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When one becomes many - alternative splicing in beta cell function and failure

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Abstract

Pancreatic beta cell dysfunction and death are determinant events in type 1 diabetes (T1D), but the molecular mechanisms behind beta cell fate remain poorly understood. Alternative splicing is a post-translational mechanism by which a single gene generates different mRNA and protein isoforms, expanding the transcriptome complexity and enhancing protein diversity. Neuron-specific and certain serine/arginine-rich RNA binding proteins (RBP) are enriched in beta cells, playing crucial roles in the regulation of insulin secretion and beta cell survival. Moreover, alternative exon networks, regulated by inflammation or diabetes susceptibility genes, control key pathways and processes for the correct function and survival of beta cells. The challenge ahead of us is to understand the precise role of alternative splicing regulators and splice variants on beta cell function, dysfunction and death and develop tools to modulate it.

Keywords

type 1 diabetes; pancreatic beta cells; alternative splicing; candidate genes; splicing factors

1. Introduction

“The door of truth was open,

but only let pass half of a person each time.

Thus, it was impossible to reach the whole truth...”.

Carlos Drummond de Andrade, translated from “A verdade dividida”¹.

The discovery that genes are discontinuous entities, constituted by coding regions (exons) interrupted by non-coding regions (introns), led to the understanding of a fundamental principle of biology, namely RNA splicing². Splicing is the editing of precursor mRNAs (pre-mRNAs) by removal of introns and joining together of the exons. Whereas some exons

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Conflict of interest

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undergo constitutive splicing, meaning that they are included in all mature transcripts originated from a specific gene, others are alternatively spliced, showing variable degrees of inclusion that lead to the production of structurally different mature transcripts or splice variants³. Genome wide studies estimate that around 95% of the human genes undergo alternative splicing, generating in average four isoforms per gene³. This indicates that alternative splicing plays a key role in amplifying the coding capacity of the genome and helps to explain how the human genome generates >200.000 proteins from only 22.000 genes⁴. Alternative splicing regulation is often species-specific and its frequency increases with species complexity, playing a major role in evolution³. Alternative splicing is controlled both spatially and temporally and contributes to cell and tissue adaptation to intra and extracellular cues³. Functionally related transcripts are often co-ordinately spliced in splicing regulatory networks controlled by master splicing regulators. These networks control complex biologic outcomes such as cell differentiation, lineage determination, tissue identity, and organ development⁵. Not all alternative splicing events lead to functional proteins. For instance, an alternative splicing event can result in a non-coding transcript through its functional coupling to the nonsense-mediated decay (NMD) system that affects mRNA stability; alternative splicing can also impact mRNA localization and the regulation of downstream mRNAs by sequestering their splicing regulators or by splicing transcription factors and/or splicing regulators⁶.

Technological advances during the last decade, particularly RNA sequencing at high depth, increased our understanding on the role of RNA splicing in biology and disease⁷. The biologic importance of this tightly regulated mechanism is underlined by the fact that mutations or variations in splicing pre-mRNA sequences or in splicing regulatory proteins are associated with several human pathologies⁸. Transcriptome analysis suggests that splicing alterations might contribute to pancreatic beta cell demise in the context of T1D and T2D^{9,10}. It is becoming increasingly evident that alternative splicing is a crucial regulatory point for beta cell identity, development, function and survival^{11–15}. This review will discuss alternative splicing regulation in pancreatic beta cells in the context of physiology and diabetes, with focus on recent advances in the field not covered by a previous review by our group¹⁵.

2. Splicing regulation – a key biological phenomenon

The discontinuous architecture of eukaryotic genes provides evolutionary advantages but requires a well-coordinated splicing regulatory machinery. The splicing events are catalysed by the spliceosome, a dynamic and flexible macromolecular complex¹⁶. The spliceosome machinery consists of five small nuclear ribonucleic acid (snRNA) molecules, called U1, U2, U4, U5 and U6, a common set of Sm proteins, and over a hundred particle-specific proteins, that together constitute the small nuclear ribonucleoproteins (snRNPs)¹⁷. These constitutively expressed snRNPs are the central players in the mRNA splicing and dictate where splicing will occur¹⁷. Structurally, alternative splicing events can be categorized into seven main subgroups: (I) skipping/inclusion of a cassette exon; (II) recognition of alternative 3' splice site (3' ss); (III) 5' splice site (5' ss); (IV) mutually exclusive exon splicing, where the splicing of two or more exons is not independent; (V) intron retention into the mature mRNA molecule; (VI) alternative first exons; and (VII) alternative last exons

¹⁸ (Figure 1A). The assembly of the spliceosome occurs in most cases in parallel with mRNA transcription, and it is highly dependent on the presence of intronic core consensus sequences such as 5' splice site (ss), branch site (A, in Figure 1B), and polypyrimidine tract (Y(n)), and the 3' splice site (ss) (Figure 1B). These core splice signals, however, are short and degenerated sequences and do not provide the complete information required for the efficient splicing of alternative exons¹⁸. Other cis-regulatory regions, namely exonic and intronic splicing enhancers (ESEs and ISEs) or silencers (ESSs and ISSs), must be recognized in order to promote positive or negative effects on splicing site recognition (Figure 1B).

Some of these regulatory sequences can modulate splicing of exons at distant locations¹⁹. The information included in these cis-elements, together with their interacting RNA binding proteins (RBP; trans-acting factors), defines the “road map” for splicing regulation, the so-called “splicing code”, that will recruit (or prevent) the association and binding of the splicing machinery to the adjacent splice site(s)²⁰. These RBPs can be grouped into two classes. One class consists of relatively broadly expressed proteins with widespread roles in mRNA biogenesis – the Serine Arginine (SR) rich proteins which, usually promote exon inclusion, and the heterogeneous nuclear ribonucleoproteins (hnRNPs), which have mostly an inhibitory effect, when bound to exons²¹. The ESE and ISE are usually recognized by SR proteins whereas ESS and ISS are often bound by splicing repressors, e.g. hnRNP proteins²² (Figure 1B). Cell type and tissue-specific differences in RBP protein expression and their post-translational modifications both contribute to alternative splicing regulation²³. The SR proteins regulate their own splicing and the splicing of other SR genes. By including or not exons harbouring a premature termination codon, the SR proteins can also regulate their own expression by triggering transcript degradation by NMD, a process named “unproductive splicing”²⁴. The other class of alternative splicing regulators comprise proteins with tissue and cell-specific alternative splicing regulatory roles. Several families have been identified, namely NOVA-1/2, TIA1/TIAR, RBFOX1/3, nPTB, STAR/GSG family proteins, Tra2α/β, CELF family proteins, and ELAVL proteins²⁵. There is a cross-talk between alternative splicing and other layers of gene regulation, such as chromatin structure, histone modifications, and DNA methylation. This adds another level of complexity and contributes to fine tune cellular responses to regulatory signals²⁶. Perturbations affecting any of the components of the pre-mRNA splicing process may have serious consequences, leading to diseases as heterogeneous as myopathies, cancers, neuropsychiatric disorders and autoimmune diseases^{15, 27, 28}. Regulation of alternative splicing and its impact on tissue development and function have been extensively explored in brain and muscle and there is an increased interest on its role in pancreatic beta cells and diabetes^{15, 29}.

3. Available tools to study alternative splicing

RNA sequencing is presently the gold standard to identify and study genome-wide alternative splicing. Structurally, what characterizes different isoforms of a gene is their specific exonic composition and combination, resulting from the different relative usages of alternative exons. The relative usage of an exon is usually described by its inclusion level, and it is measured using the “percent spliced in” (PSI or Ψ) statistics³⁰. The PSI indicates the ratio between the relative abundance of isoforms containing a certain exon over the relative abundance of all isoforms of the gene containing or not that exon³⁰. In other words,

the PSI value indicates how often an exon appears in all the isoforms of the gene. Methodologically, the analysis of alternative splicing falls into two categories: isoform-centric and exon-centric approach. The isoform-centric approach starts by an estimation of the relative amounts of each isoform by quantification tools such as Cufflinks³¹, Flux Capacitor³², or RSEM³³ (Table 1), generating a probabilistic model of the frequency of each isoform in the original sample. The exon-centric tools, such as MISO⁴⁶, VAST-TOOLS⁴⁹ and rMATS⁴⁸ quantify usage of annotated exons by calculating read densities for every exon-exon junction and computing the PSI index³⁰ (Table 1). The output from these approaches is therefore different. The isoform-centric methods attempt to capture splicing complexity by predicting and reconstructing the full-length transcripts. Since most reads cannot be unequivocally mapped to individual transcripts, further analysis is needed to identify the events⁵⁹. The exon-centric type of analysis provides information about the inclusion levels for every splicing event, allowing to identify differently spliced exons. This information can be easily used to identify sequence features in modified exons and to design oligonucleotides for further validation experiment⁵⁹. In our previous studies of alternative splicing^{9, 10, 12} we used mostly isoform-centric approaches, particularly Cufflinks and Flux Capacitor, since they are suitable for assembly and abundance quantification of isoforms from mapped reads. On the other hand, in our most recent work we used an exon-centric approach – rMATS – since it provides a more detailed description of the splicing events, a central goal of this particular study¹³.

Once the alternative splicing events have been identified, a critical issue is to interpret the profiling data, and to identify splice variants that are potentially associated with particular biological processes. For this purpose, enrichment of gene ontology and pathway terms in differentially spliced genes is a useful approach to identify affected processes⁶⁰. The challenges are to understand how specific events affect protein properties or functions and impacts on particular biological processes. Moreover, understanding how co-regulated exon networks are controlled by specific regulatory proteins to promote cell- and tissue-specific functions is a fundamental goal in the field. Structural modifications of particular protein domains and NMD-mediated transcript degradation are the main functional outcomes of splicing⁶¹. Several computational algorithms have been developed to address these questions^{56, 57} (Table 1). The recently developed ASpedia database integrates several layers of information covering genomic features as well as isoform-level functions⁵⁸. Integration of transcriptomic and proteomic data (proteogenomics) is an emerging field that can help to identify expressed splice variants at protein level⁶². It is important to keep in mind, however, that RNA sequencing is presently much more sensitive than mass-spectrometry proteomics. For instance, in our own parallel RNA-seq and proteomic analysis of human islets or EndoC-β1 cells exposed to different cytokines (using state-of-the art technology) we usually detect around 50.000 of the expected 80.000–100.000 splice variants, and around 10.000–12.000 of the expected >200.000 proteins (unpublished data). RNA-seq can be integrated with other high-throughput assay to build RNA splicing maps that allow to visualize, in a genome-wide scale, how the binding of a RBP in a given position affects the splicing of a particular exon⁶³. The most widely used method to unveil protein-RNA interactions is the UV crosslinking immunoprecipitation (CLIP) coupled with high-throughput RNA sequencing⁶⁴. Novel improvements of this method now allows to reach

nucleotide resolution, by using the individual-nucleotide resolution UV crosslinking and immunoprecipitation approach (iCLIP)⁶⁵. Computational approaches combining genomic variations with transcriptomic data enables the identification of splicing quantitative trait locus (sQTL), providing information about the association between splicing alterations and SNPs⁶⁶.

4. The “neuron specific” splicing factors that regulate beta cell function

Alternative splicing plays a central role in neuronal development and physiology⁶⁷. Mutations affecting several RNA binding proteins lead to neuronal dysfunction and disease⁶⁸. Despite different embryological origins, neuronal cells and pancreatic beta cells share many similarities, ranging from analogous signal transduction to development steps and gene expression⁶⁹. This is due, at least in part, to the absence of the inhibitory transcription factor REST (repressor element 1 silencing transcription factor) in both beta cells and neurons⁷⁰. Beta cells express several neural-enriched splicing factors, and the global analysis of splicing regulators expressed in beta cells show that they cluster together with brain as compared to other tissues¹⁴. Among the already studied splicing factors expressed preferentially in brain and beta cells, we found that NOVA 1 and 2, RBFOX1, RBFOX2, and ELAVL4 affect beta cell function and survival^{12, 14}.

The “neuron specific” NOVA proteins were first identified as neuronal antigens in an autoimmune neurologic disease characterized by failure of motor systems inhibition⁷¹. In the central nervous system (CNS), NOVA 1 and 2 have indistinguishable biochemical properties but mutually exclusive expression⁷¹. NOVA1 is highly expressed in human beta cells – to the same extent as in neurons and >10-fold higher than in liver, spleen, colon or lung¹⁰. It regulates beta cell function and survival through splicing modifications of genes involved in exocytosis, apoptosis, insulin receptor signalling, splicing, and transcription¹². Interestingly, NOVA2 knockdown (KD) by specific siRNAs impair beta cell survival but not insulin secretion¹⁴, suggesting that the two paralogs have different targets in beta cells, as suggested for other tissues⁷².

The RBFOX proteins are a highly conserved RNA binding family that regulate alternative splicing⁷³. Rbfox1 (A2BP1) and Rbfox2 (RBM9) are preferentially expressed in both human islets and brain¹⁴. In contrast with the NOVA proteins, Rbfox1 or Rbfox2 KD do not affect basal or cytokine-induced beta cell apoptosis. Instead, depletion of Rbfox proteins increases insulin secretion by modulating the splicing of voltage-gated calcium channels and the actin remodelling protein gelsolin (GSN)¹⁴. These changes augment calcium influx and lead to a faster glucose-induced actin depolymerisation, enhancing insulin release¹⁴. In line with this, the Rbfox targets in brain are involved in cytoskeleton organization and actin processes⁷⁴. Rbfox1 and Rbfox2 expression are modulated by glucose, and short term exposure of INS-1E cells to increasing glucose concentration decreases Rbfox1/2 gene expression (Figure 2A-C). These findings suggest a mechanism by which relatively short periods of high glucose exposure potentiate subsequent glucose-induced insulin release and indicate that Rbfox proteins may adapt the insulin secretory capacity to beta cells depending on the physiological demands.

Elavl4 is a master regulator of neurogenesis, modulating neuronal survival, function, and plasticity⁷⁵. In beta cells, Elavl4 has been previously shown to regulate insulin translation, autophagy, and lipid synthesis⁷⁶. Interestingly, Elavl4 inhibition increases basal apoptosis of insulin-producing cells but decreases cell death following exposure to pro-inflammatory cytokines¹⁴. This suggests that Elavl4 coordinates different transcripts and pathways depending on the functional state of the beta cells.

5. Alternative splicing, autoimmunity and the triggering of T1D

T1D is chronic autoimmune disease where pancreatic beta cells are targeted and destroyed by infiltrating immune cells and their mediators, such as cytokines and toxic free radicals, and by cell-to-cell contacts via FAS-FASL, among others⁷⁷. The mechanisms by which autoimmunity is triggered and progress to disease remain to be clarified, but T1D probably results from the interaction between predisposing genes and environmental factors (e.g. viral infections, dietary components, etc.) that lead to the breaking of tolerance to beta cell antigens^{78–80}. This cascade of events is regulated by >50 candidate genes that act on both the immune system and pancreatic beta cells^{10, 79, 80}. Genetic alterations (e.g. mutations, SNPs) in cis-regulatory elements and trans-acting factors regulating alternative splicing elements can modulate both the susceptibility to autoimmune diseases and disease severity⁸¹. Associations have been reported between autoimmune disease risk and genetic variants that affect splicing of specific mRNAs, including for instance the interleukin-7 receptor (IL7R) in multiple sclerosis (MS)⁸², protein tyrosine phosphatase non-receptor type 2 (PTPN2) in Crohn's disease⁸³, C-type lectin domain family 2 member D (CLEC2D) in T1D⁸¹, and interferon regulatory factor 5 (IRF5) in rheumatoid arthritis⁸⁴. Alternative splicing might also contribute to autoimmunity by affecting the generation and presentation of antigens and lymphocyte homeostasis⁸⁵. Through high depth RNA sequencing of human islets, we found that the pro-inflammatory cytokines IL-1 β + IFN- γ - that probably contribute to beta cell death in T1D - change the expression of >30 splicing-regulating RBP. This leads to changes in the beta cell alternative splicing repertoire, affecting the splicing of >3000 genes involved in beta cell function and survival^{10, 15} – the gene ontology terms enriched for the genes displaying alternative splicing changes following cytokine exposure are shown in Figure 3. Interestingly, the “alternative splicing signature” produced by pro-inflammatory cytokines is different from the one produced by the free fatty acid palmitate, a metabolic stressor that may contribute to T2D⁹. These observations suggest that exposure to pro-inflammatory cytokines favour beta cell expression of pro-apoptotic splice variants and potentially lead to the formation of beta cell neoantigens^{10,11,15} (Gonzalez-Duque et al. submitted for publication); these two phenomena, together, have the potential to exacerbate the autoimmune assault. Of note, characterization of splice variants present in control or stressed human beta cells may allow the identification of novel circulating biomarkers, useful to follow up beta cell death *in vivo*, and of beta cell specific surface biomarkers that can be used for beta cell imaging^{86, 87}.

6. SRSF6 (SRp55): a major splicing regulatory protein in beta cells

The SRp55 splicing regulator belongs to the SR family of proteins. As discussed above, SR proteins are essential components of the splicing machinery⁸⁸. The role of SR proteins in

autoimmune diseases remains poorly explored, but low levels of SRSF1 may contribute to the development of systemic lupus erythematosus⁸⁹, while SRp55 regulates the skipping of CD45 exon 4 during T cell activation, and alterations in CD45 isoform expression levels are associated with autoimmune and infectious diseases⁹⁰. Aberrant expression of SR proteins is present in multiple types of cancer, including leukaemia, kidney, breast, colon, pancreas and lung cancers, among others⁹¹. SRp55 has been implicated in the development of skin and colorectal cancer^{92, 93}. SRp55 is classified as a proto-oncogene, since its overexpression results in hyperplasia, increased cell proliferation and motility^{92, 93}.

In beta cells, *SRp55* gene expression is under regulation of the T1D and T2D susceptibility gene *GLIS3*, and its expression is decreased by pro-inflammatory cytokines^{11, 13}. RNA sequencing analysis of SRp55-depleted human insulin-producing EndoC- β H1 cells, followed by confirmation experiments in both EndoC- β H1 cells and human islets, indicate that SRp55 regulates a crucial splicing program in pancreatic beta cells¹³. Thus, SRp55 depletion leads to impaired insulin secretion and apoptosis through regulation of alternative splicing of genes involved in insulin secretion (e.g. *SNAP25*, *INSR*), calcium channels (e.g. *CACNA2D*, *CACNA1C*), apoptosis (e.g. BAX, BCL2L11 (BIM), Smac/DIABLO), and JNK signalling (*MAPK8*, *MAPK9*, *MAP3K7*)¹³ (Figure 4).

7. The role of alternative splicing in pancreatic beta cell death

7.1 Splicing modifications of pro-apoptotic proteins

The decrease in functional beta cell mass, mostly resulting from apoptosis, is a determining factor for T1D development⁷⁷. Most of the proteins involved in the apoptotic pathways are subject to regulation by alternative splicing, leading to different isoforms with different or even opposing functions⁹⁴. Thus, apoptosis-related proteins such as ligands and receptors (FAS/FASL), adaptor proteins and regulators (TRAF2/3, Smac/DIABLO), Bcl-2 family proteins (Bcl-x, Bak, Bim, Bax), and caspases (Casp2/9/10) are affected by alternative splicing, leading to modifications in their protein solubility, apoptotic potential and cellular localization⁹⁴. The depletion of SRp55 leads to beta cell apoptosis (see above) via activation of the intrinsic apoptotic pathway¹³, at least in part driven by splicing modifications of the pro-apoptotic proteins Bim, Bax, Bak, Bok and Smac/DIABLO¹³. In both rat and human beta cells, SRp55 regulates alternative splicing of the mRNA encoding the pro-apoptotic protein Bim^{11, 13}. *BIM* pre-mRNA is spliced in three main isoforms: Bim Extra Large (BimEL), Bim Large (BimL), and Bim Small (BimS)⁹⁵. BimS is the most potent inducer of apoptosis. The BimEL and BimL isoforms are maintained in a relatively inactive conformation through binding to the microtubule-associated dynein motor complex, while BimS is constitutively active due to the absence of this dynein-binding domain encoded by the spliced exon 2⁹⁵. Other molecular mechanism leading to Bim activation may contribute to beta cell death, such as Bim phosphorylation by JNK, which is regulated in these cells by the T1D candidate gene PTPN2⁹⁶. Bim expression is also regulated by beta cell stress-responsive transcription factors, including members of FOXO and AP-1 family^{97, 98}. Interestingly, transcriptome analysis of cytokine-modified genes indicates an enrichment for AP-1 target genes¹⁰. SRp55 also regulates the splicing of the pro-apoptotic protein Bax, and SRp55 depletion by specific siRNAs promotes the expression of a constitutively active

isoform named Bax β ¹³. Bax β results from the retention of intron 5, which modifies the encoded transmembrane domain at the Bax C-terminal end⁹⁹. This converts Bax β into a constitutively active protein that spontaneously integrates into the mitochondrial membrane, leading to the release of apoptogenic factors and cell death⁹⁹. On top of it, the BimS isoform can activate the canonical Bax, contributing to an overall activation of Bax-dependent pro-apoptotic mechanisms⁹⁹.

The c-Jun N-terminal kinase/ stress-activated protein kinase (JNK/SAPK) signalling pathway has a central role in the integration of different stress signals and promoting beta cell death¹⁰⁰. The JNK/SAPK is a component of a “three-layer” mitogen-activated protein kinases (MAPK) signalling pathway¹⁰¹. The first layer of MAPKs (the MAP3Ks, such as MAP3K7) receives the stimulus and catalyse the phosphorylation of the middle layer MAP2Ks, MKK4/7 that will in turn phosphorylate the last layer of MAPKs - the JNK proteins. JNK proteins, particularly JNK1, phosphorylate and activate downstream targets such as c-Jun, ATF2, and Elk-1 that contribute to beta cell death¹⁰⁰.

SRp55 inhibition modifies the splicing of genes from different layers of the JNK signalling cascade in human beta cells¹³. In particular, increased exon skipping in MAP3K7 and the activator JNK1 increases the pathway activity and contributes to beta cell apoptosis¹³. The MAP3K7 protein structure includes an N-terminal kinase domain, a poorly characterized linker region, and a C-terminal coiled-coil domain¹⁰². The binding of two co-activators – TAB1 and TAB2 – are probably required for MAP3K7 autophosphorylation and activation of the downstream kinase cascade¹⁰², and it remains to be determined how alternative splicing modulates these processes.

8. Splicing modulation – a novel approach to prevent beta cell loss in T1D?

Deciphering the role of alternative splicing in T1D may uncover a new line of therapeutic approaches based on splicing modulatory molecules¹⁰³ that can be targeted to beta cells by using cell surface biomarkers^{86, 87}.

RNA splicing is an interesting point for intervention since it is an early step in gene expression and represents a non-permanent (i.e. do not modify the genome) therapeutic approach. A number of tools have been developed to modulate splicing, but here we will focus on two main strategies – antisense oligonucleotides (AONs) and small molecules^{103, 104}, both already under clinical testing for treatment of two monogenic neuromuscular diseases where alternative splicing modulation is used in an attempt to rescue gene expression^{105, 106}.

8.1 Antisense Oligonucleotides (AONs)

AONs are short oligonucleotide sequences of around 20bp in length, designed as the reverse complement of a specific sequence in the pre mRNA¹⁰³. Their design relies on the principle that AONs specific hybridization to the target region on the pre mRNA blocks the access of splicing machinery components, redirecting the splicing to an adjacent region and promoting the inclusion or exclusion of specific region¹⁰³ (Figure 5).

The sequence specificity of AONs allow them to bind precisely to their target, while fidelity allows targeting of distinct RNA isoforms¹⁰³. The AONs need to be chemically modified to improve stability and internalization by the target cells¹⁰³. The AONs approach has evolved from proof of concept in *in vitro* studies and animal tests into clinical trials for Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA)^{105, 108}. AONs can also be designed to target cis-regulatory regions and promote specific alternative splicing shifts¹⁰⁹. Targeting the RNA regulatory elements has major advantages over targeting the RBP directly: while direct inhibition of an RBP will affect a broad range of downstream biological mechanisms, the AON – if adequately designed - will only affect the target mRNA.

This approach may have a potential use in T1D. Thus, CTLA-4 splicing modulation reduces the incidence of insulinitis and diabetes in NOD mice by interfering with T cell activation and proliferation¹¹⁰, while microparticles carrying AONs targeting CD40, CD80 and CD86 prevent or reverse diabetes in the same mouse model¹¹¹. In human beta cells, several isoforms that contribute to beta cell death are potential targets for AON splicing modulation, including BimS, BAX β , and JNK (our own unpublished data).

8.2 Small molecules

Small molecules that interfere with splicing are often identified by high-throughput *in vitro* screens. They either directly modify the activity/localization of spliceosomal core components and RBPs or affect them indirectly, via targeting RNA sequences that prevent their binding to pre-mRNAs¹¹². Potential limitations of these small molecules are lack of specificity and lack of detailed information regarding their biologic mechanism of action, which might lead to off target effects¹⁰³. Some of these small molecules are already used in clinical practice. One example is pentamidine, an antimicrobial that partially rescues splicing defects observed in myotonic dystrophy¹¹³. Another molecule, dantrolene, enhances by >10 fold AON-mediated exon 51 skipping in DMD myotubes¹¹⁴. Potential splicing modifiers molecules that increase SMN protein expression have also been identified¹¹⁵. Given their effect on the basal splicing machinery, it is curious that these molecules have a certain degree of specificity. For example the spliceostatin A molecule, despite targeting a component of snRNP, was shown by microarray analysis to affect preferentially alternative splicing patterns of genes involved in cell cycle¹¹⁶.

9. Conclusion and future perspectives.

Recent evidence suggests that master splicing factors and their downstream alternative exon networks have an important role in maintaining beta cell identity and phenotype, as well as in regulating their adaptation to extracellular cues such as immune-induced stress. These findings highlight the role of “neuron specific” splicing factors in beta cells¹⁴ and suggest that SRp55, a splicing regulator downstream of the diabetes candidate gene *GLIS3*, is a key splicing factor in human pancreatic beta cells¹³. This hints of a potential crosstalk between candidate genes for diabetes and alternative splicing regulation that must be further explored.

The regulation and role of alternative splicing in pancreatic beta cells remains “terra incognita”. The data discussed above describes the initial attempts to chart this new territory. Many fundamental questions, however, remain:

1. Which splicing events will actually lead to relevant biological outcomes, via modification of protein translation or other mechanisms?
2. What is the impact of immune-mediated alternative splicing changes on beta cell death in human diabetes?
3. Do changes in alternative splicing early in the course of insulinitis contribute to generate neoantigens? What is the nature of these putative neoantigens?
4. Can the identification of beta cell-specific splice variants generate useful biomarkers for beta cell imaging or to monitor, via circulating biomarkers, the progressive beta cell loss in diabetes?
5. Is it possible to modulate beta cell alternative splicing *in vivo*, and thus prevent beta cell death?
6. If yes, and considering that some of these spliced pro-apoptotic proteins (such as BimS) are expressed in other tissues and may act as anti-oncogenic proteins, how to obtain specific delivery of alternative splicing modulators to beta cells?

In conclusion, alternative splicing is an unexplored layer of regulation of beta cell function and survival. A better characterization of its role in beta cells will hopefully let the passage of more than “half of a person (or truth) each time” and lead to a comprehensive understanding of beta cells at its real level of complexity.

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ABBREVIATIONS:

AONs	Antisense oligonucleotides
AP-1	activator protein 1
BAK	bcl2 antagonist killer 1
BAX	bcl2-associated X
Bcl-x	bcl2-like 1
BIM	bcl2-like 11

BOK	bcl2-related ovarian killer
CACNA1C	calcium voltage-gated channel subunit alpha1 C
CACNA2D	calcium voltage-gated channel auxiliary subunit alpha2
CD3ζ	T-cell receptor zeta
CD45	protein tyrosine phosphatase, receptor type C
CELF	cugbp and elav-like family
CLEC2D	C-type lectin domain family 2 member D
CLIP	cross-linking immunoprecipitation
CNS	central nervous system
CTLA-4	cytotoxic t lymphocyte-associated 4
DMD	Duchenne Muscular Dystrophy
ELAVL	embryonic lethal, abnormal vision
ESE	exonic splicing enhancers
ESS	exonic splicing silencer
FAS/FASL	first apoptosis signal
FASFOX	forkhead transcription factor
GSN	gelsolin
hnRNPs	heterogeneous nuclear ribonucleoproteins
IFN-γ	interferon γ
IL-1β	interleukin 1 β
INSR	insulin receptor
IRE1	Inositol-Requiring Enzyme 1
IRF5	interferon regulatory factor 5
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
MAPK	mitogen-activated protein kinase, MS, Multiple Sclerosis
NMD	Nonsense-mediated mRNA decay
NOVA	neurooncologic ventral antigen 1

nPTB	polypyrimidine tract binding protein
PP6	protein phosphatase 6
PSI	percent spliced in
PTPN2	protein tyrosine phosphatase non-receptor type 2
RBFOX	RNA binding fox-1 homolog
RbFox	RNA-binding protein, fox homolog
REST	repressor element 1 silencing transcription factor
RBP	RNA binding proteins
SMA	spinal muscular atrophy
Smac/DIABLO	direct iap-binding protein with low pi
SMN	survival of motor neuron
SNAP25	synaptosomal-associated protein 25
SNP	single nucleotide polymorphism
snRNA	small nuclear ribonucleic acid
snRNPs	small nuclear ribonucleoproteins
sQTL	splicing quantitative trait loci
SR	serine arginine
STAR/GSG	steroidogenic acute regulatory protein
TAB1	TAK1-binding protein-1
TAB2	TAK1-binding protein-2
TIA/TIAR	cytotoxic granule-associated rna-binding protein
TRAF2	TNF receptor associated factor 2
Tra2α/β	transformer 2
T1D	type 1 diabetes
T2D	type 2 diabetes
IL7R	interleukin-7 receptor

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Figure 1.

Alternative splicing regulation and alternative splicing events. (A) The seven major categories of alternative splicing events; (B) Schematic representation of exon-intron boundaries and snRNP spliceosomal components bound to splicing signals and consensus sequences in the pre-mRNA molecule.

Figure 2.

Rbfox1 and Rbfox2 mRNA levels expression is modulated by glucose. INS-1E mRNA expression of Rbfox1 (A) and Rbfox2 (B) were measured by qRT-PCR and normalized by the housekeeping gene Gapdh, after culture for 24h at 0, 1, 5.5., 11 or 16.7mM glucose (experimental conditions and methods as in ¹⁴). (C) Schematic representation of Rbfox1/2 splicing regulatory mechanism in response glucose that may contribute to increased insulin release. Results are mean $\pm$ SEM of four independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs 0mM glucose; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$, & $p < 0.05$, && $p < 0.01$ and &&& $p < 0.001$ as indicated by bars. ANOVA followed by Bonferroni post hoc test.

Figure 3.

Inflammation activates specific splicing regulatory networks in beta cells. Gene ontology terms enriched in genes displaying alternative splicing changes in human islets after 48h exposure to cytokine (IL-1 β + IFN- γ). Data are adapted from ¹⁰ and unpublished.

Figure 4.

The impact of *SRp55* down regulation in beta cell function and survival. Exposure to pro-inflammatory cytokines (IL1 β and IFN γ) for 48h or down-regulation of the candidate gene *GLIS3* leads to down-regulation of *SRp55*. *SRp55* regulates alternative splicing of genes involved in key networks for beta cell function and survival, and *SRp55* inhibition leads to beta cell dysfunction and death. The figure is based on data published in ¹³.

Figure 5. Design of an AON targeting SRp55-predicted binding sites located in BIM pre-mRNA. (A) AON molecule designed against SRp55 predicted regulatory elements, in BIM pre-mRNA (BIM.AON); (B) Predicted binding between BIM.AON (blue line) and BIM pre-mRNA target region. The predicted structures were obtained using RNAstructure software ¹⁰⁷.

Table 1:

Relevant tools for bioinformatics analysis of RNA sequencing and alternative splicing

Workflow	Category	Package	URL	Ref
Pre-processing of raw data	Raw data Quality Control	FastQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	34
		NGSQC	http://www.ngs-qc.org/	35
	Read trimming	FASTX-Toolkit	http://hannonlab.cshl.edu/fastx_toolkit/	36
		Trimmomatic	http://www.usadellab.org/cms/?page=trimmomatic	37
Genome Alignment	Splicing-aware aligners	TopHat	https://ccb.jhu.edu/software/tophat/index.shtml	38
		MapSplice	http://www.netlab.uky.edu/p/bioinfo/MapSplice2	39
		RUM	https://github.com/itmat/rum	40
		STAR	https://github.com/alexdobin/STAR	41
		GSNAP	http://research-pub.gene.com/gmap/	42
		HISAT2	https://ccb.jhu.edu/software/hisat2/index.shtml	43
Quantification / differential gene and isoform expression		Cufflinks	http://cole-trapnell-lab.github.io/cufflinks/cufflinks	31
		RSEM	http://deweylab.biostat.wisc.edu/rsem	33
		edgeR	http://bioconductor.org/packages/release/bioc/html/edgeR.html	44
		Flux Capacitor	http://sammeth.net/confluence/display/FLUX/Home	32
Differential Exon Expression (Alternative splicing)	Isoform centric	eXpress	http://bio.math.berkeley.edu/eXpress	45
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		VAST-TOOLS	https://github.com/vastgroup/vast-tools	49
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		SplicePie	https://github.com/pulyakhina/splicing_analysis_pipeline	50
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