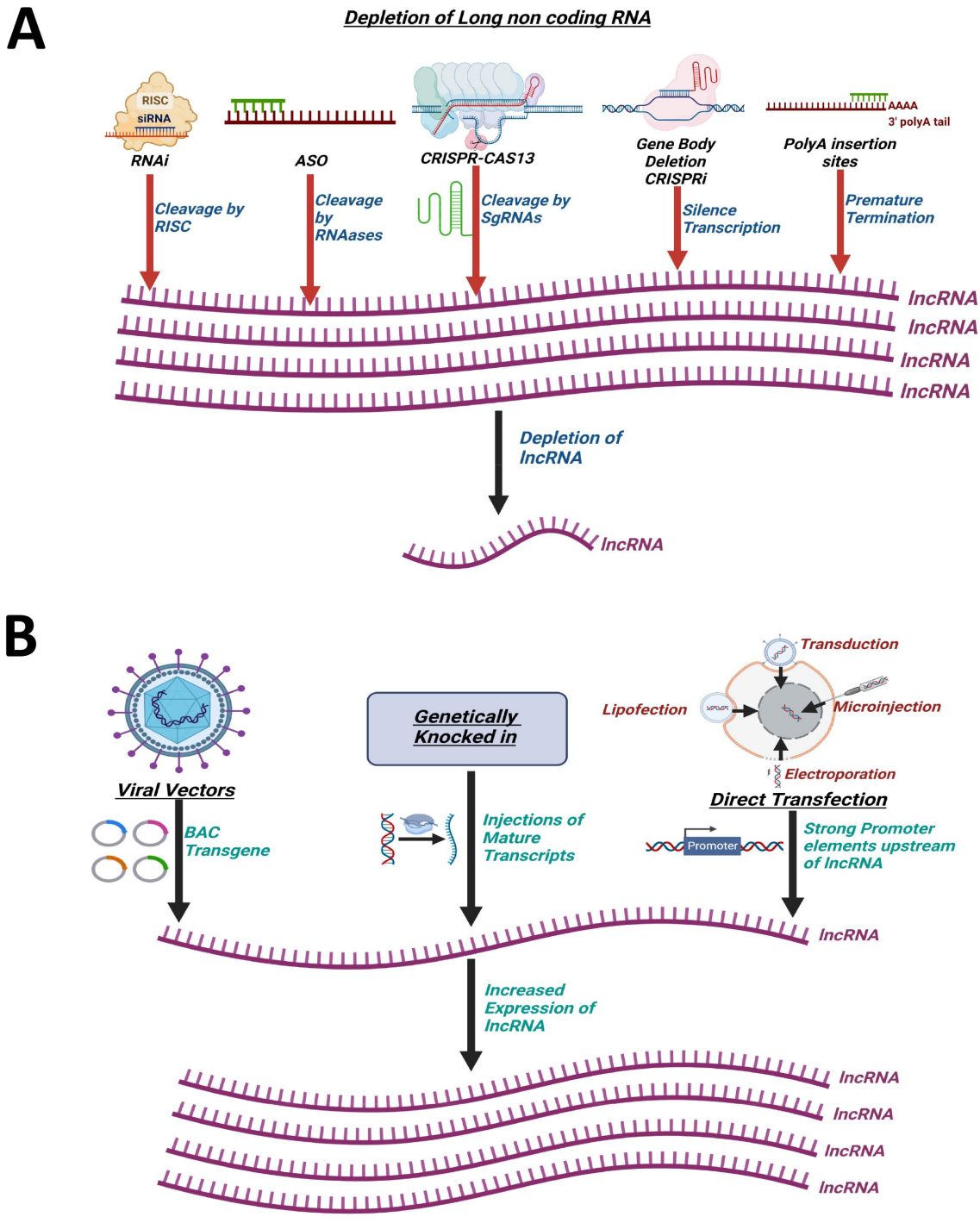

Figure 5. Modulation of long noncoding RNAs expression. (A) Schematic representation of various methods employed to deplete long noncoding RNAs (lncRNAs). (B) Schematic representation of various methods employed to overexpress lncRNAs.

Insertion of PolyA transcription termination signals into the lncRNA gene locus is another method of lncRNA depletion [200]. Knocking in polyA sites causes the transcription of lncRNAs to end prematurely, which is also a successful loss-of -function tactic that may lessen the chance of interfering with known or potential DNA regulatory functions [201]. One significant benefit of polyA site insertion is that it suppresses transcriptional elongation, making it possible to sometimes distinguish between mechanisms that depend on the intact, full-length lncRNA transcript and those that do not [182].

An alternative method involves deletion of lncRNA gene using CRISPRi [202]. This method has been used to delete up to hundreds of human lncRNAs, exposing the function of hitherto uncharacterized lncRNA loci. DoubleCas9 excision of DNA sequences that flank lncRNAs are more likely to inactivate lncRNA gene activity. The dCas9-KRAB component of the CRISPRi system, which fuses dCas9 with the KRAB repressor domain, inhibits RNA polymerase elongation and causes local H3K9me3 deposition to silence transcription [203].

Overexpression of lncRNAs

While depleting lncRNAs can be relatively straightforward, achieving their overexpression presents more challenges. Several strategies have been employed to successfully overexpress lncRNAs. (Figure 5B).

Overexpression of lncRNAs is a useful tool to discover more about the function of the lncRNA. Overexpression may reveal lncRNA functions that are not apparent under physiological conditions. This has been achieved by overexpression of the lncRNAs using viral vectors [204] and BAC transgenes [205].

Researchers have directly injected or transfected in vitro-transcribed lncRNAs, to establish lncRNA function [206]. These techniques may also be helpful for the investigation of lncRNAs that are thought to function in trans [182].

Strong promoter elements upstream of lncRNA loci can be genetically knocked in to increase lncRNA expression. Additionally, knock-in techniques can stimulate lncRNA transcription at their endogenous sites. For instance, the colorectal cancer-associated lncRNA CCAT1 has been overexpressed using TALEN-mediated knock-in of a CMV promoter upstream of CCAT1. This has boosted CCAT1 expression by 15- to 30-fold, which has elevated MYC expression and carcinogenesis in a colorectal cancer cell line [207].

Gain-of-function studies for lncRNAs have also been made possible by programmable transcriptional activation employing modified CRISPR/Cas9 systems [208].

This has been achieved by positioning gene activation domains, like VP64, directly upstream of the transcription start site of lncRNAs (or any other gene transcribed by RNA polymerase II) [208].

CRISPR display is yet another effective gain-of-function technique [209]. In order to deliver lncRNA transcripts of up to several kilobases (or smaller lncRNA domains) to ectopic regions of the genome, CRISPR display uses an engineered dCas9 that interacts with altered sgRNA-lncRNA chimaeras [209].

CRISPR display can separate the function of the transcript from the act of transcription for putative cis mechanisms in addition to testing a subset of various trans lncRNA processes.

Unanswered questions in the field

Many of lncRNAs have been shown to be associated with SFs, but the role of lncRNAs in regulating the splicing targets of the interacting SFs has not yet been studied. SFs are very difficult to regulate, due to their essential role in human physiology, so for therapeutic purposes the knowledge of SF-interacting lncRNAs might play an important role in the regulation of splicing targets in human diseases.

Do SR proteins and hnRNPs regulate lncRNAs expression? This is still an open question. Is the regulation of lncRNAs different for SR proteins and hnRNPs? What regulates the interaction of splicing factors and lncRNAs? Do some signalling pathways play a role in influencing the interaction of lncRNAs and SFs to influence AS?

SF activity has been shown to be regulated by signalling pathways. The regulation of lncRNAs expression and function, alternative splicing isoforms by generalized signalling pathways like mTORC1, mTORC2, AKT and WNT signalling requires further studies.

Theoretically, nucleus bound lncRNAs have high probability of regulating AS by interacting with SFs, since SFs reside inside the nucleus. The role of cytoplasmic lncRNAs in regulating AS by binding to the SFs needs further investigation. The degree of regulation of AS by nuclear, cytoplasmic, or cytoplasmic and nuclear both localized lncRNAs requires further investigation.

Future perspectives

It is challenging to determine the precise constellation of components required for a particular splicing choice and for the development of cancer. This is partly due to the participation of SFs in several levels of RNA processing and the redundancy of activities among some of these factors. The absence of reliable animal model systems also presents another obstacle to comprehending the role of SFs/lncRNAs under normal physiological settings. Mice lacking the SFs SRSF1 [210], SRSF2 [211] or SRSF3 [212] are not viable. Therefore, more advanced mouse model systems, such as inducible and/or tissue-specific knockouts, are required. Transgenic mice model systems that overexpress SFs and/or lncRNAs are also required. In spite of rapid increase in the number of diverse roles reported for lncRNAs, one of the greatest challenges is identifying the sequence and structural elements that are responsible for their cellular functions [213]. This is crucial in order to gain a better molecular understanding of the mechanisms by which lncRNAs contribute to AS.

Conclusion

In this review, we explored the connection of lncRNAs and SFs. Furthermore, we explored how lncRNAs, splicing factors and alternative splicing may be modulated. LncRNAs contribute to the regulation of AS through their interaction with SFs in diverse ways. To be more precise, lncRNAs can control the expression of SFs or the production of cancer-causing splicing variants of target genes. Since non-coding transcripts, primarily introns and ncRNAs, make up the majority of the eukaryotic genome, there may be a wide range of means by which ncRNAs interact with the splicing machinery to control and promote proteome flexibility. Mechanistic knowledge of the recruitment and control of lncRNAs to regulate AS may lead to new approaches for gene therapy, including cancer treatment. Due to the difficulties in correcting aberrant AS using available approaches, the modulation of lncRNAs holds hope for regulation of mis-regulated AS in cancers.

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Data availability statement

Data availability is not applicable to this article as no new data were created or analysed in this study. No datasets have been used in the current study.

Author contributions

PM and SS wrote the manuscript. MM prepared the figures. RK and JAS edited the manuscript.

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