The emerging role of splicing factors in cancer

Ana Rita Grosso*, Sandra Martins* & Maria Carmo-Fonseca[†]

Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

Recent progress in global sequence and microarray data analysis has revealed the increasing complexity of the human transcriptome. Alternative splicing generates a huge diversity of transcript variants and disruption of splicing regulatory networks is emerging as an important contributor to various diseases, including cancer. Current efforts to establish the dynamic repertoire of transcripts that are generated in health and disease are showing that many cancer-associated alternative-splicing events occur in the absence of mutations in the affected genes. A growing body of evidence reveals changes in splicing-factor expression that correlate with cancer development, progression and response to therapy. Here, we discuss how recent links between cancer and altered expression of proteins implicated in splicing regulation are bringing the splicing machinery to the fore as a potential target for anticancer treatment.

Keywords: cancer; pre-mRNA splicing; spliceosome; splicing factor; therapeutic target

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See Glossary for abbreviations used in this article.

Introduction

Removal of noncoding sequences (introns) from pre-messenger RNAs through splicing provides a versatile means of genetic regulation. Alternative splicing allows a single gene to generate multiple transcripts, thereby expanding the transcriptome and proteome diversity in metazoans. Several studies based on large-scale expressed sequence tag analysis estimated that more than 60% of human genes undergo alternative splicing; this number recently increased to more than 80% when microarray data became available (Black, 2003; Matlin *et al*, 2005). Intron excision is carried out by an assembly of snRNPs and extrinsic, non-snRNP, protein-splicing factors that are collectively recruited to pre-mRNAs and form the spliceosome. The initial events of spliceosome assembly require the recognition of specific sequences located at, and near, the 5' and 3' splice sites, which recruit the U1 and U2 snRNPs (Fig 1). In metazoan organisms,

the splice-site sequences are weakly conserved, and require specific additional RNA sequence elements that function either to enhance or to repress the ability of the spliceosome to recognize and select nearby splice sites (Maniatis & Tasic, 2002; Matlin *et al*, 2005). The multiplicity of protein–protein and protein–RNA interactions that modulate the association of the spliceosome with the pre-mRNA constitutes the basis for the control of alternative splicing (Fig 1).

A typical multiexon pre-mRNA can undergo various alternative-splicing patterns (Black, 2003). Most exons are constitutive, meaning that they are always included in the final mRNA; however, there are also regulated exons, which are sometimes included and sometimes excluded from the mRNA (Fig 1). Exons can also be lengthened or shortened by altering the position of one of their splice sites, or by a distinct splicing pattern that consists of failure to remove an intron, a process known as intron retention. Alternative splicing can also be coupled to differential promoter or polyadenylation site usage, giving rise to even larger transcriptome heterogeneity. Splicing abnormalities have an important role in human diseases such as cancer (Wang & Cooper, 2007). Several mutations are known to affect the splicing of oncogenes, tumour suppressors and other cancer-relevant genes (Srebrow & Kornblihtt, 2006; Venables, 2006); however, many splicing abnormalities that have been identified in cancer cells are not associated with mutations in the affected genes. Rather, a growing body of evidence indicates that the splicing machinery is an important target for misregulation in cancer. According to recent bioinformatics studies, changes in splicing-factor expression might have a key role in the general splicing disruption that occurs in many cancers (Kim *et al*, 2008; Ritchie *et al*, 2008).

Can splicing factors act as oncogenes?

As changes in the concentration, localization and/or activity of splicing factors are known to modify the selection of splice sites (Matlin *et al*, 2005), it is predicted that the abnormally expressed splicing factors found in tumour cells induce the production of mRNA isoforms that are either nonexistent or less abundant in normal cells. This phenomenon might contribute directly or indirectly to cancer development, progression and/or response to therapy. A recent study demonstrated for the first time that overexpression of a splicing factor can, indeed, trigger malignant transformation (Karni *et al*, 2007). The authors showed that the splicing factor SF2/ASF is upregulated in various human tumours, and affects alternative splicing of the tumour suppressor BIN1 and the kinases MNK2 and S6K1. The resulting BIN1 isoforms lack tumour-suppressor activity, the MNK2 isoform promotes MAPK-independent eIF4E phosphorylation and

Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal

*These authors contributed equally to this work.

[†]Corresponding author. Tel: +351 21 7999411; Fax: +351 21 7999412;

E-mail: carmo.fonseca@fm.ul.pt

Glossary

BCL-X	BCL-2-like 1 protein, an apoptosis regulator; alternative splicing of the BCL-X precursor messenger RNA gives rise to two isoforms encoding proteins with antagonistic functional properties, a long antiapoptotic isoform and a short proapoptotic isoform
BIN1	bridging integrator 1, a tumour-suppressor gene that is involved in apoptosis; the appearance of splicing factor 2/alternative splicing factor-dependent BIN1 splicing isoforms correlates with decreased levels of apoptosis
CDC25B	cell-division cycle 25B
FAS	an apoptosis-stimulating fragment, also known as member 6 of the tumour necrosis factor receptor superfamily; the human <i>FAS</i> gene encodes a transmembrane protein that mediates apoptosis upon ligation of the FAS ligand; alternative splicing produces either a membrane bound form of the receptor that promotes apoptosis or a soluble isoform that prevents programmed cell death
MAPK	mitogen-activated protein kinase
MNK2	mitogen-activated protein kinase-interacting serine/threonine kinase 2; as a result of splicing factor 2/alternative splicing factor-dependent alternative splicing, the MNK2 kinase is active in the absence of upstream signals from the mitogen-activated protein kinase pathway
MRP1	multidrug resistance-associated protein 1
pre-mRNA	precursor messenger RNA, the initial transcript of a protein-coding gene
PTB	polypyrimidine-tract binding protein, involved in splicing regulation
RBM5	RNA-binding motif protein 5, involved in splicing regulation
RNAi	RNA interference
RON	recepteur d'origine nantais; the RON protein belongs to the mesenchymal-epithelial transition factor proto-oncogene family of receptor tyrosine kinases
S6K1	ribosomal protein S6 kinase, involved in translational control in the mammalian target of rapamycin pathway; overexpression of splicing factor 2/alternative splicing factor induces alternative splicing of S6K1 leading to a protein isoform with oncogenic properties
SF2/ASF	splicing factor 2/alternative splicing factor, a member of the serine/arginine rich protein family; participates in constitutive and alternative splicing, and is essential for cell viability
SF3b	splicing factor 3b, an integral component of the U2 small-nuclear ribonucleoprotein particle
siRNA	small interfering RNA
snRNPs	small nuclear ribonucleoprotein particles, the building blocks of the spliceosome; each is composed of a uridine-rich small-nuclear RNA packaged with proteins
SPF45/RBM17	45 kDa-splicing factor/RNA-binding motif protein 17, involved in splicing regulation
SRP20	a member of the serine/arginine rich protein family, involved in splicing regulation
SRPK	serine/arginine rich protein kinase
U2AF	U2 small-nuclear ribonucleoprotein particle auxiliary factor is an essential splicing factor composed of two subunits, U2AF65 and U2AF35; U2AF35 assists binding of U2AF65 to the polypyrimidine tract upstream of the 3' splice site, which promotes recruitment of the U2AF to the precursor messenger RNA

the S6K1 isoform has demonstrated oncogenic properties (Karni *et al*, 2007). This study acts as a proof-of-principle and provides evidence that abnormally expressed splicing proteins can have oncogenic properties.

Fig 1 | Alternative splicing of *FGFR2*. (A) FGF signalling is mediated by four FGFR tyrosine kinases known as FGFR1–4, which have crucial roles in morphogenesis, development, angiogenesis and wound healing. FGFRs are composed of an extracellular ligand-binding portion consisting of three immunoglobulin-like domains (D1, D2 and D3), a single transmembrane (TM) helix and a cytoplasmic portion that contains protein tyrosine kinase activity. Ligand binding and specificity reside in D2, D3 and the linker that connects them. Alternative splicing in the carboxy-terminal half of D3 is an important determinant of FGF–FGFR binding specificity. (B) The transcripts (pre-mRNA) encoding FGFR2 contain two alternative exons: IIIb and IIIc. Inclusion of exon IIIb instead of exon IIIc introduces an amino-acid sequence in the second half of D3 that is less likely to form the hydrophobic core required for efficient interaction with FGF2 (Plotnikov *et al*, 2000). Inclusion of exon IIIb occurs predominantly in epithelial cells, whereas inclusion of exon IIIc is exclusively detected in cells of mesenchymal origin. A switch from exon IIIb to exon IIIc inclusion accompanies the progression of androgen-sensitive, well-differentiated prostate carcinomas to androgen-insensitive, poorly differentiated tumours (Carstens *et al*, 1997; Yan *et al*, 1993). (C) In mesenchymal cells, exon IIIb silencing depends on the combined effect of weak splice sites, an exonic splicing silencer that binds hnRNP A1, and two flanking intronic splicing silencers that bind to PTB. The binding of hnRNP A1 and PTB to the splicing silencers inhibits the recruitment of U1 and U2 snRNPs to the weak splice sites of exon IIIb. In epithelial cells, exon IIIb silencing is countered by binding of TIA-1 to an intronic activating sequence located downstream of exon IIIb. TIA-1 binding promotes the recruitment of U1 snRNP to the weak 5' splice site of exon IIIb. In addition, FOX-2 binds to intronic and exonic (U)GCAUG sequence elements, and contributes to both exon IIIb activation and exon IIIc repression. FOX-2 proteins are differentially expressed in IIIb⁺ cells in comparison to IIIc⁺ cells, and overexpression of FOX-2 is sufficient to induce a switch in splice choice from IIIc to IIIb (Baraniak *et al*, 2006 and references therein). FGF fibroblast growth factor; FGFR2, fibroblast growth factor receptor 2; FOX-2 forkhead box-2; hnRNP heterogeneous nuclear ribonucleoparticle; HSPG, heparan sulphate proteoglycan; PTB, polypyrimidine-tract binding protein; snRNP, small-nuclear ribonucleoprotein particle; TIA-1, cytotoxic granule-associated RNA binding protein.

A previous study had indicated that SF2/ASF affects alternative splicing of RON, which is a tyrosine kinase receptor involved in cell dissociation, motility and matrix invasion (Ghigna *et al*, 2005). An alternatively spliced isoform of RON that lacks exon 11 produces a constitutively active protein that is expressed in gastric, breast and colon cancers, and induces an invasive phenotype (Collesi *et al*, 1996; Ghigna *et al*, 2005). Binding of SF2/ASF to a regulatory sequence in exon 12 stimulates skipping of exon 11, and overexpression of SF2/ASF activates cell locomotion. This effect can be reversed by specific knockdown of the alternatively spliced RON isoform, suggesting that an upregulation of SF2/ASF could contribute to malignant transformation by inducing alternative splicing of RON.

Several additional splicing proteins have been shown to be upregulated in various human tumours (Table 1); however, in most cases, the effect that these changes have on splicing regulation is unknown. By contrast, the number of splicing proteins that have been shown to be downregulated in cancer is much lower (Table 1). For example, reduced expression of U2AF³⁵ was found in pancreatic cancer cells and correlated with mis-splicing of the cholecystokinin-B/gastrin receptor mRNA (Ding *et al*, 2002). Furthermore, RNAi-mediated downregulation of U2AF³⁵ in HeLa cells has been reported to alter the ratios of alternatively spliced isoforms of transcripts encoding

the oncogenic CDC25B phosphatase, and to increase the level of CDC25B protein (Pacheco *et al*, 2006).

In conclusion, there is a growing list of splicing factors that have been found to be upregulated or downregulated in cancers, as compared with the corresponding normal tissues. Nevertheless, in most cases the available data are strictly correlative. A challenge for the future will be to determine whether these changes contribute directly to the cancer phenotype, or whether they are simply among the many processes that are altered in cancer cells. A crucial issue is whether cells expressing abnormal levels of certain splicing factors are positively selected for during tumour progression, as misregulated

splicing factors might generate splice variants encoding protein isoforms that provide advantages to these cells, such as increased proliferation, anti-apoptotic or pro-angiogenic effects, enhanced cell motility or tumour-cell survival. Moreover, many RNA-binding proteins are multifunctional and their abnormal expression might have oncogenic effects that are independent from splicing. What triggers the upregulation and downregulation of splicing proteins is also unknown (Sidebar A). Consistent with the view that cancer-associated genetic instability is likely to have an important role in this process, overexpression of splicing factor SF2/ASF was shown to associate with amplification of the gene encoding it (Karni *et al*, 2007),

Table 1 | Splicing factors altered in cancer and potential splicing targets

	Name (alternative names)	Cancer tissue	Affected mRNA	References
Upregulated in cancer				
<i>SR and AR-related proteins</i>	SF2/ASF (SFRS1)	Colon, thyroid, small intestine, kidney, lung, breast and colon	RON, BIN1, S6K1, MNK2	Ghigna <i>et al</i> , 2005; Karni <i>et al</i> , 2007
	SC35 (SFRS2)	Ovary	–	Fischer <i>et al</i> , 2004; Xiao <i>et al</i> , 2007
	SRp20 (SFRS3)	Ovary	MRP1	He <i>et al</i> , 2004
	SRp40 (SFRS5)	Breast	CD44	Huang <i>et al</i> , 2007
	SRp55 (SFRS6)	Breast	–	Karni <i>et al</i> , 2007
	TRA2-β1 (SFRS10)	Breast	CD44	Watermann <i>et al</i> , 2006
	SRm160 (SRRM1)	Thymic epithelium, stomach and kidney	CD44	Cheng & Sharp, 2006; Harn <i>et al</i> , 1996; Lee <i>et al</i> , 2003; Wu <i>et al</i> , 2003
<i>hnRNP proteins</i>	hnRNP A1 (HNRNPA1)	Lung, breast and ovary	–	Patry <i>et al</i> , 2003; Zerbe <i>et al</i> , 2004
	hnRNP B1 (HNRNPA2B1)	Lung	–	Sueoka <i>et al</i> , 2001; Zhou <i>et al</i> , 1996
	hnRNP F (HNRNPF)	Colon	–	Balasubramani <i>et al</i> , 2006
	hnRNP L (HNRNPL)	Oesophageal cancer cell lines	–	Qi <i>et al</i> , 2008
	hnRNP K (HNRNPK)	Colorectal and oral	–	Carpenter <i>et al</i> , 2006; Roychoudhury & Chaudhuri, 2007
	PTB (PTBP1, HNRNPI)	Glioblastoma and ovary	FGFR1, MRP1	Jin <i>et al</i> , 2003; He <i>et al</i> , 2004
<i>Other factors</i>	YB-1 (YBX1)	Ovary	CD44	Fischer <i>et al</i> , 2004
	SPF45 (RBM17)	Bladder, breast, colon, lung, ovary, pancreas and prostate	FAS	Corsini <i>et al</i> , 2007; Sampath <i>et al</i> , 2003
	SRPK1 (SFRSK1)	Pancreas, breast, colon, T cells and chronic myelogenous leukaemia	MAP2K2	Hayes <i>et al</i> , 2006
	HuR (ELAVL1)	Breast and ovary	FAS	Denkert <i>et al</i> , 2004; Izquierdo, 2008
	HuD (ELAVL4)	T-cell acute lymphoblastic leukaemia	IK	Bellavia <i>et al</i> , 2007
	Sam68 (KHDRBS1)	Prostate	–	Busa <i>et al</i> , 2007
Downregulated in cancer				
<i>hnRNP</i>	hnRNP E2 (PCBP2)	Oral	–	Roychoudhury & Chaudhuri, 2007
<i>Other factors</i>	U2AF35 (U2AF1)	Pancreas	CCK-B	Ding <i>et al</i> , 2002; Pacheco <i>et al</i> , 2006
	SF1 (Sf1)	Colorectal	WISP1, FGFR3	Shitashige <i>et al</i> , 2007a,b
	RBM5 (LUCA15)	Lung	–	Oh <i>et al</i> , 2006

BIN1, bridging integrator 1; CCK-B, cholecystokinin-B/gastrin; FAS, apoptosis-stimulating fragment; FGFR, fibroblast growth factor receptor; hnRNP, heterogeneous nuclear ribonucleoparticle; HuD, Hu antigen D; HuR, Hu antigen R; IK, transcription factor Ikaros; MAP2K2, mitogen-activated protein kinase; MNK2, mitogen-activated protein kinase-interacting serine/threonine kinase 2; mRNA, messenger RNA; MRP1, multidrug-resistance protein 1; PTB, polypyrimidine-tract binding protein; RBM5, RNA-binding motif protein 5; Ron, recepteur d'origine nantais; S6K1, ribosomal protein S6 kinase; Sam68, Src-associated in mitosis 68 kDa; SC35, splicing component 35 kDa; SF, splicing factor; SFR, splicing factor, arginine/serine-rich; SF2/ASF, splicing factor 2/alternative splicing factor; SPF45, 45 kDa-splicing factor; SR, serine/arginine rich; SRm160, serine/arginine-related nuclear matrix protein; SRp, serine/arginine rich protein; SRPK, serine/arginine rich protein kinase; Tra2-β1, human homologue of *Drosophila* splicing regulator Transformer 2; U2AF, U2 small-nuclear ribonucleoprotein particle auxiliary factor; WISP, wingless-type MMTV integration site family, member 1-inducible signalling pathway protein 1; YB-1, Y-box binding protein 1.

whereas reduced expression of RBM5 in lung cancer correlates with deletion of its gene locus at chromosomal region 3p21.3 (Oh *et al*, 2006). Alternatively, or in addition, splicing-factor transcripts seem to be preferential targets for disrupted splicing in cancer tissues (Kim *et al*, 2008; Ritchie *et al*, 2008). Cancer-specific splicing-factor isoforms could either alter the function of the protein in the cell or reduce its level owing to the introduction of premature stop codons, thereby leading to nonsense-mediated decay of the mRNA.

Splicing factors and anticancer therapy

During the past 20 years, anticancer drug development has focused on targeted medicines that are more specifically associated with tumour cells than conventional cytotoxic drugs. More than 600 new agents are now in the development pipeline, in the hope of attaining greater anticancer activity with fewer side effects (Dancey & Chen, 2006). Several approaches are being explored for the correction of cancer-associated splicing abnormalities that are still at the preclinical stage (for comprehensive reviews, see Pajares *et al*, 2007; Wang & Cooper, 2007). One strategy uses synthetically modified oligonucleotides that are able to block spliceosome assembly at specific sites, thereby preventing the generation of cancer-associated splice variants. This approach has been successfully used to shift the ratio of anti-apoptotic to pro-apoptotic proteins produced by alternative splicing of the *BCL-X* gene, thereby sensitizing refractory cancer cells to undergo apoptosis in response to chemotherapeutic drug treatment (Taylor *et al*, 1999). Another strategy that is being explored consists of raising antibodies against epitopes that are uniquely present in the cancer-associated protein isoforms and conjugating the antibodies to tumour-cell toxins. For example, human recombinant antibodies specific to the alternatively spliced domains of tenascin-C large isoform—an abundant glycoprotein of the cancer extracellular matrix that is largely undetectable in normal adult tissues—have shown promising tumour-targeting properties (Brack *et al*, 2006).

Strategies aimed at targeting components of the splicing machinery that are abnormally expressed in cancer are expected to be less specific because they are likely to impinge on splicing regulation in normal cells. Nevertheless, many approaches have been attempted with encouraging results. Particular attention has been devoted to the development of protein kinase inhibitors that modulate the activity of splicing factors containing RS domains, which are characterized by repeats of arginine–serine dipeptides. Phosphorylation/dephosphorylation of these serine residues is thought to act as a switch that modulates the binding properties of these kinases to both RNA and proteins (Singh & Valcarcel, 2005). Although there are several known splicing-factor kinases, members of the SRPK family seem to be the most relevant in cancer (Table 1). Downregulation of SRPK1 expression by siRNA in cancer cell lines caused a reduction of cell proliferation, and increased sensitivity to gemcitabine and cisplatin, making the approach of targeting SRPK1 a promising tool that might prove to be therapeutically effective for tumours that overexpress this protein (Hayes *et al*, 2006, 2007).

In addition, the aberrant expression of splicing factors in tumour cells might be implicated in resistance to drugs that are commonly used in cancer therapy. For example, increased expression of the splicing factors PTB and SRP20 in ovarian cancer correlates with the production of alternatively spliced isoforms of MRP1, which confers increased resistance to doxorubicin (He *et al*, 2004). Another splicing factor that is highly expressed in numerous carcinomas, SPF45

Sidebar A | In need of answers

- (i) The precise mechanism underlying the induction of malignant transformation by splicing-factor overexpression remains unknown. The current working model postulates that changes in splicing-factor expression induce a switch in splice choice from target messenger RNAs, and that this, in turn, leads to production of new splicing variants with oncogenic properties.
- (ii) What triggers the upregulation and downregulation of splicing proteins in tumours?
- (iii) The ultimate remaining challenge is to integrate the different layers of gene-expression regulation that are disrupted in cancer and construct a systems view for the molecular pathology of this disease.

(RBM17), affects the alternative splicing of the apoptosis regulator FAS (Corsini *et al*, 2007), and the overexpression of SPF45 has been implicated in resistance to doxorubicin and vincristine (Sampath *et al*, 2003).

It is fully anticipated that inhibiting the function of either a splicing kinase or a splicing protein will have a pleiotropic effect, as it will alter the splicing of numerous gene products in both cancerous and normal cells. However, a well-established principle of cancer therapy is to use a combination of drugs with various mechanisms of action and resistance, at their optimal doses and according to schedules that are compatible with normal cell recovery. Therefore, it might be possible to develop and to optimize agents that temporarily inhibit a splicing regulator and partly correct abnormal splicing, resulting in enhanced tumour-cell killing by chemotherapeutic drugs.

Recently, proof-of-principle has been provided for the development of anti-tumour compounds that target the splicing machinery. Spliceostatin A (Kaida *et al*, 2007) and pladienolide (Kotake *et al*, 2007), which are two potent inhibitors of cycling cancer cells, target the essential splicing protein SF3b and inhibit the splicing of several transcripts. Both drugs are only mildly toxic to animals and a pladienolide derivative, E7107, has already progressed to clinical trials. This moderate toxicity is probably due to partial inhibition of splicing throughout the organism; however, the mechanism behind the enhanced vulnerability of cancer cells to these drugs remains unknown. Most importantly, these studies have defined a new mode of action of anticancer drugs and identified a ubiquitous core component of the U2 snRNP, SF3b, as a valuable new therapeutic target.

Conclusion

The rapid development and increasing availability of novel genome-wide tools will soon provide a catalogue of all splicing factors and all splice variants that are differentially expressed in specific cancer types and the corresponding normal tissues. Irrespective of whether changes in splicing have a direct causative role in cancer, or act as modifiers or susceptibility factors in the oncogenic process, the identification of splicing signatures is likely to provide important markers for diagnosis, prognosis and/or sensitivity to treatment. A full description of all components of the splicing machinery and splicing events altered in cancer will also identify potential new targets for therapeutic approaches. However, the most challenging goal for the future will be to integrate the different layers of gene-expression regulation altered in cancer and to acquire a systems-biology view of the many molecular mechanisms that contribute to the pathophysiology of this disease.

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From left: Maria Carmo-Fonseca, Sandra Martins & Ana Rita Grosso