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Gene Therapy

The 'pro-sense' approach to Duchenne muscular dystrophy

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It seems that a clinically applicable gene therapy for Duchenne muscular dystrophy is within our grasp, now that two recent studies show that safe and efficient systemic delivery of antisense oligoribonucleotides (AONs) to induce exon-skipping in the dystrophin gene is possible.[1,2]

After years of struggle to develop a gene therapy for Duchenne muscular dystrophy (DMD), there now seems to be a tool with which we can utilize an escape route that nature had already hinted at. A deficiency of the membrane protein dystrophin causes the progressive deterioration of muscle fibres in DMD. Sometimes however, DMD patients have rare, dystrophin-positive fibres ('revertant fibres') that originate from exon skipping in the dystrophin gene, which generates a truncated transcript with a restored open reading frame.[3] Over the last 6 years, several laboratories have shown that we can actually enhance or induce this therapeutic exon skipping,[4] using small synthetic AONs.

The simplicity of the AON approach, as well as its specificity and efficacy, has been amazing. The important issue that remained, which the two new studies by Lu *et al*[1] and Goyenvalle *et al*[2] addressed, was how to develop a safe and efficient systemic delivery method that reaches all skeletal and cardiac muscles.

AON-induced exon skipping therapy is based on the reading frame rule.[5] This rule states that frame-shifting mutations in the DMD gene cause DMD, whereas frame-conserving ones mostly cause the milder Becker muscular dystrophy (BMD). Through the skipping of exons in DMD transcripts, AONs can restore the reading-frame, and convert DMD into BMD-like fibres. AONs vary in length between 16 and 22 nucleotides and are chemically modified to be resistant to intracellular nucleases and RNaseH. It is thought that they bind to specific sequences in the pre-mRNA, and thus disturb exon inclusion signals like splice sites, intronic branch point sequences, or exonic splicing enhancer elements. This, in turn, leads to the removal of the targeted exon from the processed mRNA.

These new studies[1,2] clearly show that the AON-induced skipping of exon 23 is therapeutic for the *mdx* mouse. This is an animal model that is dystrophin-deficient due to a nonsense mutation in the in-frame exon 23. Lu *et al* use an AON that targets the 5′ splice site of this exon, in combination with a 'drug carrier' called F127. This block copolymer belongs to the group of amphiphilic Pluronics that is extensively used in the pharmaceutical industry. F127 promotes the metabolic stability and circulation time of AONs in the blood circulation and their transport across cell membranes.

In a previous study, the same authors applied intramuscular injections and detected dystrophin expression that resulted from frame-restoring exon 23 skipping in up to 20% of muscle fibres.[6] This expression persisted for 2 months and significantly improved the strength of the treated muscles. In the recent study,[1] they injected 2 mg of the same AON with F127 through the tail vein in *mdx* mice. At 2 weeks after a single injection, significant numbers of dystrophin-positive fibres were detected in all muscle groups analysed, including the diaphragm. The distribution of dystrophin-positive fibres was highly variable: a pattern that the authors attributed to the cycles of degeneration and regeneration in individual *mdx* muscle fibres that led to differential uptake of the AONs. After repeated administration, dystrophin levels accumulated up to 1–5% of normal, while the variable dystrophin levels stabilized.

Other indications for a significant role of the regenerative process on the AON uptake were the absence of dystrophin expression in the heart (an organ without regenerative capacity), and the increased dystrophin induction in older *mdx* mice (6 weeks or 6 months) *versus* younger mice (3 weeks). This is a major advance for systemic AON administration. However, since regeneration in *mdx* mice is quite different to that in DMD patients, the clinical relevance of these results remains debatable. Last but certainly not least, while the role of F127 in the systemic uptake remains to be investigated, it

appeared to be a very safe carrier. No tissue damage (including liver and kidney) or monocyte infiltration was observed and the serum enzymes and electrolytes were within normal levels.

Goyenvalle *et al* chose a more complex but also a more efficient and persistent strategy.[2] They explored a previously described antisense-plasmid system – U7SmOPT –,[7] which contains a modifiable gene for U7 small nuclear RNA (U7snRNA). This RNA component of the U7 ribonucleoprotein particle (U7snRNP) is involved in the processing of the 3′ end of histone pre-mRNAs within the nucleus, through an antisense mechanism. In the U7SmOPT plasmid, the original U7 antisense sequence was replaced by two different DMD antisense sequences, which target the 5′splice site of exon 23 and the branch point sequence in intron 22. This modified U7snRNA gene was inserted in a recombinant adeno-associated virus (rAAV). AAV is nonpathogenic and therefore considered as a safe gene therapy vehicle. Importantly, in contrast to other viral vectors, rAAV vectors have been very efficient in transducing mature skeletal muscle.

Following intramuscular injections of this engineered rAAV-U7-AON vector in *mdx* mice, the French group obtained dystrophin-positive fibres in up to 77% of the tibialis anterior muscle. After 3 months, this proportion was still 50%. The dystrophin-rescue restored normal histology of the treated muscle, without any signs of an immune response against either rAAV or the novel dystrophin. When delivered by intra-arterial perfusion of the lower limb, an even higher level (>80%) of dystrophin-rescue was observed. Moreover, this strategy completely restored the dystrophin–glycoprotein complex with which dystrophin is associated. As a result, the contractile and mechanical properties of the treated muscles, and their resistance to exercise induced damage, were improved to normal levels.

These results are obviously very promising. However, the safety concerns inherent to the use of viral vectors in gene therapy cannot be ignored. In particular, further investigation is required for the longer-term effects of this strategy in humans, the potential immunological reaction after repeated treatments, the effect of pre-existing AAV neutralizing antibodies in 10–30% of population, and the risks of integration-related mutagenesis. Although the severity of DMD, and thus the urgent need for an effective treatment, might outweigh some of these risks, they should still be assessed before we consider applying this approach in the clinic.

These studies represent increasing progress in the development of antisense-induced exon skipping for DMD. AONs have also successfully been applied in cultured human muscle cells from DMD patients.[4] The relative simplicity of AONs is for many scientists a relief, and, more importantly, offers the DMD patients and their parents new hope that we finally will be able to alleviate or even stop the progression of this terrible disease. Whereas the term 'antisense' is in accordance with their proven therapeutic applications *against* genes in cancer and viral infections, their capacity to *create* sense in DMD-associated transcripts would deserve a more positive label. Pro-sense...? ■

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Genetic Epidemiology of Cancer

Relatively risky relatives

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Epidemiological studies that demonstrate familial clustering of specific cancers in close (first and second degree) relatives are common,[1] but a new study that draws on a unique combination of databases shows that more distant relatives of those with cancer also have a higher risk of developing the disease.[2]

In principle, clustering of cancers within a nuclear family could be the result of either inherited factors or environmental and lifestyle factors that are shared within those families. If genetic factors are important, more distant relatives, which are less likely to share environmental and lifestyle characteristics, would also be at increased risk. However, few studies have been able to effectively assess familial clustering in more distant relatives. Now data from the Icelandic Cancer Registry (ICR) linked to the deCODE genealogy database have enabled Laufey Amundadottir *et al*[2] to uncover distant familial connections between cases and so estimate cancer risks for more distant relatives.

This new study's finding that relatives outside the nuclear family of a patient with cancer also have increased cancer risks indicates that genetic rather than environmental factors are important. Furthermore,