The alternative heart: impact of alternative splicing in heart disease

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ABSTRACT

Alternative splicing is the main driver of protein diversity and allows the production of different proteins from each gene in the genome. Changes in exon exclusion, intron retention or the use of alternative splice sites can alter protein structure, localisation, regulation and function. In the heart, alternative splicing of sarcomeric genes, ion channels and cell signalling proteins can lead to cardiomyopathies, arrhythmias and other pathologies. Also, a number of inherited conditions and heart-related diseases develop as a result of mutations affecting splicing. Here we review the impact that changes in alternative splicing have on individual genes and on whole biological processes associated with heart disease. We also discuss promising therapeutic tools based on the manipulation of alternative splicing.

Keywords: Alternative splicing, Molecular cardiology, Sarcomere, Cell signalling, Ion channels, Antisense oligonucleotides

Our knowledge of gene expression patterns in heart disease has increased over the years [1]. However, this information is largely incomplete, as it mainly relates to whole gene expression and does not take into account the variety of *isoforms* that are generated for each gene by *alternative splicing* (AS). Considering that most human genes are alternatively spliced and thus generate alternative isoforms with different functional and/or structural properties [2,3], the reality is that our knowledge of the molecular mechanisms that mediate heart disease is still very limited. Here we review the impact that AS has on heart development, homeostasis and disease and we discuss the manipulation of AS as a promising therapy for heart disease.

RNA Splicing and Alternative Splicing

RNA splicing is the molecular process by which introns are removed from immature pre-mRNAs and exons are linked together. Splicing is carried out by a large ribonucleoprotein complex, called the spliceosome. It is comprised of more than 100 core proteins and five small nuclear RNAs (snRNAs U1, U2, U4, U5 and U6), each of which interacts with a number of proteins to form small nuclear ribonucleoprotein particles (snRNP) [4-6]. The *core splicing signal* in the mRNA includes three elements that are present in every intron: the 5' splice site (which includes the GU nucleotides), the 3' splice site (which includes the AG nucleotides and the polypyrimidine tract) and the branch point sequence [4]. Splicing is not a rigid process. Very often variation in the final combination of exons and introns in the mature mRNAs occur (Fig. 1). This variation is due to alternative splicing (AS) and has a main role in mRNA stability, localisation and the generation of functional and structural protein diversity. AS allows the production of proteins with different function, structure, localisation and regulation, all from a single gene. It is considered that around 86% of all genes are alternatively spliced and that there are, on average, at least seven AS events per multi-exon gene in humans [2,7]. Genes tend to express many isoforms simultaneously [8], of which 70% encode important functional or structural changes for the protein [3]. Importantly, it was recently unveiled that AS is one of the major forces driving evolution and shaping species-specific features [9,10].

Alternative Splicing Regulation

Regulation of alternative splicing is not only mediated by the core splicing signal, but also by additional elements. Auxiliary *cis-regulatory sequences* found in exons and neighbouring

introns can either facilitate recruitment of the spliceosome and inclusion of an exon in the mature mRNA (splicing enhancer) or they can prevent spliceosome assembly and cause skipping of the exon (splicing silencer) [4,5]. Splicing enhancers are known as ESE or ISE and splicing silencers are known as ESS or ISS, depending on whether they work from an exonic or an intronic location, respectively [4,5]. Whereas enhancer elements are thought to participate mainly in constitutive splicing, silencers have a predominant role in AS [4]. The activity of cis-regulatory sequences can be context-dependent, changing their function when moved to a different gene or region [4]. Pre-mRNAs carry several sequences that can act as potential enhancers or silencers, but only a few of them will be associated with regulatory proteins.

Most auxiliary cis-regulatory sequences act by recruiting *trans-regulatory factors* (Fig. 2). These RNA-binding proteins orchestrate the AS of whole transcript networks in a biologically-coherent manner [11]. Traditionally, it was considered that exonic enhancers bind SR proteins (Ser/Arg-rich), which facilitate spliceosome assembly, while silencers recruit proteins of the hnRNP family to interfere with spliceosome formation and exon inclusion. On the other hand, intronic enhancers bind hnRNPL, RBFOX1, RBFOX2, RBFOX3 and NOVA, whereas silencers recruit hnRNPL, PTB and hnRNPA1, among others [4]. However, it is becoming increasingly clear that the same trans-regulatory factor can act as an enhancer or a silencer depending on its position and context [12-16]. In addition, many trans-regulatory factors act as antagonistic pairs. For instance, the balance between CUG-binding protein (CUGBP) and ETR-like factors (CELF) and PTB regulates exon inclusion or exclusion [17].

Mechanistically, SR proteins initiate spliceosome recruitment by simultaneously binding to an ESE in the mRNA and to the U1 snRNP, allowing interaction of U1 with the 5' splice site in the intron [6,15]. This process is modulated by phosphorylation of the SR protein and represents the first step in the assembly of the rest of the spliceosome, as reviewed elsewhere [6]. Although the molecular mechanism underlying exon skipping is less well understood, it has been recently shown that hnRNP proteins can inhibit exon inclusion by changing the way U1 binds to the exon, which prevents exchange of U1 for U6 and thus precludes the splicing catalytic process [18]. Although SR proteins and hnRNPs are widely expressed, changes in their stoichiometry can mediate tissue-specific AS differences [11].

In addition to direct regulatory factors, it should also be taken into account that splicing takes place as the mRNA is being transcribed and is affected by processes regulating transcription. In this context, it was recently shown that histone modifications affect the

recruitment of trans-regulatory factors and therefore the splicing outcome [19]. The precise balance of regulatory proteins in a cell and cis-regulatory elements in a gene, together with the length of exons and flanking introns, the pre-mRNA secondary structure and epigenetic cues, contribute to determine the splicing pattern [4,11,20].

The interplay between different trans-regulatory splicing factors controls whole biological processes. For instance, in parallel with the changes in gene expression taking place in the postnatal heart, several proteins switch their isoforms by AS after birth to meet the increased contraction demands. This switch is mediated by the balanced action of muscleblind-like (MBNL), RBFOX and CELF trans-regulatory factors, together with microRNAs that control the activity of these factors [21,22]. While CELF proteins decrease during cardiac development, MBNL increases. Manipulation of CELF and MBNL expression in the adult heart to replicate their levels in the embryo results in reactivation of the embryonic splicing pattern [21]. A transcriptome-wide analysis of AS in a mouse model of aortic stenosis suggested a common splicing regulatory programme in the embryonic and diseased hearts [23]. Myocardial expression of many trans-regulatory factors changes in heart failure, both in human patients and in mouse models, following a general downregulation of splicing-related factors [24,25]. Analysis of cis-regulatory motifs in alternatively spliced exons in heart failure patients showed an enrichment in hnRNPF and MBNL binding motifs together with a downregulation of hnRNPF expression itself [24]. In mouse models of myocardial infarction and aortic stenosis, a decline in RBFOX1 has also been reported [23,25]. However the functional implications of these changes and the regulatory mechanisms that control expression of trans-regulatory factors in heart disease are not well understood. It should be also noted that trans-regulatory factors are often regulated by AS themselves, although again the implications for heart disease are unclear.

Alternative Cardiac Contraction and Relaxation

Alterations in sarcomeric gene splicing were among the first to be associated with acquired and inherited heart disease (Fig. 3). A recent survey using exon microarrays showed AS of cardiac troponins T and I, β-myosin heavy chain and filamin Cγ in ischemic cardiomyopathy patients [24]. In thin filaments, a mutation in the 5’ splice donor site in intron 15 of the human cardiac troponin T gene (TNNT2) results in protein truncation and is associated with hypertrophic cardiomyopathy [26,27]. Overexpression of this protein in transgenic mice induces myocellular disarray and diastolic dysfunction, together with a reduction in

cardiomyocyte size and number [28]. AS also controls the transition between embryonic and adult cardiac troponin T isoforms [29]. The switch between both isoforms results from exon 5 exclusion, and is regulated by the trans-regulatory factors MBNL1 and CUGBP2, together with SRSF1 [30-32]. Tropomyosin (TPM) is also subjected to AS, which allows generation of several variants for each of the four tropomyosin genes often in a cell type-specific manner [33]. In the heart, AS of exon 2b in TPM1 produces a variant associated with human dilated cardiomyopathy (DCM) and heart failure [34]. Mice overexpressing this isoform in the heart show impaired cardiac function, decreased myofilament Ca^{2+} sensitivity and DCM [34].

AS also has functional effects on thick filament proteins. Mutations in different splice sites in myosin binding protein C (MyBP-C) promote exon skipping and are associated with hypertrophic cardiomyopathy [35,36]. A net reduction of full-length MyBP-C expression is observed in tissue homogenates, suggesting that splice site mutations cause MyBP-C haploinsufficiency that would be the source of the condition [37]. Also, in the M-band of the sarcomere, an alternatively spliced version of EH-myomesin has been described as a marker of heart failure. This isoform, which is abundant in the embryonic heart, is strongly induced in DCM patients and decreases after mechanical unloading with LVAD [38].

Different protein isoforms of varying sizes are generated from the titin gene by AS of some of its 363 exons. The expression of titin isoforms is developmentally regulated, with larger isoforms found in the developing heart. As the heart matures, they are substituted by smaller isoforms with lower abundance of immunoglobulin and PEVK-rich domains [39]. The reduction in the number of these spring-like elements results in increased passive stiffness. A shift towards expression of the larger more-compliant titin isoforms in the adult heart reduces passive stiffness and is associated with DCM [40]. Interestingly, about a third of the titin mutations found in patients with familial DCM affect splicing [41]. These mutations cause exon skipping, intron retention and other splicing alterations that result in truncated titin isoforms and DCM. Titin splicing is regulated, at least in part, by the RNA-binding motif protein 20 (RBM20). RBM20 is preferentially expressed in the heart and controls AS of genes involved in different processes. Lack of RBM20 in rat results in aberrant titin splicing, cardiac fibrosis, arrhythmias, diastolic dysfunction and DCM, which mirrors the phenotype observed in humans carrying RBM20 mutations [42]. Remarkably, mutations in the Arg/Ser-rich (RS) domain of RBM20 have been associated with 2-3% of all idiopathic DCM cases and up to 13% of cases with cardiac sudden death [43].

RBM20 is also responsible for alternative splicing of LIM-binding protein 3 (LDB3/ZASP/Cypher). Like other enigma proteins, LDB3 is located in the Z-disc and helps maintain the sarcomeric structure. Loss of RBM20 function results in exclusion of exon 4 from the mature LDB3 mRNA [42]. This exon encodes the protein domain that recruits the glycolytic enzyme phosphoglucomutase 1 (PGM1) to the sarcomere under stress conditions and impairment of this interaction is associated with DCM [44]. Similarly, aberrant splicing of other enigma proteins is also associated with heart disease. The splicing pattern of Enigma homologue (ENH) changes during heart development and in response to stress. Whereas some isoforms seem to be cardioprotective, others are sufficient to promote cardiomyocyte hypertrophy, at least in vitro [45].

Alternative Cell Signalling

Insulin-like growth factor (IGF-1) is an illustrative example of functional changes orchestrated by AS. IGF-1 diversity is provided at the N-terminal end of the protein by two alternative promoters that stimulate transcription either from exon 1 or from exon 2, and at the C-terminal end by AS of exons 5 and 6, which encode alternative E-peptides. The different E-peptides interact with the extracellular matrix with different affinity and determine IGF-1 bioavailability [46]. Cardiomyocyte expression of an IGF-1 isoform carrying the Ea peptide (IGF-1Ea) promotes cardiac regeneration following myocardial infarction [47]. IGF-1Ea activates mobilization of endothelial progenitors from the bone marrow to improve vascularisation of the infarct border region [48]. In addition, it reduces oxidative stress and improves cardiac function in a mouse model of DCM [49,50]. In skeletal muscle, IGF-1Ea promotes regeneration and induces expression of the calcineurin splicing variant CnAβ1, which recapitulates the regenerative effects of IGF-1Ea [51,52]. CnAβ1 contains a unique C-terminal domain that is not present in any other known protein. This unique domain is the result of intron 12-13 retention and allows CnAβ1 to interact with the mTORC2 complex and activate the Akt signalling pathway [25]. In contrast with other calcineurin isoforms, which promote pathological cardiac hypertrophy by activating the transcription factor NFAT [53], CnAβ1 has a beneficial effect on the heart. Overexpression of this isoform improves function after myocardial infarction, reducing scar size and fibrosis [25].

Like IGF-1, the biological activity of vascular endothelial growth factor (VEGF) is strongly conditioned by AS. Several different isoforms are generated from a single VEGFA gene depending on how exons 6, 7 and 8 are spliced [54]. Alternative usage of two different splice sites in exon 8 produces two different proteins, differing in just six amino acids, that are either pro-angiogenic or anti-angiogenic depending on whether the proximal or distal splice site is used, respectively. Exons 6 and 7 define the ability of VEGF-A to bind heparin and determine the spatial distribution of the molecule. Isoforms lacking exons 6 and 7 are freely diffusible and knockout mice for these exons show defects in vessel growth and capillary branching [55].

VEGF partly promotes endothelial cell growth and angiogenesis by activating splicing of the transcription factor X-box binding protein 1 (XBP1), a mediator of the unfolded protein response (UPR) [56]. The UPR is activated in infarcted hearts and in heart failure in response to ER stress, and is in part mediated by XBP1 [57]. XBP1 splicing is not carried out by the spliceosome but by IRE1α, a Ser/Thr kinase with RNAse activity that is activated in response to stress. To produce the active XBP1 form (XBP1s), IRE1α removes a short intron in the XBP1 mRNA. This promotes a shift in the reading frame that results in expression of a transcription activation domain at the C-terminal end of the protein [58]. XBP1s induces the B-type natriuretic peptide (BNP) in response to cardiomyocyte stress [59]. BNP is produced by cardiac myocytes to promote diuresis and vascular relaxation in an attempt to reduce fluid retention and blood pressure, and is widely used as a marker of heart failure. An AS isoform of BNP that retains intron 2 was identified in heart failure patients [60]. This isoform lacks the vasorelaxing activity of BNP but retains its diuretic effect on the kidneys. Expression of this variant is around 100x lower than the main BNP isoform and decreases after mechanical unloading with LVAD. Whereas XBP1 splicing can have beneficial effects on the cardiovascular system, sustained XBP1s expression in endothelial cells induces autophagy and atherosclerosis [61].

An AS isoform of the serum response factor (SRF), a transcription factor that controls a variety of cellular processes, has been associated with heart failure [62]. This isoform has little transcription activation capacity and acts as a dominant negative. The SRF coactivator myocardin is regulated by AS in a tissue-specific manner [63]. Its cardiac form carries a unique peptide in the N-terminal domain that allows it to interact with the transcription factor

MEF2 and regulate its transcriptional activity. MEF2 is also differentially spliced in different tissues and developmental stages [64], although the impact on heart disease is still unclear.

AS modulates the function of T-box transcription factors (TBX), which are involved in different aspects of heart development [65]. Two TBX5 isoforms are produced in the heart by AS. The longer isoform (TBX5a) is one of the main regulators of cardiac lineage specification and heart development. Inclusion of exon 8 in TBX5 by AS introduces a premature stop codon and results in a truncated protein with no transcriptional activity (TBX5b) [66]. Whereas there's strong TBX5a expression during cardiac development, TBX5b is the predominant form in the adult heart. Overexpression of TBX5a in the adult heart using transgenic mice induces cardiac hypertrophy, whereas TBX5b promotes growth arrest, at least in C2C12 myoblasts [66]. TBX5 interacts with the trans-regulatory splicing factor SRSF2 to modulate AS [67]. Certain mutations affecting the capacity of TBX5 to regulate splicing are associated with Holt-Oram syndrome, which is characterised by limb malformations and heart septation defects. Other TBX5 mutations with less cardiac penetrance do not affect this activity. This suggests that modulation of AS by TBX5 may be necessary for correct heart development. AS of TBX20 and TBX3 has also been detected in the heart, however the functional implications of the different isoforms are yet to be determined.

Alternative Ion Channels, Calcium Handling and Arrhythmias

Calcium entry through the cell membrane is regulated by voltage-dependent L-type calcium channels composed of $\alpha 1$ (pore), $\alpha 2/\delta$, β and γ subunits. In the heart, the main $\alpha 1$ subunit (CaV1.2) undergoes AS in various exons both during development and in heart failure [68]. Cardiac and smooth muscle express distinct CaV1.2 isoforms that have different sensitivity to dihydropyridine calcium channel blockers [68,69]. Mutations of CaV1.2 in Timothy syndrome, a multisystem disease characterised by cardiac arrhythmias, autism and other symptoms, have different functional impact depending on whether AS of the mutually exclusive exons 8 and 8a takes place [70]. AS of this exon in CaV1.2 is regulated by PTB and also partially by RBM20 [42,71].

Calcium handling in the sarcoplasmic reticulum is regulated by AS of the calcium ATPase 2 (SERCA2) and the ryanodine receptor (RyR) mRNAs (Fig. 3). RyRs facilitate

calcium release from the sarcoplasmic reticulum to induce muscle contraction. The three RyR genes comprise more than 100 exons and generate several isoforms that are differentially regulated by AS in the embryonic and adult hearts. Two splicing variants of the cardiac RyR (RyR2) carrying 24 bp or 30 bp insertions show distinct cellular localization and modulation of calcium release [72]. RyR splicing is regulated, at least in part, by SRSF2. Induction of this trans-regulatory factor has been proposed to be a compensatory mechanism in aortic stenosis patients with cardiac hypertrophy [73]. Knockout mice for SRSF2 show reduced RyR expression and develop DCM [74]. Sarcoplasmic calcium release is also affected by triadin mRNA splicing, which is regulated by SRSF10 and RBM20 [42,75]. SRSF10 knockout mice show reduced expression of triadin and calsequestrin 2 that results in calcium handling defects and embryonic lethality [75]. Calcium is pumped back into the sarcoplasmic reticulum by SERCA2 to allow muscle relaxation. Expression of the SERCA2a isoform in cardiac and slow skeletal muscle is regulated by AS. Disruption of SERCA2 splicing in knockout mice results in a switch from SERCA2a to SERCA2b, a non-muscle isoform present in other cell types. These mice show reduced calcium uptake, impaired contractility and relaxation, developmental cardiac defects and mild concentric hypertrophy [76].

The calcium/calmodulin-regulated kinase IIδ (CaMKIIδ) phosphorylates different calcium-handling proteins, including the SERCA regulator phospholamban. CaMKIIδ undergoes a postnatal isoform switch mediated by the trans-regulatory factors SRSF1 and RBM20 [32,42]. Loss of either of these factors results in exclusion of exon 14, which encodes a nuclear localisation signal, and mislocalisation of the protein. SRSF1 knockout mice show severe excitation-contraction coupling defects, cardiac fibrosis and death at 6-8 weeks of age. Transgenic expression of the alternatively spliced CaMKIIδ in cardiomyocytes phenocopies these defects, suggesting that AS of this kinase is responsible for the phenotype observed in SRSF1 knockout mice.

Sodium and potassium channels are also subjected to AS. Heart failure patients show increased expression of C-terminal truncated isoforms of the sodium channel SCN5A [77]. SCN5A splicing is altered in these patients by an increase in the splicing regulators RBM25 and LUC7L3. They favour the production of a truncated isoform and a reduction in the full-length protein, resulting in a strongly decreased Na^+ current [78]. Mutations affecting SCN5A splicing in humans are associated with ventricular fibrillation [79]. Similarly, mutations in the potassium channels KCNH2 and KCNQ2 cause intron retention and exon exclusion,

respectively, and are associated with long QT syndrome [80,81]. Besides channels themselves, AS of the K^+ channel interacting protein 2 (KChIP2) generates two splicing variants that modulate the outward K^+ current of Kv4.3 channel [82].

Alternative Splicing Associated Disorders

Several disease-causing mutations are estimated to affect splicing and directly or indirectly alter either regulatory sequences or trans-regulatory factors [83]. Myotonic dystrophy (DM) is probably the main example of an inheritable disease caused by aberrant splicing regulation. DM patients show progressive muscle wasting, muscle hypercontractility, cardiac conduction defects, insulin resistance and cardiomyopathy, among other symptoms. DM type 1 (DM1) is caused by an accumulation of a CTG repeat in the 3' untranslated region of the DM protein kinase (DMPK) gene, whereas DM type 2 is caused by a CCTG expansion in the zinc finger protein 9 (ZNF9) gene [84,85]. Aberrant repetition of these motives does not affect the proteins encoded by these genes but instead affects the splicing factors that bind these motives in their mRNAs. The CTG (CUG in the mRNA) expansion results in sequestration of MBNL1 and increased expression of CUGBP1. Transgenic mouse models carrying the CTG expansion in DMPK, lacking MBNL1 or overexpressing CUGBP1 display a similar phenotype to that observed in DM1 patients [86-88]. These mice showed that increased expression of CUGBP1 in DM1 is due to its hyperphosphorylation and stabilization by PKC [89]. Inhibition of PKC was able to improve cardiac function in these mice although it did not reduce mortality [90]. The imbalance of CUGBP1 and MBNL1 disrupts AS of other cellular mRNA targets, including cardiac troponin T, the insulin receptor or the chloride channel Clcn1, and results in muscle dystrophy, insulin resistance and myotonia [84,91-93]. In addition to splicing alterations, the toxic DMPK mRNA induces expression of the transcription factor NKX2.5, resulting in a decrease in connexin 43 and cardiac conduction defects [94].

The Hutchinson-Gilford progeria syndrome (HGPS) is another major example of a disease associated with splicing defects. Progeria patients show premature ageing and progressively develop atherosclerosis in their coronary and cerebral arteries that eventually causes their death. Progeria is caused by mutations in the gene encoding the nuclear envelope protein lamin A (LMNA) [95]. A single base mutation in exon 11 of the LMNA gene activates a cryptic splice site. This causes alternative splicing of the mRNA and production of a truncated protein (called progerin) with a 50 amino acid deletion in the C-terminal domain.

These amino acids are necessary for the processing of prelamin A into lamin A by the protease ZMPSTE24 and for translocation of lamin A to the nuclear lamina [96]. Expression of progerin results in structural defects in the nuclear envelope and affects cell division and chromatin organisation. AS of exon 11 is controlled by SRSF1 and SRSF6. Whereas inhibition of SRSF1 enhances correct splicing of the lamin A mRNA, downregulation of SRSF6 promotes progerin expression [97]. Interestingly, progerin is also expressed at low levels in healthy individuals and its expression increases with ageing [98]. Telomere shortening promotes AS of lamin A and several other genes that may also contribute to cellular senescence and ageing [99]. Modulation of lamin A splicing with an antisense oligonucleotide in a progeria mouse model has shown promising results [100].

Alternative Splicing-based Therapeutics

Besides the use of specific splicing isoforms for the treatment of heart disease, as explained above for IGF-1Ea and CnAβ1 [25,47], AS can be manipulated using small modified antisense oligonucleotides (AONs) [xref antisense therapeutics in this JCTR issue]. These oligonucleotides carry an antisense sequence that binds to a splicing regulatory sequence in the target exon, interfering with splicing and preventing incorporation of the exon into the final transcript (Fig 4). AONs carry chemical modifications in their backbone that makes them more stable, increases their target selectivity and makes the RNA-RNA interaction resistant to cleavage by RNAse H [101].

In a DM1 mouse model, a morpholino oligonucleotide targeting the CTG repeat prevents MBNL1 sequestration, corrects splicing, reduces the effects of the toxic mRNA and improves the disease symptoms [102,103]. A similar approach has been used for the treatment of Duchenne muscular dystrophy (DMD), in which several mutations in the dystrophin gene cause premature truncation of the protein. Skipping of certain exons using AONs can restore the open reading frame, generating a partially active protein [104]. Cardiac delivery of AONs has proven challenging, but new biochemical modifications in the oligonucleotides have improved splicing of the mutated dystrophin exons in the heart [105]. In fact, DMD is the disease for which an AON therapy is closest to application in humans [106]. In addition to AONs, skipping of the mutated dystrophin exons has been achieved in small and large animal models using gene therapy. An adeno-associated viral vector expressing a modified U7 small nuclear RNA targets specific cis-regulatory sequences in the

dystrophin mRNA, promotes exon skipping and improves expression of a functional dystrophin protein [107].

AONs have also been used to treat progeria in animal models. An AON designed to block the binding of the splicing machinery to the mutant splice site in prelamin A favours expression of the full-length protein and ameliorates the progeria phenotype [100,108]. Another disease for which AONs are being developed is spinal muscular atrophy, which is associated with arrhythmias and other cardiac defects, among other symptoms. This disease is caused by mutations in the spinal motor neuron 1 gene (SMN1) that result in loss of function of the protein and motor neuron disease. AON therapy to promote inclusion of exon 7 in the second SMN gene (SMN2) produces a functional SMN protein that partially compensates the loss of SMN1 [109]. These encouraging results observed in animal models have prompted a number of clinical trials using AONs to treat different diseases, including muscle dystrophies, progeria and spinal muscular atrophy [110]. Beside the treatment of inherited conditions, AONs have been used to correct splicing in other diseases like cancer [111,112]. In addition, AONs have been used to model human disease in mice [113].

Future Perspectives

Our knowledge of the involvement of individual splicing isoforms in heart disease has increased over the years; however we still lack an understanding of how global alternative splicing patterns are regulated. The advent of next-generation sequencing has paved the way to a more comprehensive view of transcriptome-wide changes and some general splicing patterns are beginning to emerge. Potential applications include the detection of novel splice sites and cis-regulatory elements, as well as the analysis of relative isoform abundances and alternative exon usage. Despite the variety of bioinformatics methods available, computational analysis of AS remains however challenging and demands the development of new ad hoc strategies and standardised protocols. Another major objective in the field is to determine how many of the alternative isoforms generated for each mRNA are actually translated into proteins. In addition, it needs to be determined whether the splicing changes observed in heart disease, besides genetic mutations, are actually a cause or a consequence of the condition and whether they are actually detrimental. Currently there's no answer to these questions and thus the exact impact of AS on heart disease remains to be determined. On the bright side, manipulation of AS using antisense oligonucleotides or the use of specific

splicing isoforms for the treatment of muscular and heart diseases is progressing fast and providing encouraging results.

ACKNOWLEDGEMENTS

E.L-P. is supported by grants from the European Union's FP7 ("CardioNeT" ITN-289600), the Spanish Ministry of Economy (SAF2012-31451) and from the Madrid Regional Government ("Fibroteam", S2010/BMD-2321). P.G-P. and E.L-P. are supported by the Instituto de Salud Carlos III (Red Enfermedades Cardiovasculares RD12/0042/0002).

CLINICAL STATEMENT

Alternative splicing is the main mechanism driving protein diversity and allows expression of different proteins from each gene. We are slowly beginning to unveil the complexity of post-transcriptional regulation in the diseased heart. Alternative splicing can change protein structure, localisation, regulation and function, leading to cardiomyopathies, arrhythmias and other pathologies. Identifying a protein that is differentially expressed in a pathological condition is not sufficient; it is essential to identify the version (isoform) of such protein, which may have particular features. It is also important to understand how alternative splicing is regulated in a global manner, so that potential therapeutic targets can be unveiled. Alternative splicing modulation using antisense oligonucleotides has recently emerged as a therapeutic option for different conditions in which splicing is altered.

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FIGURE LEGENDS

Figure 1. Alternative splicing allows the expression of different proteins with distinct functional and structural features from a single gene. As the mRNA is being transcribed, introns are removed and exons are linked together by the spliceosome in a process known as splicing. Variations in the inclusion of exons, intron retention or the usage of an alternative splicing site give rise to proteins with different length, structure, localisation, regulation and/or function.

Figure 2. Alternative splicing is controlled by cis-regulatory elements and trans-regulatory factors. Splicing is carried out by a large ribonucleoprotein complex (spliceosome) comprised of more than 100 core proteins and five snRNAs (U1, U2, U4, U5 and U6). Alternative splicing regulation is mediated by cis-regulatory sequences found in the spliced exon and in neighbouring introns. Cis-regulatory sequences can facilitate inclusion of an exon (splicing enhancers) or they can cause exon skipping (splicing silencers). Depending on their exonic or intronic position, splicing enhancers are known as ESE or ISE and splicing silencers are known as ESS or ISS. Most auxiliary cis-regulatory sequences act by recruiting trans-regulatory factors. In general, splicing enhancers bind SR proteins (Ser/Arg-rich SRSF factors), which facilitate spliceosome assembly, whereas splicing silencers recruit proteins of the hnRNP family, which can interfere with recruitment of the spliceosome or SR proteins.

Figure 3. Alternative splicing regulates calcium handling and sarcomere contraction. The figure shows proteins involved in calcium mobilisation, cell signalling and sarcomere contraction that are regulated by alternative splicing, except for phospholamban. Calcium entry through the voltage-gated L-type calcium channels (LTCC) promotes calcium release from the sarcoplasmic reticulum through the ryanodine receptor (RyR). This promotes muscle contraction until calcium is pumped back into the sarcoplasmic reticulum by the SERCA2 ATPase. Calcium also activates calcineurin (Cn) and the calcium/calmodulin-dependent protein kinase II (CaMKII), which modulate different processes in the cardiomyocyte. Some of the alternative splicing changes in the mRNAs encoding these

proteins are associated with cardiomyopathies and heart failure, while others have a positive effect on the heart.

Figure 4. Modulation of alternative splicing using antisense oligonucleotides as a therapeutic tool. A, Exon skipping of a mutated exon in the dystrophin gene (DMD) is promoted in Duchene muscular dystrophy to avoid premature truncation of the protein. Pathological splicing is indicated by a dashed line; the antisense oligonucleotide (AON) and the resulting corrected splicing are shown by thick lines; * indicates a mutation. **B**, Inhibition of an alternative splicing site due to a single mutation in Lamin A (LMNA) is blocked using AONs for the treatment of progeria. **C**, In myotonic dystrophy, a CUG expansion that sequesters MBNL1 in the DMPK and ZNF9 mRNAs is neutralised by AONs, allowing MBNL1 to reach its natural targets. **D,** Loss-of-function mutations in SMN1 cause spinal muscular atrophy. An AON targeting exon 7 in SMN2 promotes inclusion of the exon and the production of a functional SMN2 protein that partially compensates the loss of SMN1.

Figure 1
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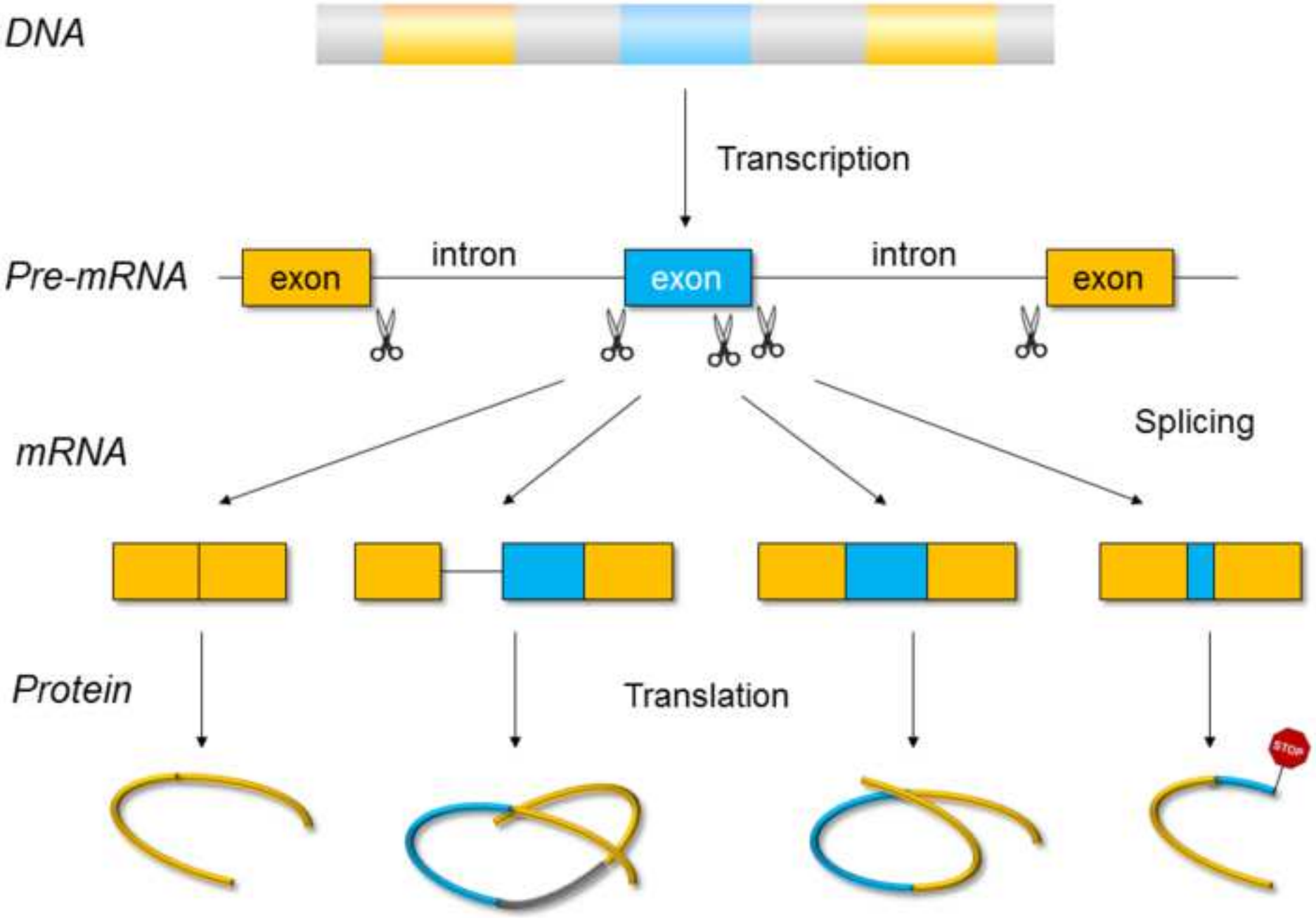

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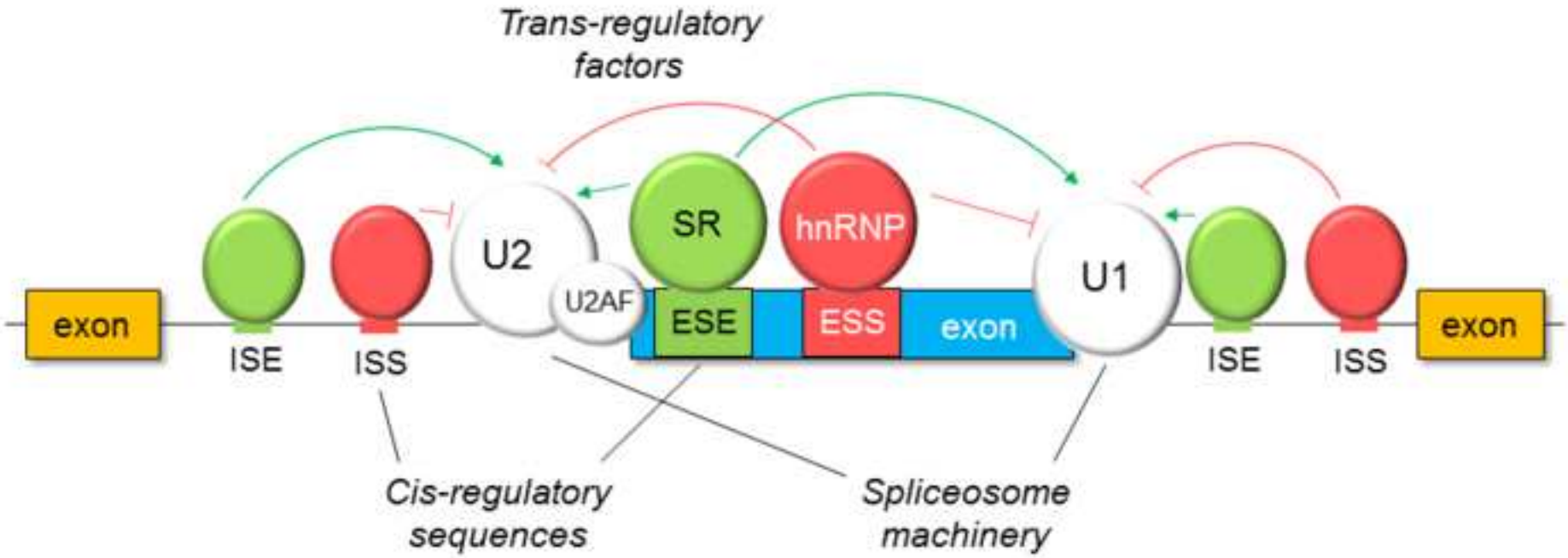

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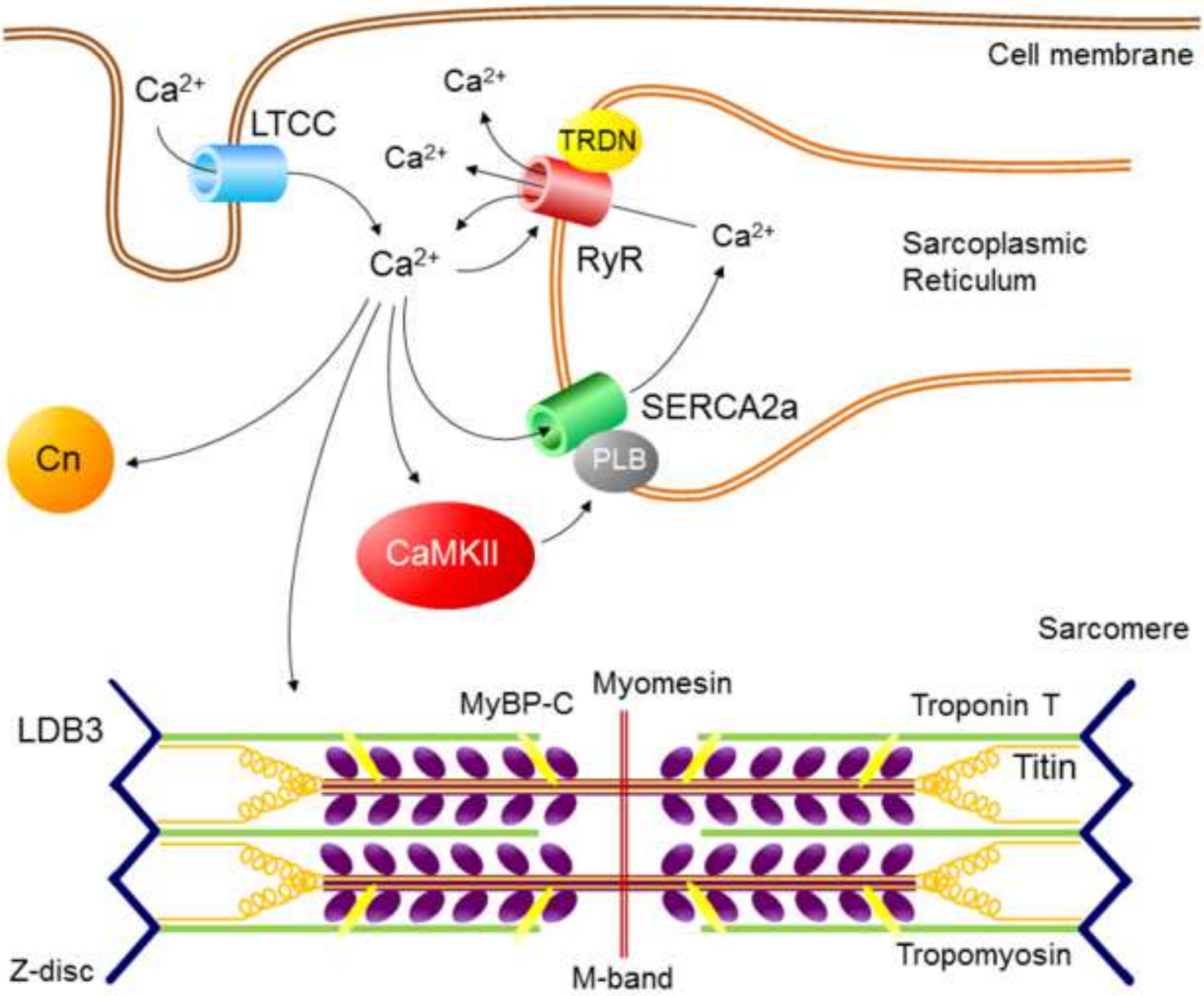

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