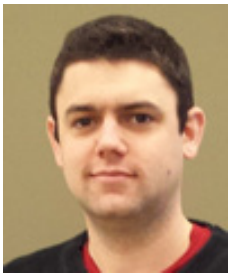

EDITORIAL

Advances and challenges of using CRISPR-Cas9 gene editing for treating Duchenne muscular dystrophy

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Editing the *DMD* gene through the CRISPR-Cas system appears to be one of the finest approaches to cure DMD patients carrying diverse mutations.

In 2016, the Food and Drug Administration (FDA) accelerated the approval of Exondys 51™ (eteplirsen), from Sarepta Therapeutics Inc., for the treatment of the Duchenne muscular dystrophy (DMD). Despite being controversial, the approval revealed the urgent need for treatment for DMD, one of the most common inherited genetic diseases, which affects boys and condemns them to a premature death between the age of 17 and 30 years. Exondys 51™ is an antisense oligonucleotide used to skip the exon 51 [1] in order

to restore the reading frame of the dystrophin (Dys) protein, encoded by the *DMD* gene. If Exondys 51™ works as predicted, it can be used to treat approximately 13% of the mutations known to cause DMD [2]. Thus Exondys 51™ might help some, but not all patients.

The *DMD* gene covers 2.22 megabases at locus Xp21. It is the largest gene of the human genome (0.08% of the genome), encoding 79 exons and a 14 kb cDNA coding for the very complex Dys protein [3]. It therefore represents a noteworthy challenge for a potential gene replacement therapy. Promising increases in muscle force have been obtained in a dog model of DMD by delivering a micro-dystrophin with a modified adeno-associated virus serotype 9 (AAV9) [4]. However, trials in human patients did not reach the same level of improvement, possibly due to an immune reaction that shut down the expression of the micro-dystrophin, as suggested by the presence of auto-reactive T cells against truncated dystrophin expressed in revertant dystrophin fibers [5]. Another therapeutic approach to treat patients is the graft of cultured normal myoblasts into muscle tissues [6,7]. The administration of myogenic cells is a potential treatment for a specific muscle or a muscle group, for example to restore functionality of the hand. Although this treatment does not expose the patient to immunogenic viral vectors [8], a life-long immunosuppressive treatment is required to prevent the immune response to the allogeneic donor cells. An alternative investigated by many groups, including ours, is to derive myogenic cells from the patient's own genetically corrected induced pluripotent stem cells (iPSCs) [9]. The main disadvantage of this approach is that it is very labor intensive and would thus be very costly.

There is currently a new promising therapeutic approach aiming to correct genes directly. The convenient CRISPR-Cas system brings to scientist minds, what seems to be, an infinite range of possibilities. Indeed, the editing of the *DMD* gene appeared to be one of the finest approaches to cure DMD patients, carrying diverse mutations.

70% of DMD patients have a deletion of one or more exons within the *DMD* gene that leads to a premature stop codon and to the absence of the dystrophin protein [10]. Patients with a milder form of muscular dystrophy, called Becker muscular dystrophy (BMD), carry deletions that do not cause a frame shift but the expression of an internally deleted dystrophin [11]. BMD patient symptoms may vary depending on the structure of the remaining dystrophin protein [12]. Thus different combinations of guided RNAs (gRNAs) could be used to delete complete exons to restore a normal reading frame. Approximately 70–90% of DMD patients could benefit from single exon or multiple exons deletion strategies [13]. Since it has been estimated that even low-level expression of dystrophin (3–15% of wild-type) could be sufficient to ameliorate cardiomyopathy and skeletal muscle symptoms [14–16], the effects of a gene editing therapy can be substantial for the patient.

The beginning of the year 2016 has been a great one for DMD patients. With the publication of many papers in late December 2015 reporting the gene editing of the *DMD* gene and the restoration

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of the dystrophin expression came hope in the development of new treatments. Three groups have shown the targeted deletion of the mouse exon 23 in the mdx mouse model and the resulting *de novo* mouse dystrophin protein expression [17–19]. All these groups delivered the CRISPR component using AAVs. A direct *in vivo* injection of AAVs encoding the *S. aureus* Cas9 and gRNAs into the *Tibialis anterior* (TA) of the mdx mouse allowed the re-localization of the multimeric dystrophin-glycoprotein complex and the neuronal nitric-oxide synthase at the sarcolemma [19]. This "myoedition" [17] was also proven to be beneficial for AAV9-injected mice as the grip strength test showed a significant increase in strength at 4 weeks post-injection [17]. All these papers were able to show an efficient *in vivo* delivery and a strong evidence of a dystrophin restoration, as well as the first evidence that gene editing can improve the phenotype of an animal model of muscular dystrophy [20].

The latter single exon deletion strategy represents certainly an excellent proof-of-principle that CRISPR-Cas technology can be used to correct the *DMD* gene *in vivo*. However, exon deletion in general might not be the best therapeutic option because the resulting dystrophin protein will not fold into a proper structure. Le Rumeur's group showed that the expression of a dystrophin protein with an inadequate spectrin-like repeats (SLR) leads to a severe BMD phenotype [12], especially when the interaction with nNOSμ is abrogated [3]. The dystrophin protein has a central rod domain containing 24 SLRs, each comprising three α-helices (A, B and C) forming a coil–coil structure [10,21]. Since the limits of the coding sequences of these helices do not correspond precisely to the limits of the exons, exon deletions are more likely to produce a protein where helices are not aligned. In that case, what appeared to be a great treatment will maybe end up to be disappointing because of the inadequate structure of the edited dystrophin protein, even when the absence of the nNOSμ interaction is compensated by the administration of PDE5 inhibitors [22].

> Among the remaining challenges to cure DMD is certainly the absence of an appropriate animal model.

Our group has developed an alternative approach in which the formation of a hybrid exon not only restores the normal reading frame but also codes for a dystrophin protein with an adequate SLR containing a normal succession of helices A, B and C [23]. Selection of targets is made in the existing exon sequences flanking the mutated or deleted exons and introns. This selection should reduce the "stochastic indel-derived frame shifting" [24] by forcing the creation of an hybrid exon. This precise reframing would potentially be beneficial for the overall structure of the protein, as suggested by software predictions [23]. However, the functionality of the protein remains to be demonstrated in an *in vivo* model. This CRISPR-induced deletion (CinDel) approach, as we named it, could also be used to remove an exon or part of exon containing a non-sense mutation.

The CRISPR-Cas9 gene editing approach, which is being tested in a human for the first time, will have answers about the feasibility, toxicity and long-term effect of this approach in treating human diseases.

Among the remaining challenges to cure DMD is certainly the absence of an appropriate animal model. In the mdx mouse, the nonsense mutation present in the exon 23 prevents the expression of an internally truncated dystrophin protein. Also, only the SLR 6 is affected by exon 23 removal while most of the mutations in DMD patients are located in the hot-spot region containing exons 45–55 and corresponding to SLR 16–22 [25]. In the humanized DMD (hDMD) mouse model, a complete human DMD transgene is expressed while the mouse DMD gene is knockout [26]. This model can be used to test gRNAs *in vivo* [23] but the wild-type protein is preferentially expressed, making this model useless for functional and phenotypic analyses. Many other mdx-derivative mice and other models of dystrophic mice have been generated [27]. If the closest DMD phenotype is preferred, it would be best to also have a good representation of DMD mutations. To do so, an hDMD-derivative model has been recently generated in our group [Unpublished data] in order to test gRNA combinations that can be transferred more rapidly into human clinical trials.

Another challenge, yet partially addressed, is the delivery of CRISPR components. This includes the shuttle vector design, i.e., the specificity of the promoter, the choice of the nuclease species, gRNA multiplexing, the packaging size and so on, and the vehicle to deliver these molecules. "Owing to a favorable set of characteristics, recombinant AAVs (rAAVs) are particularly suited for testing genome-editing strategies *in vivo*" [28] and some groups have already successfully delivered AAVs in skeletal muscles of mdx mice using AAV8 and AAV9 serotypes [17–19]. Other fruitful attempts have been made *in vivo* and *in vitro* using adenoviruses [8,29]. *Ex vivo* and *in vivo* approaches to edit the *DMD* gene have both pros and cons, as reviewed recently by Maggio *et al.* [24]. A potential problem of gene editing therapies is the immune response against the immunogenic components of vectors and gene editing tools. The immune reactivity will have to be addressed in detail *in vivo* and vectors modified accordingly. A strong immunoreaction would potentially require co-treating patients with immunosuppressive drugs or, as indicated by VandenDriessche and Chuah [30], the Cas9 protein will probably have to be expressed only transiently to avoid an immune response and accumulation of off-target mutations. These off-target mutations can be created by the expression of the Cas9 nuclease, tailored to induce double-stranded DNA breaks (DSBs), which increases the risk of unwanted events such as off-target DSBs, inversions and translocations [24], and the co-expression of gRNAs, which can target essential genes and potentially knock them out. Therefore, putative off-targets will have to be addressed in human cells, and DMD-derived hiPSCs represent a great advantage in that matter [31].

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Although there are still a lot of challenges, a *DMD* gene editing therapy is on the way. With many of the hurdles shared among scientists working on other CRISPR-treatable genetic diseases, hopefully these problems will be solved one by one. At the time of writing, the first clinical trial in humans, involving cells treated *ex vivo* with CRISPR-Cas9 to disabled PD-1 and being sent back into the patient with a non-small-cell lung cancer, has begun [32]. This offers hope that answers about the feasibility, toxicity and long-term effect in humans will soon be available for all.

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