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Antisense oligonucleotides, exon skipping and the dystrophin gene transcript

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Antisense oligonucleotide induced exon skipping has recently emerged as a potential therapy to by-pass the consequences of many, but not all dystrophin mutations that lead to Duchenne muscular dystrophy. Targeted removal of one or more exons, to restore a disrupted reading frame, or omit a nonsense mutation, could lessen the consequences of an estimated 80% of dystrophin gene mutations. Promising *in vitro* and *in vivo* experiments in animal models of dystrophinopathies, as well as demonstration of induced exon skipping in cultured human myogenic cells have prompted considerable enthusiasm. Furthermore, advances in antisense oligonucleotide chemistries have resulted in the development of more stable and less toxic compounds, some of which are currently in Phase III clinical trials for selected antiviral applications. This review will summarize developments in induced exon skipping that have paved the way to clinical trials and some of the challenges and possible limitations.

Keywords: Alternative splicing, Revertant Fibres, Mutation suppression, Duchenne muscular dystrophy

Introduction

Mutations in the dystrophin gene that preclude the synthesis of a functional protein lead to Duchenne muscular dystrophy. In developing a treatment for DMD, compensating for the defective dystrophin gene has now been recognised as a much greater challenge than originally anticipated. Potential therapies have included cell (myoblast, satellite and stem cell) or gene replacement (viral and non-viral delivery) (for review see [1,2], read-through of nonsense mutations [3,4], corticosteroids [5-8] or inhibition of specific proteolysis [9,10] and the subject of this review, antisense oligonucleotide (AO) induced exon skipping. Of all these approaches, it is only the latter that has any natural precedent.

Dystrophin positive revertant fibres [11] in dystrophic tissue arise from an unknown exon skipping mechanism [12-14], while the variable phenotypes observed in Becker muscular dystrophy patients clearly demonstrate that some in-frame, internal deletions of dystrophin, particularly in the rod domain, can result in a protein of near normal function [15-17].

Furthermore, although chemically synthesised antisense oligonucleotide (AO) analogues cannot be regarded as natural compounds, small, naturally occurring, non-coding RNAs have been identified and implicated in the control of a variety of cellular processes [18]. Small RNAs have been shown to silence selected genes [19] and modify gene expression at the level of splicing or translation [20]. Therefore, the application of AOs to modify gene transcripts for therapeutic outcomes should not be regarded as whimsical.

Natural precedents for an Exon Skipping approach to address dystrophin mutations

Revertant fibres were reported in the *mdx* mouse [11] and in DMD patients [21] and so named because of 'reversion' to the normal dystrophin staining pattern. Various dystrophin mRNA transcripts excluding the primary genetic lesion, and in which the reading frame has been restored or maintained have been described in human, canine and murine dystrophic tissue [12, 22, 23]. It is now clear that revertant fibres result from an exon skipping mechanism, and that not all have the same exonic combination [13]. *In situ* hybridization studies using a dystrophin intron 21-exon 25 genomic probe on *mdx* mouse muscle showed that the dystrophin gene was intact in the majority of revertant fibres, and RT-PCR and antibody epitope mapping indicated that the most common exon skipping rearrangements involved 20 or more exons [13].

With the apparent exclusion of secondary somatic genomic deletions within the dystrophin gene being the cause of revertant fibres, the mechanism

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responsible for generating dystrophin is most likely to involve a localized alteration in splicing. Since revertant fibres occur singly or in small clusters, suggesting a clonal origin [11,13, 24], the events that bring about exon skipping must only occur within the dystrophin–positive fibres and not in the surrounding muscle. Small non-coding RNAs have recently been credited with controlling aspects of gene expression, from splicing to translation [19,20]. The possibility exists that the revertant fibres express novel microRNA variants that interfere with dystrophin pre-mRNA processing.

Apart from confirming the existence and utility of exon skipping in the dystrophin gene transcript, another important property of revertant fibres is that they not only demonstrate immune tolerance to dystrophin, but may also play a causative role in the development of this tolerance [25,26]. Depending upon the nature and position of the mutation, production of amino terminal fragments and dystrophin isoforms from internal promoters would also expose the immune system to various dystrophin epitopes. Consequently, an immune response to any induced dystrophin in individuals who have revertant fibres is considered unlikely, although the possibility of novel epitopes encoded by the induced exon junctions cannot be excluded.

The dystrophin gene rearrangements in mildly affected BMD patients clearly demonstrate that some domains are not essential for near-normal function. The reading frame rule [27] holds true for the majority of dystrophin mutations. Nonsense or frame shifting mutations result in premature termination of translation and the absence of a functional protein leads to DMD while in-frame deletions cause BMD [27]. In some cases, the consequences of a deletion are so mild that the individual is asymptomatic and may only be diagnosed later in life [16,17,28,29]. There appears to be an upper limit to the size of in-frame deletions that may be tolerated, where the loss of 34 or more exons is invariably associated with a severe phenotype [30].

Exceptions to the reading frame hypothesis and the need for precise mutation detection

Apart from rare mis-sense mutations in crucial binding domains of the dystrophin gene, many of the apparent exceptions to the reading frame rule may be explained when the responsible secondary mechanisms are identified. Some dystrophin nonsense mutations do not lead to DMD, since the base change compromises motifs involved in pre-mRNA processing [31,32]. In these cases, the nonsense mutation may prevent efficient exon recognition by the splicing machinery and the exon is variably excluded from the mature dystrophin mRNA. If loss of the exon does not disrupt the reading frame, the nonsense mutation is removed from the mature dystrophin gene transcript and a slightly shorter, BMD-like protein can be produced. The amount of functional dystrophin generated, and hence the severity of the phenotype, reflects the degree to which the exon is excluded [32]. If the effect of the base change were to marginally weaken splicing, generating only a small percentage of the transcripts missing the mutation, a more severe phenotype would be predicted. Conversely, if exon skipping as a result of the nonsense mutation was complete and assuming that the lost coding domain was not essential, the patient could be asymptomatic.

It has been estimated that some 15% of human mutations alter splicing [33]. Changes in primary splicing motifs that may be readily identified include, the branch-point, acceptor and donor splice sites. Other DNA changes that may alter splicing can be less obvious, particularly when a single base change deep within an intron results in the inclusion of a pseudo exon [34]. Intronic changes over 10 kilobases from the nearest coding sequence have been shown to alter the processing of dystrophin exons [35]. Exonic splicing enhancers, motifs recognised by splicing factors such as the SR-proteins can be predicted *in silico* [36] but accurate identification occurs when a particular exonic base change modifies the splicing pattern [37]. An apparently neutral polymorphism (C>T change at the third base of codon 608 in the lamin A/C gene) is responsible for Hutchinson-Gilford Progeria Syndrome [38-40]. This *de novo* substitution activates a cryptic splice site 5 bases upstream that leads to the loss of 150 nucleotides from the gene transcript [40].

It is examples such as these that emphasize the need for detailed molecular characterization in disease diagnosis, so that not only are DNA changes detected, but the consequences of the alterations are considered. Furthermore, precise mutation detection will be essential prior to the application of targeted therapies such as splicing manipulation. The boundaries of the genomic deletions or duplications must be clearly defined so that the appropriate target site can be characterized for the design of AOs to restore the reading frame. Similarly, any exon carrying a

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