Brosseau *Applied Cancer Research* (2018) 38:18
https://doi.org/10.1186/s41241-018-0068-6

Applied Cancer Research

REVIEW Open Access

Splicing isoform-specific functional genomic in cancer cells

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Abstract

Alternative splicing is a regulated process whereby one gene can generate multiple mRNA isoforms susceptible to be translated into protein isoforms of various functions. Several publications report the aberrant expression of splicing isoforms in cancer cells and tissues. However, in most cases, their function remains to be established. In this review article, I will discuss the molecular tool available to perform isoform-specific functional genomics, the methodologies to quantify their effectiveness and the resulting isoform-specific phenotype in human cancer cell lines.

Introduction to the splicing molecular toolbox

Generalities about alternative splicing

Alternative splicing is a regulated process whereby one gene can generate multiple mRNA isoforms susceptible to be translated into protein isoforms of various functions. In human, most multi-exon genes undergo alternative splicing [1]. As illustrated in Fig. 1, two 5'ss (5′ splice site) could compete for a single 3'ss (3′ splice site) (Fig. 1a) or inversely two 3'ss for a single 5'ss (Fig. 1b). These examples are alternative 5′ (alt5') or 3′ (alt3') splicing events (ASEs), respectively. Complete exon exclusion (cassette exon) (Fig. 1c) is the most frequent ASE type in human. There are also cases where two or more consecutive exons are excluded as a whole (multiple exon skipping, Fig. 1d) or mutually exclusive (Fig. 1e). Alternative splicing can also be coupled to other RNA regulatory mechanism such as polyadenylation [2, 3] (Fig. 1f) or non-sense mediated decay (NMD) [4] (Fig. 1g). The later happen when a stop codon is introduced too early in the mRNA sequence relative to the last exon. It is predicted that up to one third of splicing isoforms is linked to the NMD mechanism [5, 6]. Of note, even if the ASE is located outside the coding region, the untranslated region still contains regulatory sequences [7]. Alternative initiation of transcription does produce mRNA isoforms but is not due to alternative splicing (Fig. 1h). Thus, the average gene is transcribed into several mRNA isoforms and the majority of them are susceptible to be translated into protein isoforms with widely different functions. Although there is no consensus nomenclature on splicing isoforms, this review will use the convention where, for a particular ASE, the resulting isoform without the alternative sequence is named "short isoform" and the isoform with the alternative sequence is named the "long isoform" [8–10].Whereas the consensus sequence defining the 5'ss, 3'ss and branch point define constitutive splicing, less well defined exonic and intronic regulatory sequence *in cis*, as well as trans-acting splicing factors, dictate the final outcome of an ASE [11].

Several publications report the aberrant expression of splicing isoforms in cancer cells and tissues [12, 13]. However, in most cases, their function remains to be established [14]. At least 15% of DNA mutations disrupt splice site and hence interfere with splicing regulation and gene expression [15]. It is likely that this percentage is higher but evaluating the functional consequence of gene mutation on RNA isoforms and ultimately protein isoforms remain a significant challenge [16]. Aberrant splicing isoforms level may also be the indirect results of unbalanced splicing factors expression or mutated splicing factors rather than the direct cause of DNA mutation in cis [17–19]. Importantly, splicing modulators (SMs) and isoform-specific inhibitors (ISIs) can be used to either correct splicing defect due to DNA mutation or to shift splicing due to expression change in trans acting factor.

Generalities about antisense oligonucleotides

Theoretically, interfering with any gene function (enzymatic activity, protein-protein interaction) is amenable to rational drug design by providing a molecule that

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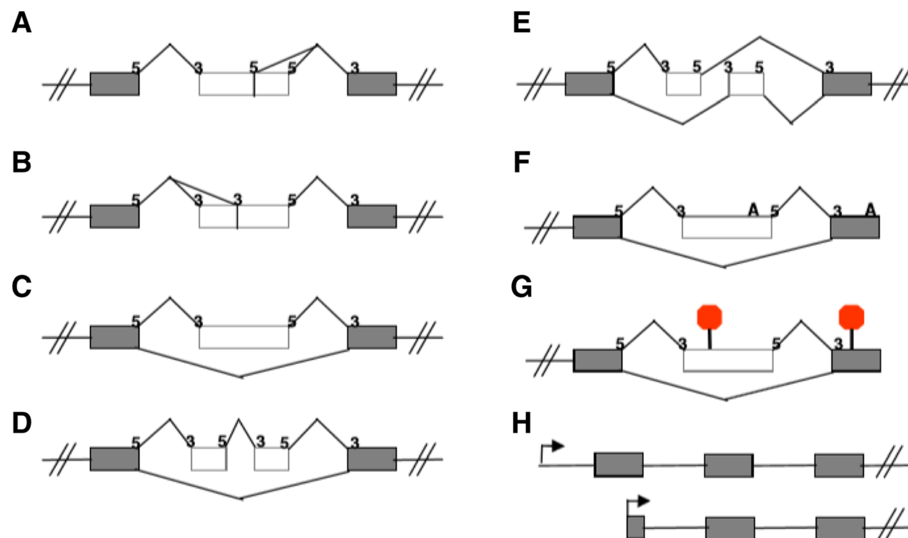

Fig. 1 Type of alternative splicing event. **a** Alternative 5′ splice site. Two 5′ss are in competition for a single 3′ss. **b** Alternative 3′ splice site. Two 3′ss are in competition for a single 5′ss. **c** Cassette exon. An exon may be skipped. **d** Multiple exon skipping. A stretch of exons may be skipped together. **e** Mutually exclusive exon skipping. A choice between two alternative exons. **f** Alternative splicing event coupled to alternative polyadenylation **g** Alternative splicing event coupled to non-sense mediated decay. **h** Alternative transcription initiation

ultimately decreases its protein level or activity. In practice, designing a small molecule inhibitor is a daunting task. In contrast, designing an antisense oligonucleotide is straightforward thanks to the base pairing rule. One simply need to design an oligonucleotide having a complimentary sequence to the mRNA target. The mechanism of action of antisense depend mainly on its binding location on the mRNA (or pre-mRNA) and the oligonucleotide backbone chemistry used. The RNA strand of a DNA/RNA duplex can be recognized and cleaved by endogenous and ubiquitously expressed RNase H [20, 21]. Therefore, targeting specific mRNA with a DNA molecule (or a DNA-like oligonucleotide retaining the capacity to form a B type helix such as phosphorothioate [22] leads to decreased level of this mRNA and ultimately a lower protein level (for protein-coding genes). Since the minimal requirement for the RNase H is a stretch of 4 base pair [23], a novel class of antisense with as few as 4 DNA bases flanked by modified bases (to increase nuclease resistance) was developed and named “gapmer” [24]. Most if not all modifications of the oligonucleotide backbone known to date [2’O-alkyl, morpholinos, peptide nucleic acid (PNA), locked nucleic acid (LNA)] confer resistance to various RNase (including RNase H) as well as increase the binding affinity to RNA [25–27] and use therefore RNase H-independent mechanism. Oligonucleotide targeting the start codon interfere with ribosome initiation and hence reduce the protein translation of specific mRNA [28]. Antisense targeting alternative splice site or splicing regulatory sequence may affect splicing regulation. To reach its target, the antisense have to cross the cellular membrane and localized to the cytoplasm (mRNA) or nucleus (pre-mRNA). The challenge is even greater in vivo where other physiological barriers (vasculature, blood-brain barrier, epithelial layer) and pharmacokinetic issues restrict the efficient delivery of oligonucleotides. Several strategies are currently investigated [29, 30].Thus, the binding location on the mRNA (or pre-mRNA) and the oligonucleotide backbone chemistry used dictate the mechanism of action of antisense oligonucleotides.

Splicing modulators (SM)

Splice-switching oligonucleotides (SSO) are antisense oligonucleotides that reprogram splicing of endogenous pre-mRNA target. Initially, SSOs were designed to correct aberant splicing defect due to DNA mutation. For example, b-thalasemia is caused by a mutation in the second intron of b-globin, which result in defective hemoglobin and hence anemia. Most b-globin mutations expose a cryptic 3’ss and a new 5’ss favoring the inclusion of a novel exon. Targeting the cryptic splice site redirect splicing toward the original splice site and restore protein function [31–33]. In the case of the neuromuscular disease such as Duchenne muscular dystrophy (DMD), nonsense mutation drastically decreases the level of dystrophin; whereas in the mild version, the Becker muscular dystrophy, mutation generates a truncated form of dystrophin that retains partial activity [34, 35]. This suggests that DMD patients would benefit from a strategy that would skip the premature stop codon-containing exon to restore the reading frame.

This idea implies the establishment of antisense design rule. Intron-exon (Fig. 2a) or exon-intron (Fig. 2b) boundary-spanning antisense over the alternative cassette exon promote exon exclusion. Targeting an exclusively exonic sequence generally promote exon exclusion (Fig. 2c) although it also promotes exon inclusion in some cases, most likely due to masking of an exonic splicing silencer (Fig. 2e) [8, 36]. Targeting an exclusively intronic sequence is less common but may be approached rationally by targeting putative intronic splicing regulatory sequence (Fig. 2d, f) [37–39]. Importantly, bioinformatic prediction of splicing regulatory sequences are used as guidelines for finding a robust SSO usually require testing several antisense sequences [40, 41]. Thus, in most cases, SSOs favor the production of one isoform over another without significant impact on the overall gene expression [8].

The “Targeted Oligonucleotide Silencer of Splicing” (TOSS) is a bifunctional SSO (Fig. 2g). An antisense targeting the alternative sequence is used to artificially display a non-hybridizing exonic splicing silencer sequence recruiting HNRNPA1 [42]. Initially used to reprogram the alt5’ of Bcl-x [42], the TOSS design was later generalized to cassette exon, alternative 3’ ASE, and complex ASEs [8, 43, 44]. In all cases, the TOSS is designed to promote the short isoform. Mechanistically, it probably acts by blocking the upstream proximal 3’ss and the downstream proximal 5’ss [45]. The main advantage is the high success rate of TOSS as most of the time there is no need to design and test multiple oligonucleotides [8], most probably because the strong silencer of the TOSS overrides the context-dependent regulatory sequence mask by the antisense platform. Theoretically, one can conceptually imagine the complementary tool: the “Targeted Oligonucleotide Enhancer of Splicing” (TOES) (Fig. 2h). Indeed, this concept was reported in the literature [46–48]. However, the generalization of the TOES concept as remain elusive so far [8, 36, 44].

Isoform-specific inhibitors (ISI)

As opposed to SMs, RNase H-dependent antisense (e.g. gapmer) trigger the degradation of their target mRNA. If designed to target a splicing isoform, it becomes an isoform-specific inhibitor (ISI) (Fig. 2h, i). Similarly, siRNAs targeting specific splicing isoform fall in the same category. These tools are powerful and complementary to the use of SM. Importantly, they are intended to decrease the expression of specific isoform and indirectly, to alter the ratio of one isoform over another in addition to the overall gene expression. One of the challenges is

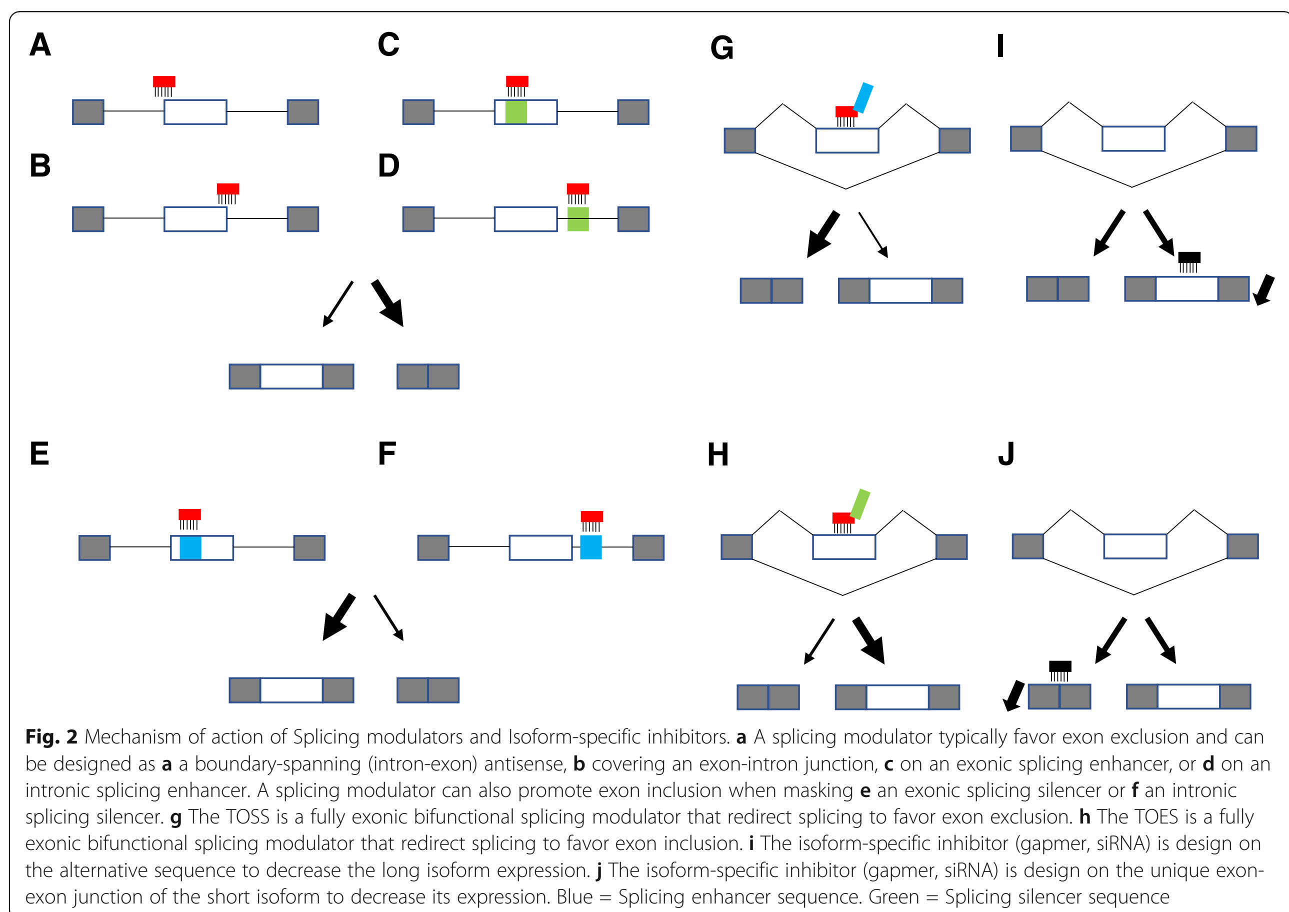

Fig. 2 Mechanism of action of Splicing modulators and Isoform-specific inhibitors. **a** A splicing modulator typically favor exon exclusion and can be designed as **a** a boundary-spanning (intron-exon) antisense, **b** covering an exon-intron junction, **c** on an exonic splicing enhancer, or **d** on an intronic splicing enhancer. A splicing modulator can also promote exon inclusion when masking **e** an exonic splicing silencer or **f** an intronic splicing silencer. **g** The TOSS is a fully exonic bifunctional splicing modulator that redirect splicing to favor exon exclusion. **h** The TOES is a fully exonic bifunctional splicing modulator that redirect splicing to favor exon inclusion. **i** The isoform-specific inhibitor (gapmer, siRNA) is design on the alternative sequence to decrease the long isoform expression. **j** The isoform-specific inhibitor (gapmer, siRNA) is design on the unique exon-exon junction of the short isoform to decrease its expression. Blue = Splicing enhancer sequence. Green = Splicing silencer sequence

to design ISIs against the short isoform as the only unique sequence is an exon-exon junction (Fig. 2i). Studies on siRNA distinguishing single nucleotide polymorphism revealed the critical role of an RNA helix type A integrity. In brief, one mismatch is generally not sufficient to discriminate between highly similar sequences [49] unless it is positioned at the cleavage site [50, 51].

Methodologies to measure the effectiveness of isoform-specific tool

End-point PCR

The gold standard method to quantify splicing isoform modulation is end-point PCR (Fig. 3a). Briefly, two primers are designed on flanking constitutive exons in such a way that it will generate two PCR products. Fractionation on an agarose gel will allow the quantification of each band. Capillary electrophoresis allows to reduce hazards and increase the throughput significantly [52]. The ratio or the "percent splicing index" (Psi) is calculated as the ratio of the long isoform over the sum of the long and short isoforms [52].

To determine the splicing shift of a SM or an ISI, Psi values are compared over a control oligonucleotide of the same length and same chemistry ($Psi_{modulator}$ − $Psi_{control}$ = splicing shift). Importantly, the end-point PCR assay is a competitive PCR reaction meaning that the individual PCR products (Long PCR $product_{modulator}$ vs Long PCR $product_{control}$) can't be directly compared. Thus, it is impossible with this technique to assess the impact of a SM on the overall gene expression.

Real-time PCR

Real-time PCR is routinely used to monitor gene expression but much less frequently to quantify splicing isoforms (Fig. 3b). This is partly due to the challenge of designing isoform-specific PCR primers [53]. In this case, each isoform needs to be amplified in a separate tube. For the long isoform, at least one primer needs to be located in the alternative sequence. For the short isoform, the only unique region is the exon-exon sequence and hence, boundary-spanning primer.

The extent of modulation can be quantified in two ways. The first method is to run one PCR reaction per isoform plus one PCR reaction for a normalizing gene (long, short and normalizing gene) for each sample (control and SM) and apply the deltadeltaCt relative quantification method [54, 55]. This analysis will give the extent of modulation as a fold change for each isoform. However, for the purpose of comparison to the gold standard, a real-time PCR version of the Psi was derived (QPsi) as a second method [43, 44]. To determine the splicing shift, QPsi values are compared over a control ($QPsi_{modulator}$ − $QPsi_{control}$ = splicing shift). Of note, the QPsi formula doesn't include any normalizing gene. Although antisense oligonucleotides should have a minimal off-target effect, confirmation by unbiased and genome-wide methodologies such as mass spectrometry and RNA sequencing have not been reported [56, 57].

Western-blot

In most cases, alternatively spliced isoforms are predicted to be translated into protein. Therefore, it is theoretically possible to detect the splicing shift at the protein level by western-blot. Indeed, the effectiveness of SMs to modulate the splicing ratio can be performed using antibodies that recognize both isoforms [43, 44, 58]. In practice, the resulting protein size of the isoforms is often approaching the resolution limit on a polyacrylamide gel. Alternatively, this issue can be circumvented using isoform-specific antibodies [59]. Conceptually similar to the Real-time PCR technique, the ratio or absolute level of each isoform can be estimated by quantification of individual bands.

When designing an experiment using oligonucleotide, proper control is needed to assess the on-target effect. The ideal control is an oligonucleotide of the same

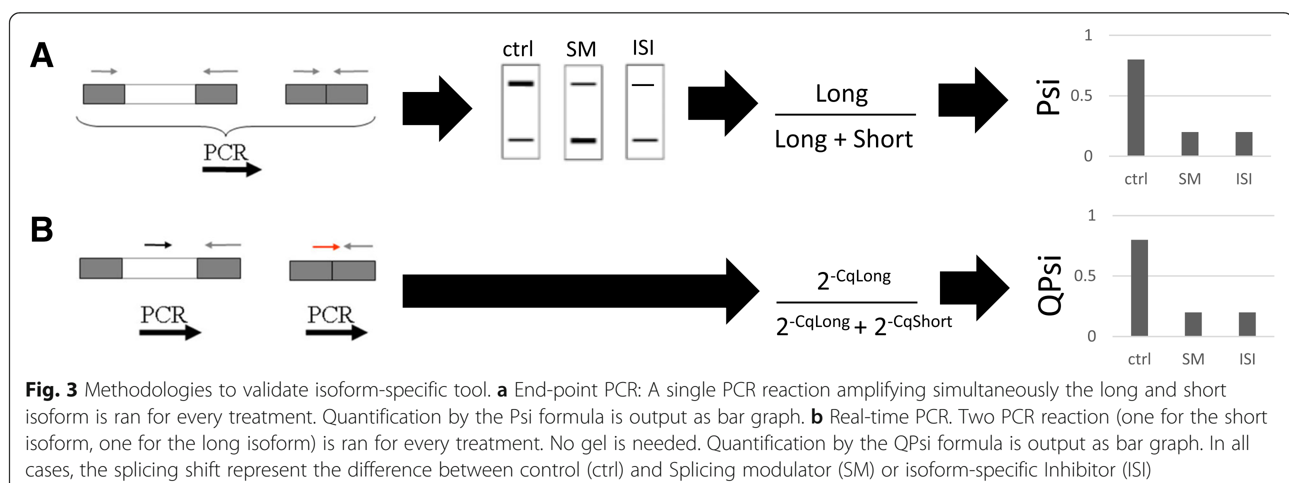

Fig. 3 Methodologies to validate isoform-specific tool. **a** End-point PCR: A single PCR reaction amplifying simultaneously the long and short isoform is ran for every treatment. Quantification by the Psi formula is output as bar graph. **b** Real-time PCR. Two PCR reaction (one for the short isoform, one for the long isoform) is ran for every treatment. No gel is needed. Quantification by the QPsi formula is output as bar graph. In all cases, the splicing shift represent the difference between control (ctrl) and Splicing modulator (SM) or isoform-specific Inhibitor (ISI)

length and same chemistry. This could be a scrambled version of the oligonucleotide or a version with few mismatches. This is particularly useful when a phenotype is evaluated. To control for the off-target effect, splicing shift to unrelated ASEs can be examined.

Initially, antisense was developed to block the elongation of reverse transcriptase [60]. If remaining antisense post-treatment can survive the RNA extraction, then it is possible that some antisense may bloc the cDNA synthesis step of the PCR assay required for splicing shift evaluation. Since the antisense is hybridized to the long isoform, it may lead to an underestimation of the long isoform and hence, artefactual splicing shift. Indeed, a dose-response curve of SM spiked on total RNA extract (without cell transfection) is sufficient to deduce artefactual splicing shift [44]. However, it was demonstrated that RNA extraction using Trizol® reagent, as opposed to column-based strategy, remove any residual oligonucleotide. A column-based approach is attractive to perform high throughput SM validation but typically overestimate the splicing shift. Confirmation by further Trizol extraction is recommended, particularly for moderate SM (deltaPsi < 25) [44].

Prior to any phenotypic analysis, the induced-splicing shift of a SM or an ISI have to be validated (Fig. 4). The first step is to develop an assay to monitor the level of splicing isoform. In brief, RNA is extracted from a cell line of interest and the ASE target is monitored. Then, SMs and ISIs are designed and transiently transfected. Finally, the splicing shift is evaluated based on the detection method previously established in step 1. Validated SM and ISIs can then be used for functional genomics.

Splicing isoforms specific phenotype in human cancer cell lines

The functional relationship between splicing isoforms of a gene

Bioinformatic analyses and experimental evidence suggest that the majority of ASEs in coding region will ultimately produce protein isoforms [61]. To uncover the functions of endogenous splicing isoforms in vitro using transient transfection of an oligonucleotide, three complementary class of tools can be used. ISIs, SMs and global targeting of all isoforms (Global siRNAs or global Gapmers) [62]. Directly comparing the phenotype of an ISI (induce a splicing shift and a decrease in global expression) vs a SM (induce a splicing shift) or vs a strategy targeting all isoforms (decrease global expression) may shed light on the functional relationship of splicing isoforms of a gene [43]. In theory, there are at least three possible scenarios to illustrate the relationship between two splicing isoforms (Table 1). In scenario 1, alternative splicing produces an isoform without a functional domain or triggering NMD. In this case, only one of the two isoforms have a function and its abundance will drive the phenotype. Therefore, SMs, ISIs and global targeting of all isoforms should all yield similar phenotypic results. Of note, similar results are expected if the two isoforms have a widely different function because only one of them will respond in a defined functional assay. In scenario 2, alternative splicing produces an isoform with antagonistic functions or one isoform act as a dominant negative. In this case, the relative abundance of one isoform over the other (i.e. the isoform ratio) will dictate the phenotype. Therefore, SMs and ISIs should yield similar results to scenario 1 except that they can't be discriminated by an inhibitor targeting all isoforms. In scenario 3, alternative splicing produces an isoform with compensatory function. In this case, the overall expression level will drive the phenotype. Therefore, SM should not produce a phenotype as they do not impact on the overall gene expression level. This framework may help to decipher the role of splicing isoforms with unknown function.

Functional genomic of splicing isoforms

Although there is example of splicing isoforms promoting each hallmark of cancer [13, 63, 64], the phenotype of a

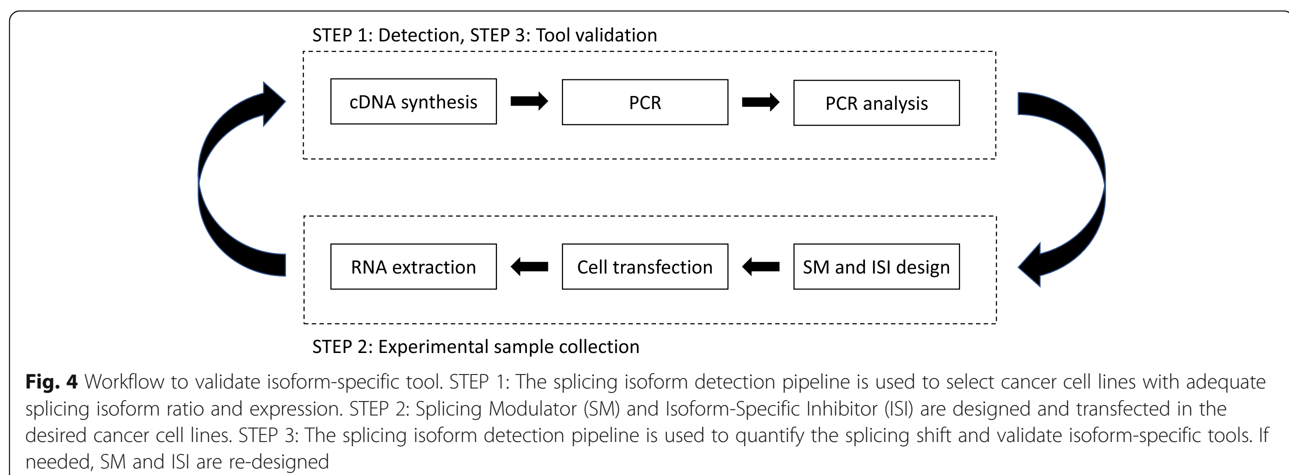

Fig. 4 Workflow to validate isoform-specific tool. STEP 1: The splicing isoform detection pipeline is used to select cancer cell lines with adequate splicing isoform ratio and expression. STEP 2: Splicing Modulator (SM) and Isoform-Specific Inhibitor (ISI) are designed and transfected in the desired cancer cell lines. STEP 3: The splicing isoform detection pipeline is used to quantify the splicing shift and validate isoform-specific tools. If needed, SM and ISI are re-designed

Table 1 Functional relationship between splicing isoforms of a gene

	Scenario 1 (Monofunctional)	Scenario 2 (Antagonistic)	Scenario 3: (Redundancy)
Isoform A	Function A	Function A	Function A
Isoform B	No function	Function anti-A	Function A'
Phenotype dependent on	[A]	[A] / [B]	[A] + [B]
SM targeting Isoform A	Yes	Yes	No
SM targeting Isoform B	No	Yes (anti A)	No
ISI targeting A	Yes	Yes	Yes
ISI targeting B	No	Yes (anti A)	Yes
ISI targeting A and B	Yes	No	Yes

Three theoric scenarios that fits most ASEs generating two isoforms. *Yes* phenotype, *No* no phenotype

limited number of cancer-related genes targeted by SMs (Tables 2 and 4) or ISIs (Table 3) were reported. Most of these splicing isoforms are functionally linked to the apoptotic pathway. Apoptosis is a regulated programmed cell death to clear out undesired cells. The Bcl-2 family of proteins contains about 20 members; some having anti-apoptotic and other pro-apoptotic properties. Interestingly, the pro- vs anti-apoptotic function of some Bcl-2 members such as Bcl-2, Bcl-x and Mcl-1 is finely regulated through alternative splicing (MCL-1S/MCL-1 L; BCL-2α/BCL-2β and BCl-xS/BCl-xL [65, 66].

RAP1GDS1 is a guanine nucleotide exchange factor that activates RhoA and RhoC in vitro. Skipping of exon C generate isoforms (smgGDS-558 and smgGDS-607) with different biochemical properties for small GTPases. To uncover their role in breast cancer, Hauser et al. systematically target the short, long and all isoforms using siRNAs in multiple breast cancer cell lines [67]. Very consistently, they observed a decrease in proliferation using siRNAs against the short isoform or all isoform but not siRNAs against the long isoform. These results indicate that the phenotype is driven by the quantity of the short isoform (scenario 1 in Table 1).

Protein kinase C (PKC) is a family of a protein involved in cellular proliferation and apoptosis. During all-trans retinoic acid-induced differentiation of NT2 teratocarcinoma cells, Jiang et al. observed that generation of a new PKCdelta isoform [68]. Usage of an alternative 5'ss downstream of the constitutive 5'ss of exon 10 produces PKCdeltaVIII. Jiang et al. extensively optimized multiple oligonucleotides to finally obtain antisense strategies that promote almost exclusively either the distal 5'ss (ASO 71) or the proximal 5'ss (ASO 75) on exon 10 [68]. Consistently, favoring the production of PKCdeltaVIII through ASO71 decrease apoptosis whereas targeting PKCdeltaVIII increase apoptosis of NT2 cells [68]. Thus, a splicing shift is sufficient to induce an apoptosis phenotype. Altogether, the PKCdelta exon 10 ASE generate isoforms with antagonistic function (scenario 2 in Table 1).

There is experimental evidence suggesting that the exon 69 of *SYK* encode a nuclear localization signal [69]. Consequently, reprogramming *SYK* splicing to favor the short form (without exon 69) redistribute the protein to the cytoplasm and hence affect cell survival [43]. Indeed, a SM or ISI is sufficient to induce apoptosis in multiple cancer cell lines but not a strategy targeting all isoforms [43]. Therefore, alternative splicing of *SYK* leads to isoform with antagonistic functions (Table 1, scenario 2). Interestingly, the SYK isoforms do not appear to act antagonistically in the context of adherent independent growth in soft agar. Indeed, both ISIs targeting the long isoform as well as siRNAs targeting all isoforms reduce the growth of cancer cells. The theory predicts that targeting *SYK* with siRNAs against the short isoform or with SMs would allow to delineate between scenario 1 (monofunctional) or scenario 3 (redundancy). Unfortunately, the particular exon-exon junction was refractory to siRNA design and the duration of the assay prevent the use of SM, respectively [43]. Thus, isoform functional relationship is not an intrinsic property and may vary depending on the context.

One of the well studied model gene in alternative splicing is the gene *BCL2L1* (best known as *Bcl-x*). Two 5'ss are in competition in the second exon to produce a shorter and pro-apoptotic version (Bcl-xS) or a longer and anti-apoptotic molecule (Bcl-xL). Several groups have successfully targeted the Bcl-xL isoform and witnessed a concomitant increase in apoptosis in multiple cancer cell lines (Table 4). Since both isoforms possesses opposite function; the isoform ratio should dictate the phenotypic outcome and hence either a SM or ISI targeting Bcl-xL should be effective at inducing apoptosis. However, SMs stimulating the use of the distal 5'ss was not as robust at inducing apoptosis despite an obvious splicing shift (Table 4). This can be reconciled if one takes account of the much more transient effect of a SMs compared to ISIs. Indirect support to this theory comes from sustain Bcl-xS overexpression that sensitizes cancer cells to apoptosis [70, 71]. In addition, there is a strong correlation between the overall Bcl-x expression level in a defined cell line and the efficiency of apoptosis induction, suggesting that generation of Bcl-xS and hence the Bcl-xL / Bcl-xS ratio drives the apoptotic phenotype [72]. Also, worth mentioning is that phosphorothioate-modified oligonucleotides can lead to false positive splicing and phenotypic effects [44,

Table 2 Phenotypic analysis of human cancer cell lines transfected with SM targeting cancer-related genes

Gene	ASE type	Backbone chemistry	Relative location	Splicing effect	Phenotype	Ref.
MDM2	cassette	PNA	Spanning the 3′ss intron-exon junction	Promote intron retention and exon exclusion	Sensitize JAR cells to camptothecin	[81]
PRKCD	Alt5′	2′-MOE-PS	Covering the proximal 5′ss	Promote usage of the distal 5′ss	Induce apoptosis in NT2 cells	[68]
FLT1 (VEGFR1)	cassette	morpholino	Spanning 5′ss exon-intron junction	Induce exon exclusion	Suppress laser-induced choroidal neovascularization	[82]
KDR (VEGFR2)	cassette	morpholino	Spanning 5′ss exon-intron junction	Induce exon exclusion	Suppress laser-induced choroidal neovascularization	[83]
MCL1	cassette	morpholino	Spanning 3′ss intron-exon junction	Induce exon exclusion	Induce apoptosis in BCC and AGS cells	[84]
		morpholino	Spanning 5′ss exon-intron junction	Induce exon exclusion	Induce apoptosis in BCC and AGS cells	[84]
VEGF	cassette	morpholino	Spanning 3′ss intron-exon junction	Induce exon exclusion	Induce angiogenesis in PC-3	[85]
TAF6	Alt5′	PS-2′OMe	Fully exonic between both 5;ss	Promote the proximal 5′ss	Induce apoptosis in HeLa cells	[86]
SYK	cassette	2′OMe	Fully exonic	Induce exon exclusion	Induce apoptosis in SKOV3ip1	[43]
ATR	cassette	PS-2′OMe	Fully exonic	Induce exon exclusion	Induce apoptosis of Eu-myc B cells	[58]
EP400	cassette	PS-2′OMe	Fully exonic	Induce exon exclusion	Induce apoptosis of Eu-myc B cells	[58]
DVL1	Intron retention	PS-2′OMe	2 oligonucleotides One Spanning 5′ss exon-intron junction One Spanning 3′ss intron-exon junction	Promote intron retention	Induce apoptosis of Eu-myc B cells	[58]
ERBB4	cassette	LNA, 2′OMe	Fully exonic	Induce exon exclusion	Inhibit growth in MCF7 and T47D	[87]
PKM	Mutually exclusive two exon cassette	PS-2′MOE	Fully exonic	Induce concommittant exon 9 inclusion and exon 10 exclusion	Induce apoptosis in U87-MG and A172 cells	[88]
STAT3	Alt3′	morpholino	Spanning 3′ss intron-exon junction	Promote distal 3′ss	Induce cell death in MDA-MB-435 and MBA-MB-468	[89]
MDM4	cassette	morpholino	Spanning 5′ss exon-intron junction	Induce exon exclusion	Inhibit cell growth in melanoma cell lines	[90]
GLDC	cassette	PS 2′OMe	Fully exonic	Induce exon exclusion	Inhibit cell growth of A549 cells	[91]
USP5	Alt5′	morpholino	Covering the proximal 5′ss	Promote usage of the distal 5′ss	Inhibit cell growth and migration in U251 and LN229	[92]
BRCA1	cassette	morpholino	Fully exonic	Induce exon exclusion	Inhibit cell growth in combination with PARP inhibitor in MCF-7 and MDA-MB-231	[93]
MKNK2	Alt3′	PS-2′MOE and PS-2′OMe	Spanning distal 3′ss junction	Promote proximal 3′ss	Sensitize cells to chemotherapy	[94]
BIM	cassette	PS-2′MOE	Fully exonic	Induce exon exclusion	Restored imatinib-induced apoptosis in KCL22 cells	[95]

The ASE type, backbone chemistry of the oligonucleotide used, Relative location on the ASE, splicing effect and resulting phenotype is described. *2′-MOE* 2′ OMethoxyethyl, *PS* Phosphorothioate, *2′OMe* 2′OMethyl, *Ref.* Reference

73]. Thus, Bcl-xS and BclxL possess antagonistic apoptotic functions (Table 1, scenario 2).

Perspectives

This review article focusses on studies that investigate the phenotype of cancer-related splicing isoform through the use of SMs and/or ISIs. Doing so, it excludes studies where the functional role of splicing isoform was uncovered through other approaches such as overexpression of specific isoforms through expression vectors in vitro and isoform-specific shRNA expressing vector [74]. It also excludes reports where

Table 3 Phenotypic analysis of human cancer cell lines transfected with ISIs targeting cancer-related genes

Gene	ASE type	Isoform targeted	Phenotype	Ref.	Gene	ASE type	Isoform targeted	Phenotype	Ref.
NEK2	Intron retention	Long (NEK2B)	Induce mitotic delay in HeLa cells	[96]	*CCND1*	Alt5′	Long (isoform b)	Suppress proliferation and invasiveness of SBTB1A and T24 cells	[97]
H-Ras	cassette	Long (p19)	Induce cell proliferation in HeLa cells	[98]	*CPEB2B*	cassette	Long (Exon 4)	Sensitized the AnR cell lines to detachment-induced cell death.	[99]
BIRC7	Alt3′	short (isoform β)	Decrease cell proliferation in HeLa cells	[100]	*PKM2*	Mutually exclusive	M2	Induce apoptosis of multiple cancer cell lines	[101]
CCND1	Intron Retention	Long (isoform b)	Decrease cell invasion in T24 cells	[102]	*RREB1*	cassette	Long (exon 9)	Decrease UMUC-3 cell growth	[103]
EIF4H	cassette	Long (isoforme 1)	Decrease cell proliferation in LOVO cells	[104]	*WT1*	Alt5′	Long (Exon4a)	Attenuate apoptosis	[105]
RPAP3	cassette	Short (isoform 2)	Attenuate doxorubicin-induced cell death in T47D cells	[106]	*RPS6KB1*	Multiple exon cassette	short	Inhibit growth and induce apoptosis	[107]
BRD8	cassette	Long (p120β)	Decrease thyroid receptor activation in PC-3 cells	[108]	*GHRL*	cassette	Ln1-ghrelin	Reduce cell viability	[109]
KLF6	Alt5′ and cryptic 3′ss	short (isoform SV1)	Induce apoptosis in A549 cells	[110]	*CD44*	cassette	Exonv6	Reduce MBM cell migration	[111]
PKC	Alt5′	Long (PKCδVIII)	Induce apoptosis in NT2 cells	[68]	*TP53*	cassette	9by	Increase MCF-7 cell growth	[112]
CXCR3	Alt3′	Long (isoform B)	Increase proliferation in MDA-MB-435 cells	[113]	*RAP1GDS1*	cassette	smgGDS-558	Decrease proliferation in multiple breast cancer cell lines	[67]
CD99	cassette	short	Decrease invasion of MDA-MB-435 cells	[114]	*UGT1A*	cassette	i2s	Sensitize HT-115 cells to drug-induced cell death	[115]
MDM2	Complex	Long (DMX211)	Decrease proliferation of ARO cells	[116]	*MYLK*	cassette	Long (Isoform 1)	Increase trans-epithelial resistance	[117]

The ASE type, isoform targeted and resulting phenotype is described. *Ref.* Reference

SMs were used but the consequence on the phenotype was not examined in cancer cells [37, 48, 75, 76].

Initially, antisense oligonucleotides were used to correct splicing defect due to a mutation in the splicing regulatory sequence (e.g. b-thalassemia) [32]. It was later applied to reprogram alternative splicing of cancer-related genes independently of the mutational status of the gene (Table 2, Table 4). As high-throughput genomic studies continue to reveal mutations directly causing splicing defect [16, 19, 77], it would be beneficial to

Table 4 Oligonucleotide-based strategies targeting Bcl-x and their capacity to induce apoptosis in human cancer cell lines

Human cancer cell line	Backbone chemistry	Induce apoptosis following a splicing shift from Bcl-x_L to Bcl-x_S	Induce apoptosis following an isoform specific inhibition of Bcl-x_L
A549	2′-MOE	No [118]	Yes [24]
MCF-7	PS-2′OMe	No [119]	Yes [120]
MDA-MB-231	PS-2′OMe	Yes [72]	Yes [120]
HeLa	PS-2′OMe PNA-peptide	No [72] Yes [121]	N/A
CaOV3	N/A	N/A	Yes [122]
PC-3	PS-2′OMe 2′OMe (TOSS)	Yes [119] No [44]	N/A
U87	PS	Yes [123]	N/A

N/A Data not available

restore the expression and splicing pattern of some of those genes through antisense oligonucleotides to uncover their functional role in cancer.

Recently, re-analysis of TCGA datasets indicates that intron retention is abundant in cancer [78–80]. Their enrichment in tumor suppressor gene suggests that intron retention and hence the inclusion of premature stop codon is an effective way of shutting off gene expression. Therefore, efforts aiming at restoring tumor suppressor genes expression level through antisense-based strategies, including enhancing intron splicing are worth pursuing.

Conclusions

In summary, SMs and ISIs are powerful molecular tool to dissect the functional contribution of cancer-related splicing isoform in cancer cell lines. End-point PCR and isoform-specific real-time PCR are complementary and scalable assays to evaluate the performance of SMs and ISIs. Direct comparison of SM, ISI and siRNA targeting all isoforms help to deduce the functional contribution of individual splicing isoforms. Importantly, there is a need to develop antisense-based strategies promoting splicing (TOES-like strategies) as there is no design rule established so far.

Abbreviations

2'-MOE: 2' OMethoxyethyl; 2'OMe: 2'OMethyl; alt3': Alternative 3'; alt5': Alternative 5'; ASE: Alternative splicing event; DMD: Duchenne muscular dystrophy; ISI: Isoform-specific inhibitor; LNA: Locked nucleic acid; NMD: Non-sense mediated decay; PNA: Peptide nucleic acid; PS: Phosphorothioate; Psi: Percent splicing index; QPsi: Quantitative Psi; Ref: Reference; siRNA: Small interfering RNA; SM: Splicing modulator; SSO: Splice-swithing oligonucleotide; TOES: Targeted oligonucleotide enhancer of splicing; TOSS: Targeted oligonucleotide silencer of splicing

Acknowledgements

The author would like to thank Jean-Pierre Perreault, Benoit Chabot and Sherif Abou Elela.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Authors' contributions

The author read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that he has no competing interests.

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Received: 14 August 2018 Accepted: 19 November 2018
Published online: 14 December 2018

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