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Alternative RNA splicing and cancer

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Abstract

Alternative splicing of pre-messenger RNA (mRNA) is a fundamental mechanism by which a gene can give rise to multiple distinct mRNA transcripts, yielding protein isoforms with different, even opposing, functions. With the recognition that alternative splicing occurs in nearly all human genes, its relationship with cancer-associated pathways has emerged as a rapidly growing field. In this review, we summarize recent findings that have implicated the critical role of alternative splicing in cancer and discuss current understandings of the mechanisms underlying dysregulated alternative splicing in cancer cells.

Introduction

Alternative splicing is a tightly regulated RNA processing event that contributes to protein diversity in mammals. It has been estimated that up to 95% of human multi-exon genes are alternatively spliced[1, 2], adding yet another layer of complexity to our understanding of the human genome. Splicing involves the recognition of exons and introns followed by exons being joined together and introns being removed. Figure 1 illustrates different types of alternative splicing. These include exon skipping (or cassette exon), mutually exclusive exon splicing, alternative 3' or 5' splice site usage, and intron retention. Among these different modes of alternative splicing, exon skipping is most common, accounting for 40% of the entire alternative splicing events[3–5]. This mode of alternative splicing produces different messenger RNAs that translate into protein isoforms with distinct coding sequences.

Splicing occurs through the concerted actions of multi-subunit complexes. Splice sites are recognized by the spliceosome, a catalytic splicing machine comprised of five small nuclear ribonucleoprotiens (snRNPs) and over 100 individual proteins[6–9]. The recognition of exons is determined by consensus sequences at the 5' and 3' exon boundary and a polypyrimidine track located 20 – 40 nucleotides upstream of the 3' splice site (Figure 2). Alternatively spliced exons often contain weak splice sites that diverge from the consensus sequence and are poorly recognized by the spliceosome. The recognition of these weak exons and hence alternative splicing is facilitated by *trans*-acting splicing regulators bound on *cis*-acting pre-mRNA sequences. The *cis*-acting elements can be located in the variable exons and adjacent introns and, depending on their functions, these regulatory elements are categorized as Exonic Splicing Enhancers (ESEs), Exonic Splicing Silencers (ESSs), or Intronic Splicing

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Enhancers and Silencers (ISEs and ISSs). The most well-characterized classes of splicing regulators are the families of SR proteins (serine/arginine-rich proteins) and hnRNPs (heterogeneous nuclear ribonuceoproteins). These two classes of splicing regulators are ubiquitously expressed, although their relative abundance can vary among different tissues[10]. SR proteins are characterized by the presence of at least one Serine/Arginine-rich domain, termed an RS domain at the carboxy terminal. The SR proteins are generally thought to promote alternative splicing by binding to ESE-sequences located in the variable exons and interacting with components of spliceosome, thereby promoting variable exon inclusion[10–13]. hnRNPs, on the other hand, generally bind to ESS- or ISS-elements, resulting in inhibition of variable exon inclusion by interfering the contact between the spliceosome and weak splice sites[10, 14, 15]. However, accumulating evidence has also suggested that both SR proteins and hnRNPs can function reciprocally to repress and activate variable exons, respectively[16–21]. The precise function of these splicing factors can be influenced by the location and sequence context of *cis*-elements that recruit them[22–27]. In many cases, these splicing factors exert a combinatorial effect of positive and negative regulations for the control of alternative splicing[28–33].

In addition to the ubiquitously expressed splicing factors, several tissue-specific RNA-binding proteins have been characterized. Examples include the neuronal-specific protein NOVA, nPTB, the brain- and muscle-specific FOX1 and FOX2, and the epithelial-specific ESRP1 and ESRP2[34–40]. Aided by advanced RNA-sequencing technology, splicing targets of many of these tissue-specific factors have been identified, providing explanations of tissue-specific alternative splicing events [41–46]. These findings suggest that the extent of alternative splicing is tightly controlled in a spatial- and temporal-dependent manner.

Given that the majority of our genes are alternatively spliced, it is inevitable that among them there will be a large number that have well-established roles in cancer. Indeed, tumor suppressor genes, such as p53 and BRCA1, and oncogenes, such as Ras and EGFR, have all been shown to undergo regulated alternative splicing[47–51]. Recently, a number of studies suggested that dysregulation of alternative splicing plays a pathogenic role in cancer[52–55]. Furthermore, alternatively spliced genes may be useful biomarkers for diagnosis and prognosis and their protein products may represent specific therapeutic targets in malignancy[56–59]. Below, we will describe functional impacts of alternatively spliced isoforms in cancer. We will then summarize our understanding on mechanisms by which alternative splicing is regulated in cancer cells.

Functional impacts of splice isoforms in cancer

Cancer is a complex disease that is associated with a variety of genetic and epigenetic aberrations. As summarized by Hanahan and Weinberg[60], in order for cancer to develop, cells acquire eight common traits. These include sustaining proliferative signaling, resistance to cell death, evasion of growth suppressors, acquisition of the ability to invade normal tissues and metastasize, enabling replicative immortality, induction of angiogenesis, reprogramming of energy metabolism, and avoidance of immune destruction. Many of these traits can be directly linked to aberrant gene regulation resulting from dysregulation of alternative splicing during cancer progression (Figure 3).

Sustaining proliferative signaling

A critical feature of tumorigenesis is uncontrolled cell proliferation, including the ability to grow in the absence of external growth stimuli[61–63]. Homeostasis of growth control in cancer is often disrupted by constitutive activation of the Ras/MAPK signaling pathway. Approximately 25% of human tumors contain Ras mutations that cause constitutive activation of the MAPK pathway[64]. Alternatively, aberrant Ras activity may occur by disruption of negative feedback loops that limit Ras or MAP kinase activity or by the establishment of aberrant positive feedback loops that augment Ras signaling. We previously demonstrated that alternative splicing of the CD44 gene serves as a critical mechanism for a feed-forward loop regulation that sustains Ras/MAPK activation[65] (Figure 4). The CD44 gene undergoes extensive alternative splicing and generates CD44 variants (CD44v) that contain one or more of a set of variable exons. When the variable exons are excluded, the CD44 standard isoform (CD44s) is produced. Interestingly, CD44v promotes cell growth by forming co-receptor complexes with receptor tyrosine kinases (RTKs) and activating Ras/MAPK signaling[66]. By contrast, CD44s mediates cell contact inhibition[67]. Upon mitogenic activation, Ras/MAPK pathway promotes alternative splicing of CD44, generating a variable exon 6 containing CD44v6 isoform[65, 68–70]. The newly synthesized CD44v6 isoform augments the action of RTKs, including Met and EGFR, and further promotes Ras/MAPK signaling. These actions constitute a positive feedback circuit that amplifies Ras/MAPK signaling, stimulating cell proliferation by controlling G1-S transition[65]. When utilized by tumor cells, this CD44 alternative splicing-mediated positive feedback loop could cause uncontrolled tumor cell proliferation and oncogenic transformation. The mechanisms by which Ras/MAPK stimulates CD44 alternative splicing have been studied. Ras/MAPK signaling facilitates the activity of splicing factors Sam68 and SRm160, most likely via phosphorylation of these splicing factors, to stimulate CD44 variable exon inclusion.[70, 71] Moreover, using a mouse model of Kras-induced lung adenocarcinoma, we found that CD44v expression is preferentially upregulated in lung adenocarcinomas. Ablation of the CD44 gene attenuates lung tumor formation and prolongs the survival of these mice.[72] These observations suggest that an aberrant set point of alternative splicing could alter intracellular signaling, engage cell proliferation, and influence tumor progression.

The critical role of alternative splicing in promoting sustained proliferation signals in cancer is further demonstrated by Cyclin D1. Cyclin D1 controls cell cycle progression by regulating cyclin-dependent kinase activities. Alternative splicing of Cyclin D1 generates Cyclin D1a and D1b[73]. Cyclin D1b is produced by intron 4 retention, which contains a premature stop codon, resulting in production of a C-terminal truncated version of Cyclin D1. Cyclin D1b is upregulated in several types of cancer. Its constitutive localization in nucleus is likely to contribute to Cyclin D1b's function in allowing anchorage-independent growth in tumor cells[74–76]. Notably, Cyclin D1b production is stimulated by splicing factors Sam68 and SRSF1[77, 78], and its expression correlates with the levels of these splicing factors in clinical prostate cancer specimens[77, 78], emphasizing the importance of Cyclin D1 alternative splicing in cancer development.

Evading growth suppressors

In addition to abnormally activating proliferative signals, cancer cells must also overcome or even eliminate tumor suppressor programs that negatively regulate cell proliferation such as the p53 pathway that plays an indispensible role in maintaining genome stability. The production of p53 mRNA is controlled by alternative promoter usage and alternative splicing. There are three types of alternative splicing events occurring at the C-terminal region of p53, resulting in p53α, β, and γ isoforms. p53β in particular is upregulated during replicative cellular senescence, and when overexpressed, p53β induces senescence[79, 80]. While the mechanism by which p53β promotes senescence is not fully understood, the production of p53β is inhibited by SRSF3 (SRp20), a member of highly conserved family of splicing factors[79]. SRSF3 binds to p53 exon i9 and prevents inclusion of the p53β-unique exon, resulting in inhibition of cellular senescence. Notably, SRSF3 is highly elevated in various cancers[81, 82], suggesting a mechanism by which cancer cells eliminate p53β-mediated senescence through alternative splicing regulation. Furthermore, two p53-related proteins, p63 and p73, are also extensively regulated by alternative splicing[83]. While p63 and p73 mutations are rare, aberrant expression of their splice isoforms was frequently observed in human cancers[84], however, the functional consequences of this are not yet well studied.

Resisting cell death

Apoptosis is a programmed cell death that serves as a natural barrier to cancer cells. However, as tumors progress, cancer cells become insensitive to apoptotic signals, eventually leading to advanced malignancy and chemo-resistance[85, 86]. The apoptotic pathway consists of both upstream regulators and downstream effectors. The death receptor FAS is an upstream regulator that receives extracellular death signals induced by the Fas ligand and processes the signals to the intrinsic apoptotic pathway that carries out the final execution. One of the initial executioners of apoptosis is Caspase-9. Activation of Caspase-9 initiates a cascade of proteolysis leading to consumption and clearance of the cell. Importantly, both Fas and Caspase-9 are regulated by alternative splicing. Splice isoforms of these proteins can have opposing functions to either stimulate or inhibit apoptosis[87, 88].

In the case of the death receptor Fas, inclusion of variable exon 6 results in the production of the membrane-bound Fas that promotes apoptosis, whereas skipping of variable exon 6 produces a soluble form of Fas that inhibits apoptosis[89, 90](Figure 5). The splicing factors TIA-1 (T-cell intracellular antigen 1) and TIAR (TIA-1-related) promote the inclusion of exon 6 by facilitating the U1 snRNP-mediated 5’ splice site recognition and the binding of U2AF to the upstream 3’ splice site, resulting in generation of the pro-apoptotic FAS isoform[91]. In contrast to these activities, several splicing factors were found to promote the skipping of exon 6. PTB (polypyrimiding tract-binding protein) binds to an ESS of exon 6 and promotes exon 6 skipping by inhibiting the binding of U2AF and U2 snRNP to the upstream 3’ splice site[91]. More recently, HuR, hnRNPC1/C2, and RBM5 were shown to inhibit exon 6 inclusion by antagonizing the function of TIAR and preventing the spliceosome assembly, resulting in the production of the anti-apoptotic Fas isoform[92–94]. These findings suggest that splicing factor-regulated of alternative splicing of the Fas gene may directly control the degree of cell apoptosis.

Caspase-9 serves as yet another example by which alternative splicing controls cell apoptosis. Alternative splicing of Caspase-9 produces Caspase-9a and Caspase-9b that differ by inclusion or exclusion of a 4-exon cassette (exons 3,4,5, and 6), respectively[95, 96]. Inclusion of the 4-exon cassette results in production of the pro-apoptotic Caspase-9a, whereas exclusion of this cassette generates the anti-apoptotic Caspase-9b. It has been reported that the ratio of Caspase-9a to Caspase-9b is greatly decreased in non-small cell lung cancer (NSCLC)[97, 98]. Ectopic expression of Caspase-9b caused an increase in anchorage-independent growth and tumorigenic capacity of NSCLC cells, while depletion of Caspase-9b resulted in decreased tumorigenicity. Examination of the mechanisms regulating Caspase-9 alternative splicing led to the identification of the heterogeneous nuclear ribonucleoprotein L (hnRNP L). hnRNP L binds to an purine-rich ESS in exon 3 of Caspase-9 and facilitates skipping of the 4-exon cassette. This favors the production of Caspase-9b in NSCLC cells, thereby contributing to tumorigenesis[98]. By contrast, SRSF1 (SF2/ASF) promotes Caspase-9 exon inclusion, thus generating the Caspase-9a isoform and increasing chemosensitivity of NSCLC cells[97, 99].

Many other apoptotic-associated genes are also subjected to alternative splicing regulation. For example, the Bcl-x gene is alternatively spliced to produce the pro-survival Bcl-x(L) and the pro-apoptotic Bcl-x(s)[86, 100, 101]. Caspase-8 alternative splicing generates the pro-apoptotic factor Caspase-8a and its antagonizer Caspase-8L[102]. In both cases, the pro-survival isoforms, Bcl-x(L) and Caspase-8L are upregulated in cancer[103–106]. Collectively, these data emphasize the prevalence of alternative splicing dysregulation in apoptotic genes in cancer and suggest that the manipulation of alternative splicing favoring an apoptotic direction of these genes could be used as a unique therapeutic strategy to induce cancer-specific cell death.

Enabling replicative immortality

Unlike normal cells, cancer cells are capable of unlimited replication and division that allow them to bypass senescence and cell death, and eventually grow into macroscopic tumors. One of the key features that enable tumor cells to overcome senescence and cell death is telomere maintenance, which is the result of expression of telomerase[107]. The protein component of telomerase, human telomerase reverse transcriptase (hTERT), catalyzes the synthesis of telomere and is found highly expressed in normal stem cells as well as cancerous cells[108–110]. There are seven alternative splice sites within the *hTERT* transcript, which theoretically can generate multiple alternative transcripts[111, 112]. Several splice variants of *hTERT* were demonstrated to regulate telomerase activity and their expression is associated with certain types of cancers[113–116]. For instance, the hTERTα splice isoform contains an in-frame deletion of 36 nucleotides that lies within the reverse transcriptase domain. This isoform acts as a dominant negative inhibitor of endogenous telomerase activity and causes telomere shortening and chromosome end-to-end fusions, resulting in cell death or senescence[116]. The hTERTβ splice isoform, which skips exons 7 and 8, creats a premature stop codon that is subjected to nonsense-mediated decay (NMD), an RNA surveillance pathway by which premature termination codons trigger mRNA degradation[117]. Very recently, *cis*-elements located in introns 6 and 8 were reported to modulate the production of hTERTβ by alternative splicing[118]. Interestingly, utilizing an antisense-

oligonucleotide complementary to the intron 8 *cis*-element increases the production of this non-functional hTERTβ, suggesting a strategy for cancer therapeutics by manipulating hTERT alternative splicing[118].

Inducing angiogenesis

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing ones. The tumor-associated neovasculature, generated by the process of angiogenesis, gives tumors the access to blood circulation and facilitates tumors to grow beyond just a few millimeters in size[119]. In contrast to physiological processes, such as wound healing and female reproductive cycling, in which angiogenesis is only turned on transiently, tumors remain activated angiogenesis, enabling sustained growth of new vessels and neoplastic tissues. The best studied and probably most important growth factor that promotes angiogenesis is the vascular endothelial growth factor-A (VEGF or VEGF-A). Accumulating evidence has shown that VEGF is regulated by alternative splicing[120, 121]. The VEGF gene is comprised of eight exons. Exon 8 contains a proximal 3' splice site and a distal 3' splice site[122](Figure 6). When the proximal splice site is used, cells generate VEGF mRNAs that encode pro-angiogenesis VEGF proteins. By contrast, the usage of the distal 3' splice site of exon 8 results in the production of the VEGFb isoforms that exhibit anti-angiogenic activities[123]. For example, VEGF165 and VEGF165b are two isoforms that differ in the C-terminal region as a result of exon 8 alternative splicing. Although both isoforms bind to VEGFR, binding of VEGF165b to VEGFR induces differential phosphorylation and intracellular trafficking as compared to VEGF165, resulting in angiogenesis blockage[123–125]. Additionally, exons 6 and 7 can be alternatively spliced, increasing the numbers of VEGF isoforms, and thus, the functional diversity of VEGF[120]. Mechanistic studies on the alternative splicing of VEGF demonstrated that splicing regulators SRSF1 and SRSF5 (SRp40) promote the usage of VEGF exon 8 proximal 3' splice site, thus favoring the production of VEGF[126]. Insulin-like growth factor (IGF-1) promotes the activity of SRSF1 by activating PKC signaling, which stimulates SRPK1, SR Protein-Specific Kinase 1, that phosphorylates SRSF1[127]. By contrast, SRSF6 (SRp55) and SRSF2 (SC35) facilitate the selection of the distal 3' splice site, resulting in VEGFb production[126]. These results suggest that signaling-mediated VEGF alternative splicing controls the balance of pro-angiogenic VEGF and anti-angiogenic VEGFb. This view was further supported by a recent finding showing that mutations in WT1, the Wilm's tumor suppressor gene, suppress the production of VEGF165b, causing abnormal activity of angiogenesis and Wilms' tumors[128]. WT1 represses the transcription of SRPK1 by directly binding to its promoter. SRPK1 phosphorylates SRSF1 that enhances the ability of SRSF1 to promote the production of VEGF. Thus, in WT1 mutant cells SRPK1 is highly expressed, resulting in hyperphosphorylation of SRSF1, which in turn favors the production of VEGF and renders the WT1 mutant cells proangiogenic[128].

Currently, the available anti-VEGF cancer therapeutics, such as the anti-VEGF antibody Bevacizumab, does not distinguish between different spliced isoforms of VEGF[129]. This poses a dilemma in clinics as VEGF165b competes with VEGF165 for binding to Bevacizumab, resulting in drug resistance and side effects[129]. Therefore, understanding the

mechanisms to manipulate the production of VEGFb may lead to a novel therapeutic strategy for reduction of tumor angiogenesis.

Activating invasion and metastasis

As tumors progress to higher pathological grades of malignancy, cancer cells typically begin to develop alterations in cell shapes and the ability to attach to other cells and to extracellular matrix (ECM). These series of discrete changes prepare cancer cells for local invasion and distal metastasis[130]. Recent studies have shown that a developmental process epithelial-mesenchymal transition (EMT) is hijacked by cancer cells to disseminate to distant organs[131–134]. When EMT occurs, the tightly packed epithelial cells become loosely connected and transit to spindle-shaped mesenchymal cells that show high degree of migratory ability. It was recently shown that alternative splicing represents a novel mechanism that causally controls EMT[135]. Work from our group has demonstrated that alternative splicing of the CD44 gene is dynamically regulated during EMT[135](Figure 7). In epithelial cells, the variable exon-containing CD44v is predominant. When cells undergo EMT, there is a gradual loss of CD44v and gain of the short CD44s isoform, resulting in a nearly complete switch in expression to CD44s in mesenchymal cells. Importantly, this CD44 isoform switching is required for cells to undergo EMT and for the formation of breast tumors that display EMT characteristics in mice. Analysis of breast cancer patient specimens showed that CD44s is up-regulated in high-grade breast tumor tissues and positively correlates with the mesenchymal status of these tumors[135]. This study demonstrated that cells utilize alternative splicing as a means to regulate EMT by producing a specific CD44 isoform that acts as a key mediator of EMT. Studies on the role of CD44s revealed that CD44s potentiates Akt signaling and promotes cell survival[153], an activity that differs from the proliferative advantage mediated by CD44v shown in Figure 4. These data reflect the plasticity of alternative splicing that could allow tumor cells to generate distinct splice isoforms in response to the need for cell proliferation or survival at different stages of tumor progression.

The insight into the regulation of alternative splicing during EMT has been advanced by the identification of the Epithelial Splicing Regulatory Protein 1 and 2 (ESRP1/ESRP2)[40]. ESRP1/2, reflected by their names, are highly expressed in epithelial cells. We and others have shown that downregulation of ESRP1 is necessary in order for cells to transit to a mesenchymal phenotype[45, 135]. When ESRP1 is ectopically expressed, cells lose their ability to undergo EMT in response to EMT stimuli, such as Twist, Snail, or TGFβ. Strikingly, ESRP1 prevents EMT by inhibiting the production of the CD44s splice isoform[135, 136], again reinforcing the importance of splice isoform specificity in modulating EMT. Moreover, aided by large-scale RNA sequencing analysis, a subset of alternative splicing events has been identified to associate with the EMT phenotype[44, 45, 137], and a functional role of these specific splice isoforms in EMT and tumor metastasis awaits further investigation.

Deregulating cellular energies

Warburg first observed an unusual characteristic of cancer cell energy metabolism in 1930: most cancer cells predominantly produce energy by a high rate of glycolysis even in the

presence of oxygen, leading to a state that has been termed 'aerobic glycolysis'[138]. It was later shown that less efficient energy-producing metabolism of aerobic glycolysis in tumor cells favors various biosynthetic pathways, which in turn facilitates biosynthesis of macromolecules for rapid proliferation[139, 140]. This metabolic switch exhibited in tumor cells is partially governed by alternative splicing of pyruvate kinase (PK), an enzyme that catalyzes the conversion from phosphoenolpyruvate (PEP) to pyruvate[141]. PKM1 and PKM2 are two isozymes of PK in mammals, which are generated by alternative splicing of the PKM gene (Figure 8). PKM1 and PKM2 differ in a 56-amino acid stretch by including mutually exclusive exons, exon 9 for PKM1 and exon 10 for PKM2[142–144]. The PKM1 isoform promotes oxidative phosphorylation and is expressed in most adult tissues, especially in brain and muscle that require a large amount of energy production through the TCA cycle. The PKM2 isoform, on the other hand, is expressed in embryonic cells and tumor cells and promotes aerobic glycolysis and lactate production allowing for high rate of biosynthesis[145–147]. PKM2 converts PEP to pyruvate less efficiently than PKM1[148]. As a result, tumor cells that have high levels of PKM2 accumulate glycolytic metabolites from anabolic metabolism[148]. PKM2 level is elevated in glioblastomas[142]. When PKM2 was replaced by PKM1 in lung tumor cells, there was a significant reduction in lactate production and increase in oxygen consumption, which was correlated with impaired tumor formation in mouse xenografts[141].

It was later shown that a group of hnRNP proteins, hnRNPA1, hnRNPA2, and PTB, control PKM alternative splicing[144]. These hnRNP proteins directly bind to sequences flanking PKM exon 9 and repress exon 9 inclusion, resulting in exon 10 inclusion and PKM2 production[142, 144]. Knockdown of these hnRNP proteins resulted in an increase in PKM1, concomitant with a decrease in lactate production. Interestingly, the oncogenic transcription factor cMyc upregulates the expression of these hnRNPs, ensuring high production of PKM2 in tumor cells[142, 144]. These findings provide convincing evidence illustrating the importance of alternative splicing in regulating tumor metabolism.

Avoiding immune destruction

Immune system could be a double-edged sword in tumor initiation and progression[60]. On one hand, immune escape is a critical gateway for malignancy. The immune surveillance theory proposes that cells and tissues are constantly monitored by immune system to eliminate cancer cells[149]. On the other hand, a compelling body of evidence suggests that tumor-associated inflammation caused by infiltration of immune cells, especially those from innate immune system, enhances tumorigenesis. These infiltrating immune cells secret growth factors and cytokines to foster incipient neoplasia[150–152]. In both cases, our host bodies often initiate activation of T and B lymphocytes in response to the growth of cancer cells.

It is interesting to note that one of the mechanisms for T cell activation is by regulating alternative splicing of the *CD45* gene. CD45 is a transmembrane tyrosine phosphatase that mediates T Cell receptor signaling[153, 154]. Dimerization of CD45 leads to inhibition of its phosphatase activity, possibly due to steric hindrance of the catalytic site[155]. *CD45* is expressed in all nucleated hematopoietic cells and can be alternatively spliced by inclusion

of variable exons 4, 5, and 6, also called variable exons A, B, and C[156, 157]. Naïve T cells express high levels of CD45 isoforms that include at least one of the variable exons 4–6, such as CD45RA, whereas activated T cells express predominately the smaller CD45RO isoform, which excludes all of the variable exons. The CD45RO isoform shows a high tendency of dimerization and dampens signaling for T cell activation in response to extracellular stimuli. Thus, an increase in CD45RO production will eventually lead to a termination of T cell response following T cell activation[158]. Evidence from Lynch and colleagues showed that hnRNP L represses exon 4 inclusion by binding to an ESS element in this exon and subsequent recruitment of hnRNPA1[159, 160]. hnRNPA1 traps U1 snRNP at the 5' splicing site and prevents U6 snRNA from binding to the 5' splicing site, thus blocking proper spliceosome assembly and subsequent splicing events[159]. CD45 alternative splicing is also regulated by signaling cues. The PTB-associated splicing factor (PSF) binds to exon 4 and represses its inclusion[32]. In resting T cells, PSF is phosphorylated by GSK3. This allows for a complex formation between PSF and TRAP150, sequestering PSF from binding to exon 4 and thus leading to exon 4 inclusion[161]. Upon T cell activation, GSK3 activity is reduced, thus PSF is no longer phosphorylated, releasing PSF from TRAP150 and allowing PSF to repress exon 4 inclusion[161]. The net result of this is to stimulate the production of the CD45RO isoform. Apart from regulating protein phosphorylation, signaling-stimulated T cell activation also elicits an upregulation of hnRNP LL expression, a homolog of hnRNP L, that plays a critical role in mediating signal-induced increase of *CD45* exon skipping in both cell-culture and mice [162–164]. Furthermore, a mechanism of epigenetic regulation of CD45 splicing is emerging. A recent study showed that DNA methylation directly inhibits the binding of the CTCF DNA-binding protein to the CD45 variable exon 5, which in turn impairs CTCF-mediated local RNA polymerase II pausing, resulting in inhibition of CD45 variable exon inclusion[165]. Hence, these data demonstrate that alternative splicing of CD45 is tightly regulated at multiple levels in order to precisely control T cell activation.

Chronic inflammation and infiltration of T lymphocytes are common in the tumor microenvironment. One of the main questions to investigate is whether these T cells inhibit or promote cancer progression. It was recently reported that, in a mouse model of pancreatic cancer, inflammation promotes EMT and tumor cell dissemination to distant organs. Intriguingly, suppression of antigen-specific T cell response is required for cancer-associated inflammation and tumor formation in mice[166, 167]. It will be particularly interesting to investigate the role of CD45 alternative splicing in regulating T cell response during cancer progression.

Collectively, studies described in this section demonstrate that aberrant alternative splicing is observed in each of the hallmarks of cancer and their splice isoforms play critical roles in promoting tumorigenesis. Encouraged by these observations, increasing considerations have been shown for the use of a cancer-specific splice isoform as a prognostic marker for detection and diagnosis of certain types of cancer [59, 168–170] . An example of such is the p53 inhibitor HDMX. The ratio between HDMX short and full-length isoforms was demonstrated as a more effective biomarker than the status of p53 for poor prognosis of sarcomas in patients[168]. Efforts have also been made for targeting splice isoforms or

redirecting splicing events including the aforementioned VEGF alternative splicing to VEGFb, as a therapeutic strategy[56, 57, 121, 129, 171–173]. Research in these areas will undoubtedly advance our knowledge in the diagnosis and treatment of cancer.

Of note, the above-described alternative splicing events are just exemplars from an incomplete list of genes for which alternatively spliced products promote cancer-associated pathways. With advances in large-scale RNA sequencing and isoform-specific gain-and-loss of functional approaches, it is tempting to speculate that an increasing number of alternatively spliced events of tumor suppressor genes and oncogenes will be identified to serve as an essential mechanism for the regulation of cancer hallmarks.

Regulatory mechanisms of aberrant alternative splicing in cancer cells

As described above, dysregulated alternative splicing in cancer cells produces cancer-promoting splice isoforms. We also showed examples on how splicing factors impact on the choice of splice sites resulting in production of different protein isoforms that elicit distinct biological consequences. In this section, we focus on discussing various layers of regulation that lead to aberrant alternative splicing in cancer cells. For the mechanisms that affect splice site recognition and spliceosome assembly in alternative splicing, the reader is referred to references cited in the Introduction section of this review.

Regulation of alternative splicing through mutations in cis-elements

Mutations in *cis*-acting splicing elements can disrupt or create splicing regulatory elements, such as splicing enhancers and silencers, causing aberrant alternative splicing. Additionally, genomic mutations can generate a cryptic splice site along with disruption of a canonical one. These mutations, which lead to production of aberrant splice isoforms, could have a profound impact on tumor development and progression.

The Kruppel-like factor 6 (KLF6) gene encodes a Zn-finger transcription factor and functions as a tumor suppressor. Interestingly, a germline single nucleotide (G/A) polymorphism in intron 1 of the KLF6 gene is associated with high risk of prostate cancer[174]. This point mutation generates a binding site for the SR protein SRSF5 (SRp40), resulting in preferential usage of cryptic splicing sites in KLF6 exon 2[174]. Consequently, the produced splice variants, KLF-SVs, antagonize wild-type KLF6, promoting prostate cancer progression[174, 175]. Moreover, ectopic expression of the KLF6-SV1 splice isoform promotes an EMT phenotype and breast cancer metastasis, and its expression correlates with poor survival of breast cancer patients[176].

The germline mutation of BRCA1 can also cause aberrant alternative splicing in cancer. The tumor suppressor gene BRCA1 is involved in DNA damage repair by forming a BRCA1-associated genome surveillance complex (BASC) through protein interactions[177]. Individuals with BRCA1 mutations show high risk of ovarian and breast cancers. An inherited G-to-T nonsense point mutation in BRCA1 exon 18 may disrupt an SRSF1-binding site necessary for the inclusion of exon 18[178–180]. At the same time, this mutation creates a binding site for splicing inhibitors hnRNPA1/A2 and DAZAP1, resulting in exon skipping[181]. Exon 18 exclusion eliminates the first BRCT (BRCA1 C-terminus) domain,

through which BRCA1 interacts with various DNA damage proteins[182, 183], thus generating a non-functional BRCA1 mutant protein. These observations illustrate that *cis*-element mutations can cause aberrant alternative splicing that affects the function of coding genes.

Regulation of alternative splicing through trans-acting factors

In addition to *cis*-acting element mutations, *trans*-acting regulators, i.e. splicing factors, can also be aberrantly regulated at multiple levels, including genomic mutation, transcriptional regulation, post-transcriptional regulation, and post-translational regulation.

Mutations in splicing factors—Exome sequencing has demonstrated great power in uncovering somatic mutations that are associated with diseases. Recent identifications of mutations in the SF3B1 and U2AF35 genes in hematopoietic malignancies and other solid tumors suggested a novel means of RNA splicing deregulation that could be a driver for the development of various types of tumors[184–189]. Especially in myelodysplastic syndromes and myelodysplasia, as many as 45–85% of patients have mutations in the RNA splicing machinery[184]. These mutations occur in a mutually exclusive manner, and the mutated genes are involved in the 3'-splice site recognition during splicing. Importantly, introducing the U2AF35 mutant found in patients into cancer cells resulted in enrichment in unspliced introns and increased expression of members of the NMD pathway[184]. It was suggested that these spliceosomal pathway mutations compete with normal splicing machinery, leading to pathogenesis. It will be interesting to investigate the functional connections between these mutations and disease phenotypes.

Transcriptional regulation—Splicing factors can also be transcriptionally regulated. As noted earlier, ESRP1 promotes an epithelial cellular state[40, 45, 135]. In response to EMT stimuli, ESRP1 level is markedly decreased, allowing cells to transit to a mesenchymal state. Interestingly, the transcription repressors and EMT inducers, Snail and Zeb1/2, can directly bind at the promoters of ESRP1 or ESRP1's paralogous ESRP2 to suppress their expression[136, 190]. Given that ESRP1 inhibits EMT via preventing CD44 variable exon skipping[136], these results illustrate a mechanism by which a transcription factor promotes EMT through transcriptional repression of a splicing factor that controls alternative splicing of key genes whose splice isoform is critical for EMT.

Post-transcriptional regulation—Splicing regulators are subject to many types of post-transcriptional regulations. One of such regulation is NMD. The splicing regulators SRSF2 and PTB autoregulate their expression by NMD[191, 192]. It was later found that highly conserved stop codon-containing exons frequently exist in genes that encode splicing regulators[193, 194]. Interestingly, genes encoding splicing activators such as SR proteins undergo splicing activation-triggered NMD. By contrast, splicing inhibitors including hnRNPs are regulated by NMD through a splicing repression event. Such observations suggest that cells utilize NMD regulation to control the homeostasis of splicing regulators[194, 195].

Studies of the SRSF1 splicing factor revealed that SRSF1 is tightly controlled by autoregulation[195]. SRSF1 produces several splice isoforms, including the full length

functional SRSF1 and other isoforms that are either retained in the nucleus or degraded by NMD. More interestingly, SRSF1 autoregulation also occurs at the translational level. SRSF1 inhibits its translation by reducing the polysome association of its own mRNA, possibly mediated by micro-RNAs[195]. Given the oncogenic role that SRSF1 plays[196–199], it is conceivable to speculate that cancer cells must have disrupted SRSF1 autoregulation to account for its overexpression observed in many types of cancers[198].

Post-translational regulation—Post-translational regulation of splicing factors, such as protein phosphorylation, acts as a critical mechanism for controlling splicing factor activity, and thus alternative splicing. Splicing factor phosphorylation has been shown to control their binding affinity to RNA *cis*-elements, the interaction with other protein components, and their sublocalization[200–202]. Splicing factor phosphorylation is often stimulated by extracellular cues via signaling cascades, bridging extracellular environmental signaling to alternative splicing regulation[203]. An excellent example was illustrated by work from Fu and colleagues[204]. They recently demonstrated that SR-protein specific kinases, SRPKs, mediate SR protein phosphorylation in response to EGF stimulation[204]. By systemically dissecting EGF-induced global changes in alternative splicing, they found that the Akt signaling pathway plays a major role in activating SRPKs through inducing SRPK auto-phosphorylation, resulting in switched binding of SRPK from HSP70- to HSP90-containing complexes. Hsp90/SRPK interaction allows SRPK translocation to the nucleus, thus enhancing SR protein phosphorylation and SR protein-regulated alternative splicing[204]. In addition to the EGF-Akt-SRPK-SR axis, previous work also suggested other signaling cascades that impinge on alternative splicing. As described earlier, EGF- and HGF-stimulated Ras/MAPK signaling promotes CD44 alternative splicing through phosphorylation of splicing regulators[65, 69–71]. Furthermore, Akt promotes Fibronectin EDA inclusion by phosphorylating SRSF1[205, 206]. These findings revealed that signaling-controlled alternative splicing is mediated by phosphorylation of splicing factors.

In addition to phosphorylation, splicing factors are subjected to other types of protein modification, such as protein methylation and SUMOylation. SRSF1 methylation was shown to be essential for its localization in nucleus[207]. Mutations that block SRSF1 methylation lead to its accumulation in the cytoplasm, preventing its function as a splicing regulator. Furthermore, recent large-scale proteomic studies identified several hnRNPs to be modified by SUMOylation[208, 209], emphasizing the potential importance of post-translational modification of splicing factors in controlling RNA-processing events.

Other emerging regulatory mechanisms of alternative splicing

Splicing factors can also be regulated by microRNAs. MicroRNAs are a large class of noncoding RNAs present in diverse organisms. MicroRNAs target the 3’UTR of mRNAs that leads to inhibition of translation and ultimate degradation of the mRNA[210–213]. Several splicing factors have been reported to be targets of microRNAs. For example, miR-133 downregulates nPTB level during muscle differentiation[214], and miR-1 induces tumor cell apoptosis by direct inhibition of SRSF9 (SRp30c)[215].

Accumulating evidence has also suggested that transcription and splicing are intimately coupled[216–221]. Alternative exon usage is modulated by different transcriptional rates of RNA polymerase II and the status of chromatin structure and modification (for recent comprehensive reviews, see references 222–225). For example, fast-moving RNA polymerase II favors the deposit of spliceosome to the strong 3' splice site located downstream of a weak splice site, resulting in exon skipping[218, 220, 221]. On the contrary, slow-paced RNA polymerase II allows for the recruitment of spliceosome and splicing regulators to enhance the upstream weak exon splice site recognition, facilitating exon inclusion[221, 226]. Thus, malfunction of transcriptional regulation, resulting from epigenetic alterations in cancer cells, may lead to aberrant alternative splicing, resulting in production of cancer-promoting splice isoforms.

Conclusions

This review article summarizes provocative evidence demonstrating that dysregulation of alternative splicing influences all aspects of cancer hallmarks. The production of splice isoforms that exert distinct and sometimes opposing functions also suggests that the function of a gene is not a fixed property of a cell, but is dynamically regulated by alternative splicing in a spatial and temporal manner. The complexity provided by alternative splicing will allow cells to rapidly convert to or adapt a specific cellular phenotype in response to environmental cues.

Notably, current knowledge on the regulation and function of alternative splicing in cancer is only a tip of the iceberg. Especially, our understanding on the functional consequences and mechanisms of alternatively spliced isoforms in cancer progression is very limited from studies of a handful of genes. Thus, the field of alternative splicing in cancer is wide open for rigorous investigation. In addition to using a specific gene model to investigate alternative splicing regulation and consequences in cancer, a systematic approach aided by high-throughput sequencing is becoming a powerful tool for understanding alternative splicing in cancer at a global setting. Considering the high frequency of alternative splicing in humans, it is anticipated that a large number of cancer-associated alternative splicing events and new regulatory mechanisms will be identified.

In summary, alternative splicing is a prevalent and tightly regulated process that occurs in nearly all human genes. Given the pivotal role of alternative splicing in modulating all aspects of cancer processes, alternative RNA splicing adds a new mode of fundamental mechanism in gene regulation that controls cancer phenotypes.

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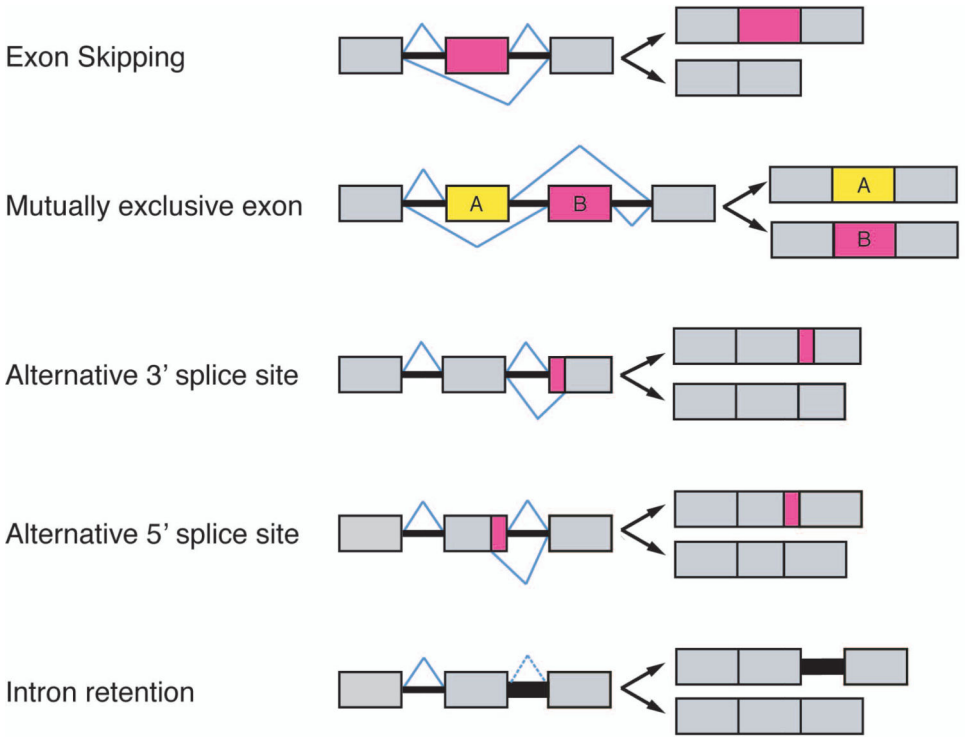

Figure 1. Schematics of different modes of alternative splicing

Exons are denoted as boxes and introns are depicted as thin lines in black.

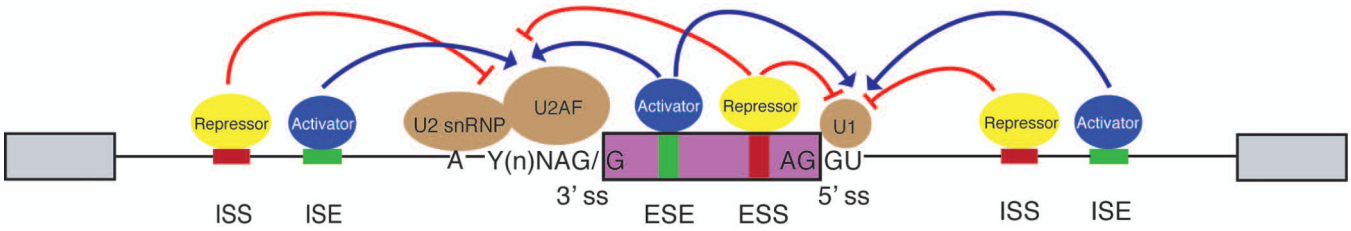

Figure 2. A schematic representation of alternative splicing regulation

Three core splicing sequences are recognized by components of the spliceosomes: U1 binds to a 5' splice site (5'ss) that contains a GU dinucleotide. U2AF binds to a 3' splice site (3'ss) that contains an AG dinucleotide. U2 snRNP binds to a branch site, where adenosine is indicated. ESE and ESS denote exonic splicing enhancer and silencer, respectively. ISE and ISS represent intronic splicing enhancer and silencer. Splicing activators and repressors bind to these *cis*-acting elements for alternative splicing regulation.

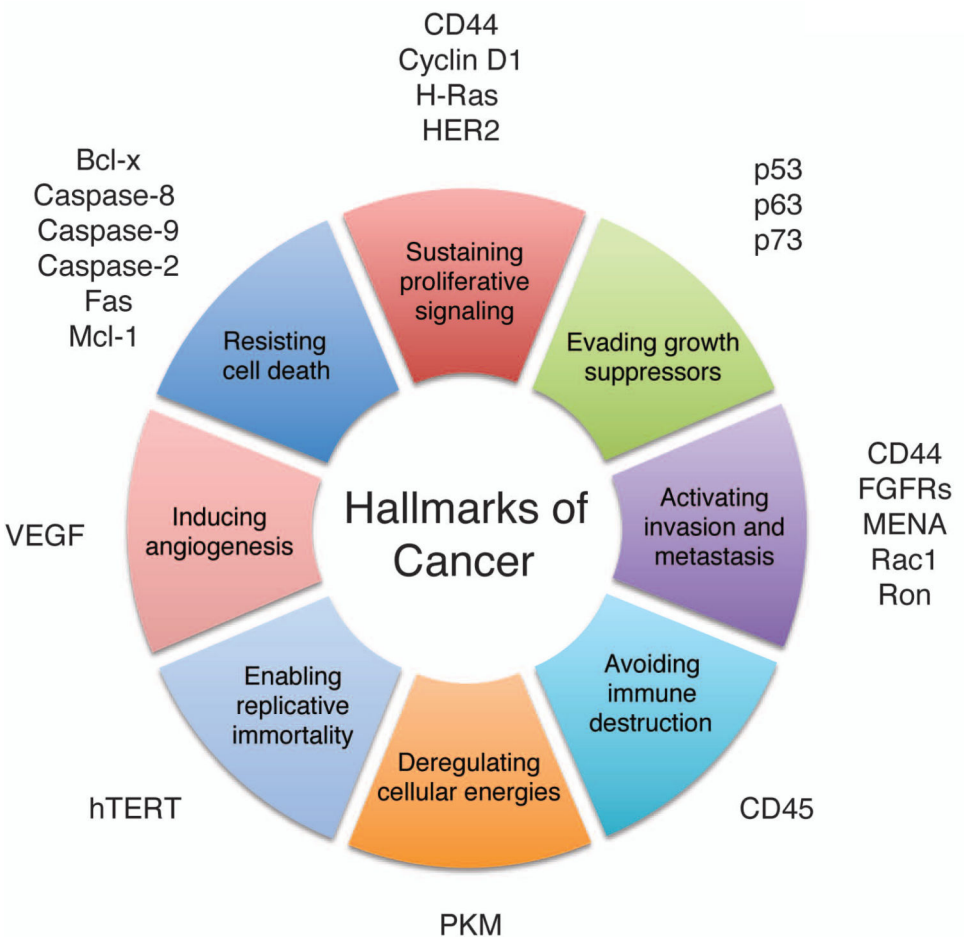

Figure 3. Alternative splicing occurs in every category of cancer hallmarks

Examples of genes whose alternative splicing controls a cancer phenotype are shown next to their corresponding hallmarks.

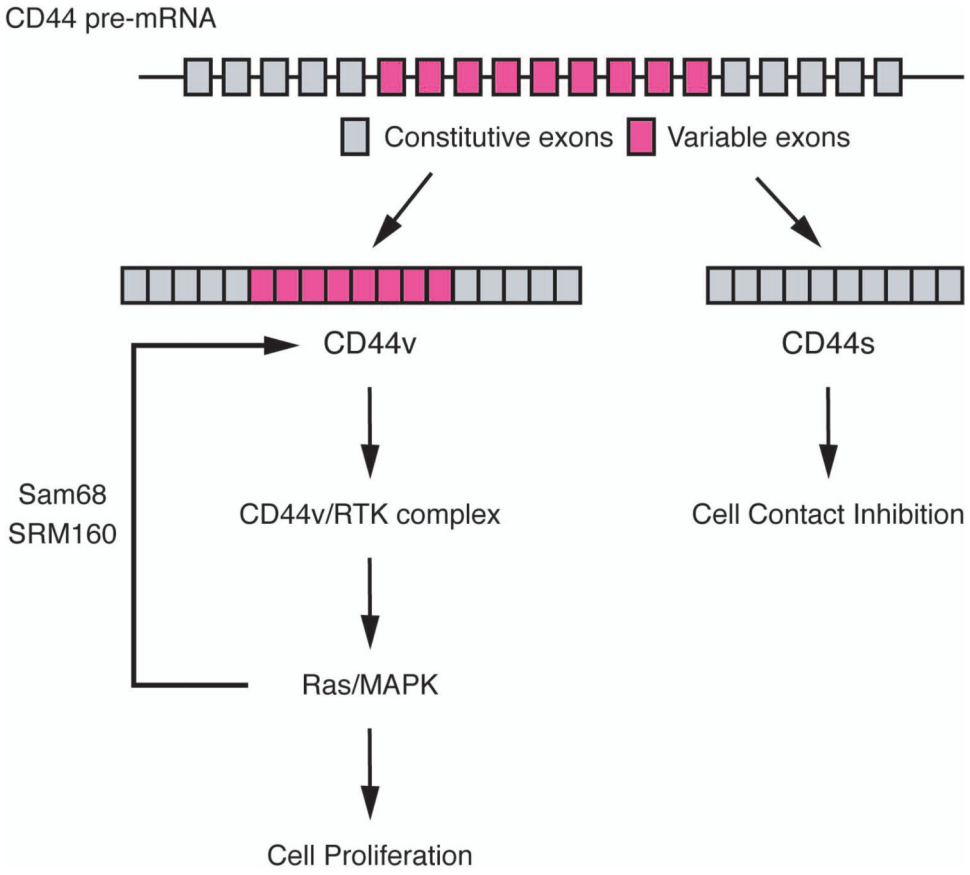

Figure 4. A positive feedback loop couples CD44 alternative splicing and Ras/MAPK activation

A schematic of the CD44 pre-mRNA is shown with constitutive and variable exons depicted as gray and magenta boxes, respectively. Introns are shown as thin lines. Inclusion of one or more of the variable exons produces CD44v. A human CD44 variant containing variable exons v3 to v10 is frequently detected in CD44v–expressing cells and is shown to represent CD44v. Variable exon v6-containing CD44v activates Ras/MAPK signaling by forming a co-receptor complex with RTK. Activation of the Ras/MAPK signaling cascade in turn stimulates the production of CD44v isoforms through splicing factors, Sam68 and SRM160. These actions form a positive feedback loop that sustains Ras/MAPK signaling, critical for tumor cell proliferation. By contrast, the CD44s isoform that is devoid of all variable exons promotes cell contact inhibition.

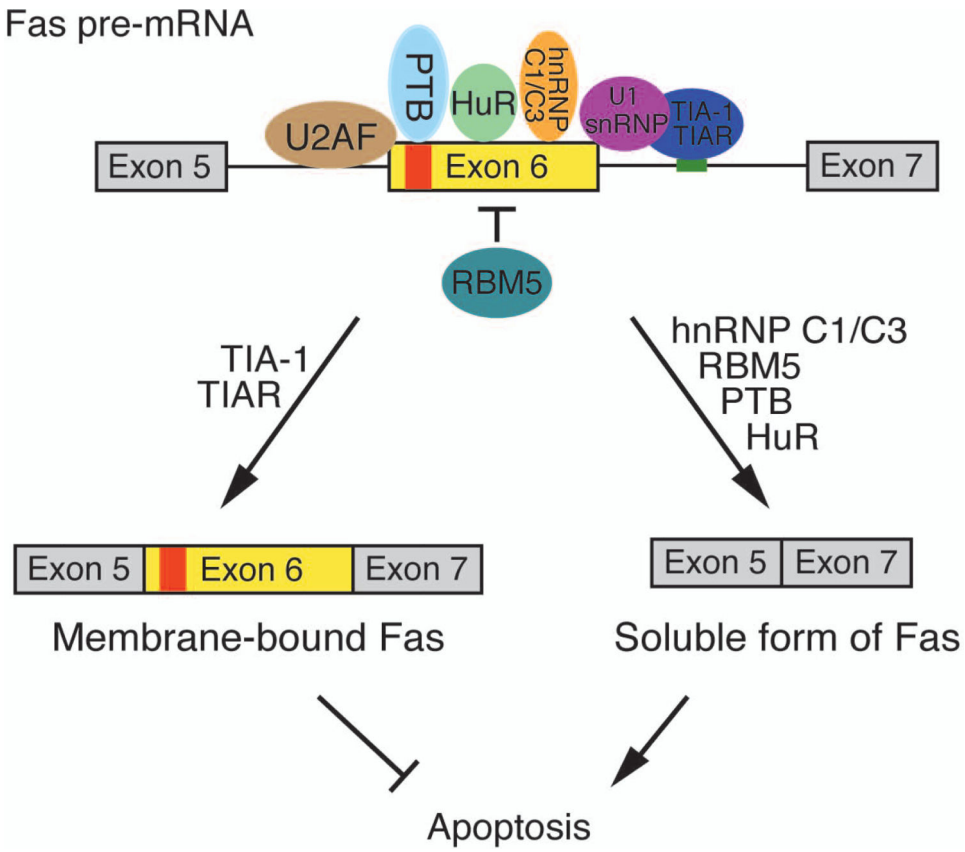

Figure 5. Alternative splicing of the death receptor FAS controls the degree of apoptosis

Usage of the FAS variable exon 6, shown in yellow, is controlled by splicing factors as shown. Inclusion of exon 6 results in a membrane-bound form of FAS that promotes apoptosis. This inclusion event is mediated by TIA-1 and TIAR binding at an ESE motif downstream of exon 6 that facilitates U1 snRNP recognition to the 5' splice site and U2AF binding to the upstream 3' splice site. In contrast to the membrane-bound form, exon 6 exclusion produces a soluble form of FAS that inhibits apoptosis. PTB, RBM5, hnRNPC1/C3, and HuR prevent exon 6 inclusion through binding to exon 6 *cis*-elements or preventing the spliceosome assembly. An ESS of exon 6 that recruits PTB is shown in red.

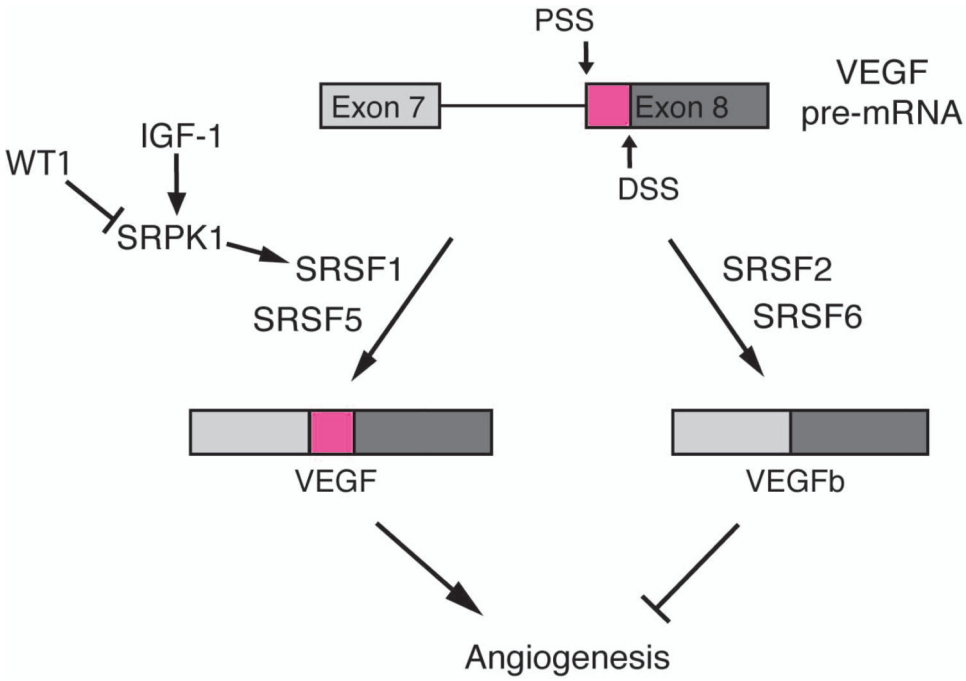

Figure 6. VEGF alternative splicing regulates angiogenesis in tumor cells

VEGF exon 8 contains a proximal 3' splicing site (PSS) and a distal 3' splicing site (DSS). Splicing factors SRSF1 and SRSF5 promote the usage of 3' PSS, generating wild-type and functional VEGF. By contrast, SRSF2 and SRSF6 facilitate the selection of 3' DSS, resulting in production of the VEGFb isoform that is anti-angiogenic. The activity of SRSF1 is regulated by SRPK1 through phosphorylation. IGF-1 promotes SRPK1-mediated SRSF1 activity and WT1 inhibits the transcription of SRPK1, thus usage of 3' PSS and the production of VEGF.

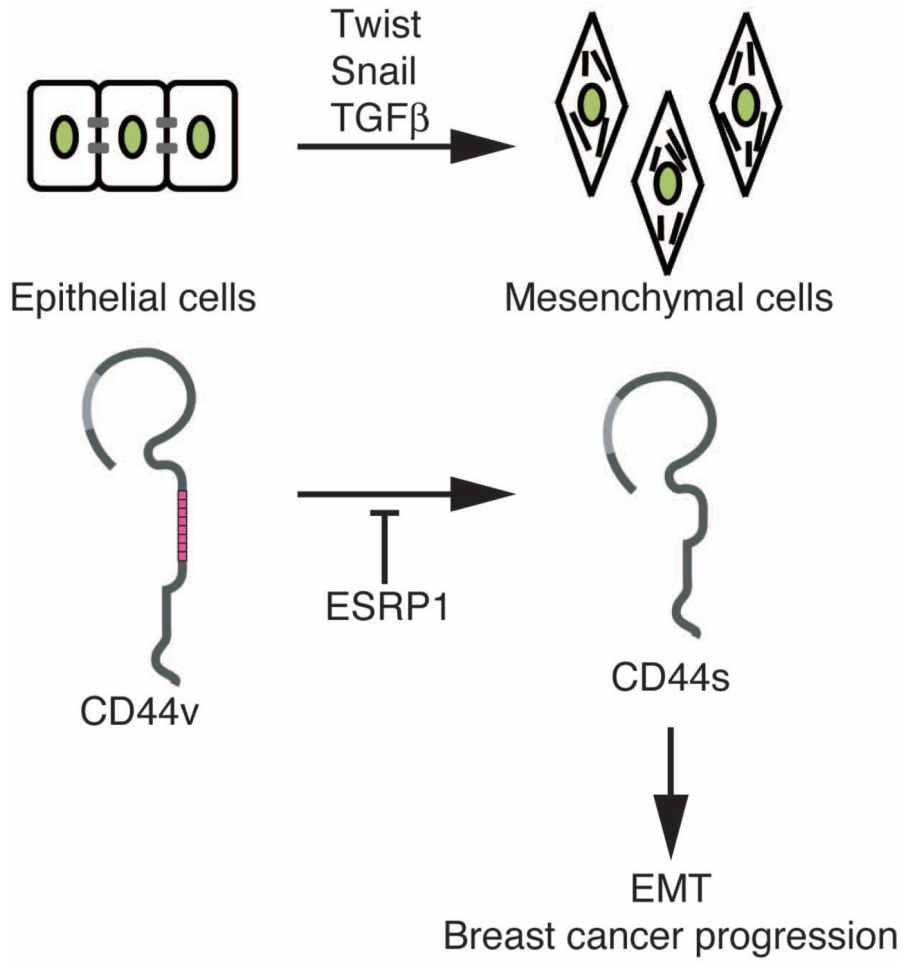

Figure 7. CD44 splice isoform switching is critical for EMT and breast cancer progression

Top panel shows a schematic of EMT that involves the change from a cobble-stone-like epithelial phenotype to a spindle-shaped morphology of mesenchymal cells. EMT can be induced by transcription factors Twist, Snail, or the cytokine TGFβ. Middle panel illustrates that CD44 isoform switching from CD44v in epithelial cells to CD44s in mesenchymal cells occurs during EMT. The switched expression to CD44s, which is inhibited by ESRP1, is critical for cells to undergo EMT and form a more aggressive breast cancer phenotype. The CD44 variable exon-coding region is shown in magenta.

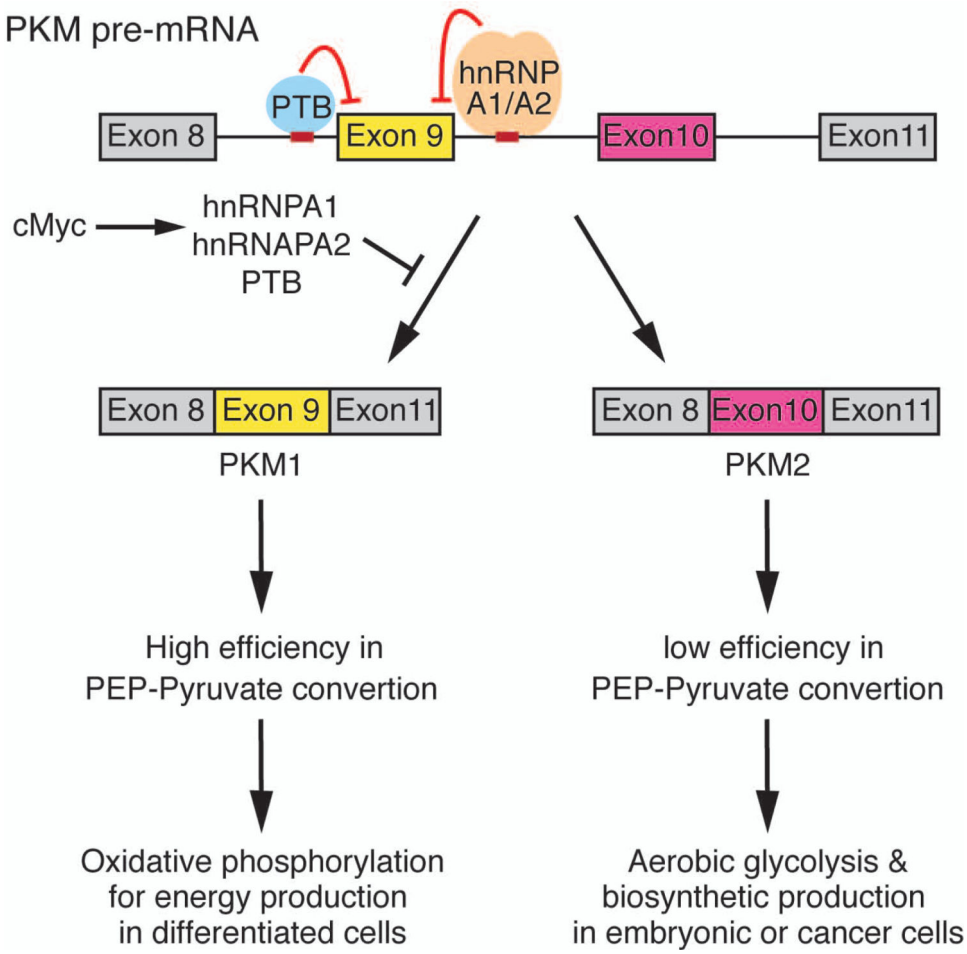

Figure 8. The PKM2 splice isoform is aberrantly upregulated in tumor cells for a metabolic switch favoring biosynthesis

Mutually exclusive exon splicing of exon 9 (yellow) and exon 10 (magenta) of the PKM pre-mRNA gives rise to two protein isoforms PKM1 and PKM2, respectively. PKM1 is highly efficient in converting PEP to pyruvate, allowing cells for maximal energy production through the TCA cycle. PKM2, on the other hand, has low efficiency in PEP-Pyruvate conversion. Cells that express high levels of PKM2 undergo aerobic glycolysis, fulfilling the need of biosynthesis in embryonic or tumor cells. Splicing factors PTB and hnRNP A1/A2 repress exon 9 inclusion by binding to ISS-elements (red) that flank exon 9, thus promoting the production of PKM2. The cMyc oncogene upregulates the expression of hnRNP A1/A2 in tumor cells.