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# Emerging Gene Correction Strategies for Muscular Dystrophies: Scientific Progress and Regulatory Impact

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Additional information is available at the end of the chapter

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## Abstract

Muscular dystrophies comprise a heterogeneous cluster of inherited muscle degenerative disorders with the common feature of progressive muscle weakness. These represent good candidates for treatment with gene-based therapies. Progress in gene transfer technologies has raised hopes for successful therapeutic restoration of mutated genes such as dystrophin in Duchenne muscular dystrophy. Delivery to enough muscle cells, however, remains a challenge for a successful gene replacement therapy. Other approaches based on exon skipping to correct mutant dystrophin's pre-mRNA splicing patterns have been tried, and partial restoration of dystrophin expression was reported in late-stage clinical trials, but full therapeutic efficacy is yet to be confirmed. The emergence of gene editing and its recent success in AIDS have opened a new therapeutic era for muscular dystrophies. This chapter will cover new gene correction strategies for muscular dystrophies and their regulatory challenges before they can become routine treatment modalities in the clinic.

**Keywords:** gene therapy, muscular dystrophies, regulatory impact, inherited muscle degenerative disorders, progressive muscle weakness

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## 1. Introduction

Muscular dystrophy refers to a range of conditions of progressive muscle weakening generally due to genetic defects in proteins that are critical for muscle functioning. The most common form of the disease, and one of the more common of any seriously debilitating genetic disorders, is Duchenne muscular dystrophy (DMD), which is caused by the mutation of the

dystrophin gene on the X chromosome and found in approximately 1 in 3600 of male births. The dystrophin gene product is a large protein with repeated elements and is involved in connecting the muscle fibres to the extracellular matrix [1]. Other proteins, mutations of which may cause forms of muscular dystrophy, include poly(A) binding protein nuclear 1 (PABPN1), myotonic dystrophy (DM) protein kinase (DMPK), or the product of the Emery-Dreifuss muscular dystrophy gene.

This chapter focuses on opportunities to develop treatments for these conditions with therapies that target the genetic defect that is the cause of the disease. The promise shown by new technologies for gene therapeutics makes this a particularly propitious time to consider the impact that such scientific progress may have on this clinically important set of conditions.

Notable among the new technological advances of relevance are the development of methods of specifically changing the sequence of the human genome, either by introducing a new gene sequence, which may repair the function of a defective gene, or by correcting the defect in the endogenous gene. Although the means to deliver genes to human cells still rely on the use of viral vectors, the methodology for the effective manufacture of these complex biologicals has developed. The approvals by the European Union (EU) of Glybera® and Imlygic® show that viral products can be manufactured to the quality standards acceptable for commercial products, with acceptable profiles of safety and efficacy. Another transformative technology is the derivation from stem cell precursors of different differentiated cell types with the potential to repair damaged or otherwise defective tissue. Putting these technologies together results in a possibility to create and manufacture to GMP standard, well-characterised patient-specific (or at least patient-compatible), genetically modified cells, a combination that may be anticipated in the near future to enable the viable therapeutic treatment of many previously intractable genetic diseases.

This chapter looks at the current status and future prospects of how the latest gene-based technologies are being applied to the alleviation, or even cure, of inherited muscular dystrophies. Furthermore, we consider the challenge of translating these treatment modalities into medicines that can be approved for commercial use by the regulatory authorities, notably within the EU. To do this, developers must go beyond the scientific mechanisms of efficacy to establish how the products will be manufactured to consistent quality standards and to demonstrate that they are clinically safe.

## 2. Muscular dystrophies

Muscular dystrophies comprise a heterogeneous cluster of inherited muscle degenerative disorders, each caused by a distinct gene mutation. More than 30 genes have been identified, each causing a different type of muscle pathology with different patterns of muscle weakness and disease progression (**Table 1**). This includes but is not limited to Duchenne, Becker, congenital, myotonic, Emery-Dreifuss, facioscapulohumeral, oculopharyngeal, and limb-girdle muscular dystrophies. Each of these varies in terms of pattern of inheritance, age of

disease onset, biochemical markers (such as creatinine kinase's upper limits), types of muscles affected, and complications at other organ sites, including cardiac and pulmonary problems. These give rise to varied symptoms, including muscle weakness and wasting (a common feature of a number of muscular dystrophies), joint stiffness, and scoliosis in addition to respiratory complications (such as chest infections and shortness of breath). Other symptoms include ankle swelling often linked with cardiomyopathy, fainting, eyelid drooping, and dysphagia. Ophthalmological symptoms, such as myopia in facioscapulohumeral muscular dystrophy and severe congenital muscular dystrophy (CMD) variants, cataracts in DM, and eyelid drooping in oculopharyngeal muscular dystrophy (OPMD), have also been reported. Hearing loss and skin lesions are also common in facioscapulohumeral muscular dystrophy and Ullrich CMD, respectively. Overall, these cause profound impairments in physical activity and quality of life [2, 3].

Disease type	Affected protein	Genetics	Age of onset (years)	Main pathological features	Current treatment
Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD)					
DMD	Dystrophin	Xp21.2 (X-R)	<5	Progressive muscle weakness (proximal to distal); cardiomyopathy; respiratory impairment.	No cure available to date. Symptomatic treatment to control the onset of symptoms and maximise the quality of life (corticosteroid therapy, physiotherapy, orthopaedic assistance, respiratory support).
BMD	Dystrophin	Xp21.2 (X-R)	5–40	Milder disease phenotype compared to DMD; variable progression; cardiomyopathy.	No cure available to date. Symptomatic treatment to control symptoms and improve the quality of life.
Limb girdle muscular dystrophy (LGMD)					
LGMD type 1B	Lamin A/C	1q21.2 (AD)	10–30	Variable symptoms.	No cure available to date.
LGMD type 1C	Caveolin-3	3p25 (AD)		Progressive muscle weakness and atrophy; cardiomyopathy	Symptomatic care to control cardiac and
LGMD type 2A	Calpain-3	15q15.1 (AR)		and respiratory impairment observed in sarcoglycanopathies (LGMD	respiratory symptoms with medications and/or devices; physical

Disease type	Affected protein	Genetics	Age of onset (years)	Main pathological features	Current treatment
LGMD type 2B	Dysferlin	2p13 (AR)		types 2C, 2D, 2E, and 2F),	activity and orthopaedic assistance.
LGMD type 2L	Anoctamin 5	11p14.3 (AR)		types 2I, 2K, 2M, and 2N), and LGMD type 2L.	
<i>Sarcoglycanopathies</i>					
LGMD type 2C	$\gamma$ -Sarcoglycan	13q12 (AR)	10–30	Progressive muscle weakness and atrophy; cardiomyopathy and respiratory impairment observed in	Symptomatic care to control cardiac and respiratory symptoms with medications and/or devices; physical
LGMD type 2D	$\alpha$ -Sarcoglycan	17q12-q21.33 (AR)		sarcoglycanopathies (LGMD types 2C, 2D, 2E, and 2F),	activity and orthopaedic assistance.
LGMD type 2E	$\beta$ -Sarcoglycan	4q12 (AR)		types 2I, 2K, 2M, and 2N), and LGMD type 2L.	
LGMD type 2F	$\delta$ -Sarcoglycan	5q33 (AR)			
<i>Dystroglycanopathies</i>					
LGMD type 2I	Fukutin-related protein	19q13.3 (AR)	10–30		
LGMD type 2K	Protein-1-O-mannosyl-transferase 1	9q34 (AR)			
LGMD type 2M	Fukutin	9q31 (AR)			
LGMD type 2N	Protein-O-mannosyl-transferase 2	14q24 (AR)			
Congenital muscular dystrophy (CMD)					
MDC1A	Laminin $\alpha$ 2 chain of merosin	6q2 (AR)	Congenital onset	Progressive muscle weakness; severity of disease progression varies; respiratory impairment observed in CMD with merosin deficiency, WWS, MDC1C, Ullrich syndrome.	No cure available to date.
MDC1C	Fukutin-related protein	19q13 (AR)			No specific treatment existent. Current
MDC1D	Like-glycosyl transferase	22q12 (AR)			pharmaceutical management includes a combination of
FCMD	Fukutin	9q31-q33 (AR)			orthopaedic supportive care, regular monitoring, and intervention to treat and

Disease type	Affected protein	Genetics	Age of onset (years)	Main pathological features	Current treatment
Fukutin-defective WWS	Fukutin	9q31-q33 (AR)			control respiratory insufficiency.
Protein-O-mannosyl-transferase 1 defective WWS	Protein-1-O-mannosyl-transferase 1	9q34 (AR)			
Protein-O-mannosyl-transferase 2 defective WWS	Protein-O-mannosyl-transferase 2	14q24 (AR)			
Protein-O-linked mannose $\beta$ 1,2-N-aminyltransferase 1 defective WWS	Protein-O-linked mannose $\beta$ 1,2-N-aminyltransferase 1	1p34 (AR)			
Fukutin-related protein defective WWS	Fukutin-related protein	19q13 (AR)			
Ullrich syndrome-collagen type VI subunit $\alpha$ 1 defect	Collagen type VI, subunit $\alpha$ 1	21q22.3 (AR)			
Ullrich syndrome-collagen type VI subunit $\alpha$ 2 defect	Collagen type VI, subunit $\alpha$ 2	21q22.3 (AR)			
Ullrich syndrome-collagen type VI subunit $\alpha$ 3 defect	Collagen type VI, subunit $\alpha$ 3	2q37 (AR)			
Integrin $\alpha$ 7-defective CMD	Integrin $\alpha$ 7	12q13 (AR)			
Integrin $\alpha$ 9-defective CMD	Integrin $\alpha$ 9	3p21.3 (AR)			
Facioscapulohumeral muscular dystrophy	Unknown	4q35 (AD), 18 (AD)	7–30	Slowly progressive disease with facial and shoulder muscle weaknesses; uncommon/mild cardiac impairment and infrequent respiratory problems. Hearing loss reported.	No cure available to date. Symptomatic treatment and physiotherapy.

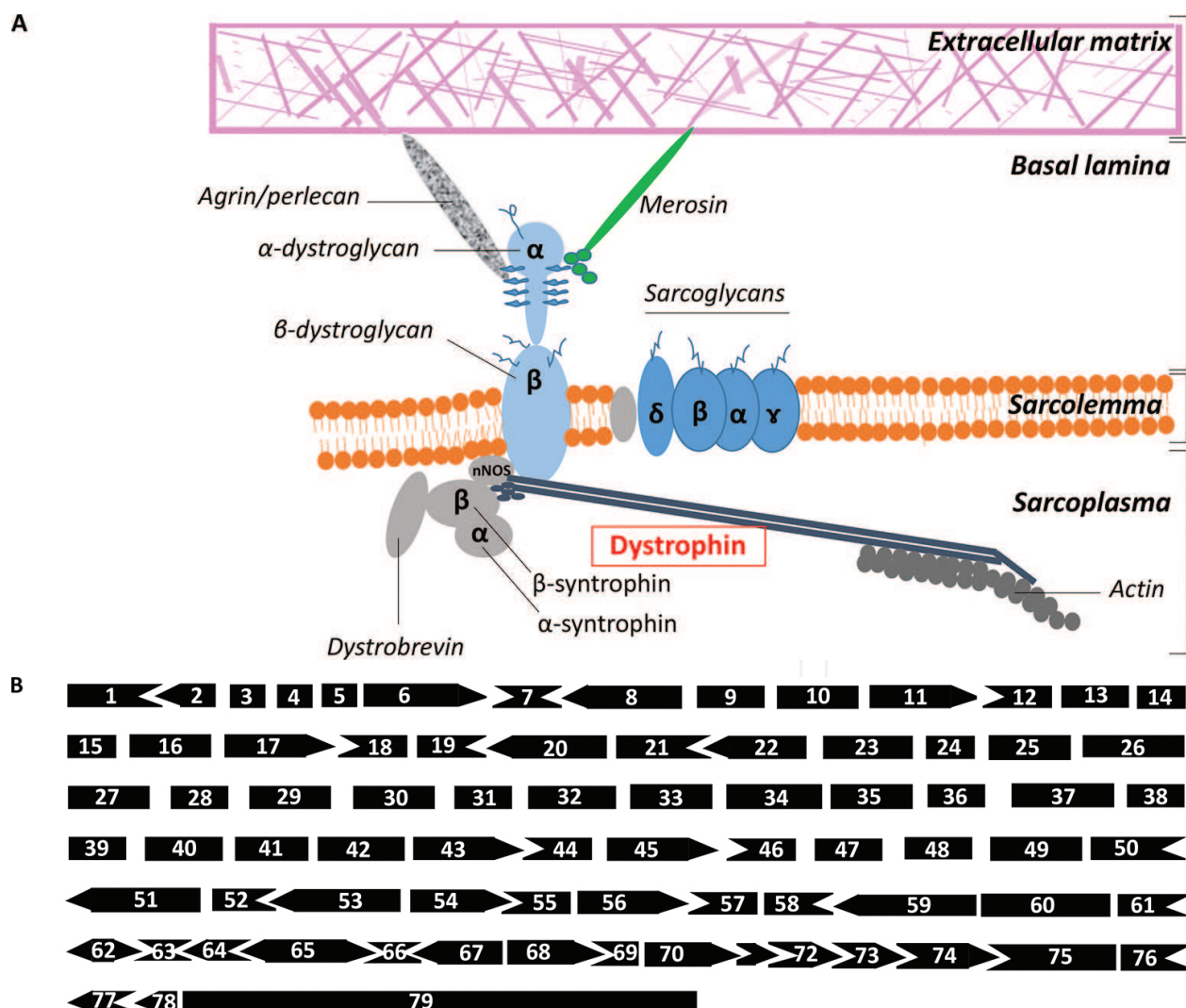
Disease type	Affected protein	Genetics	Age of onset (years)	Main pathological features	Current treatment
Emery-Dreifuss	Emerin	Xq28 (XLR, AD)	4–20	Slow-progressive muscle weakness and wasting affecting upper arms, lower legs, shoulder, and hips; cardiac problems (arrhythmia, conduction block); infrequent respiratory problems.	No cure available to date. Current management aims to prevent sudden cardiac death (use of pacemakers, cardiac transplantation) and correction of skeletal complications to maintain ambulation (Achilles tenotomy, surgery, orthopaedic support).
OPMD	PABPN1	14q11.2 (AD, AR)	30–60	Slow-progressive muscle weakness affecting muscles of the eyes (ptosis), throat (dysphagia), and proximal limb. Aspiration pneumonia reported. No cardiac problems reported.	No cure available to date. Symptomatic and supportive care management (nasogastric feeding, surgery).
Myotonic dystrophy (DM)					
DM1	DMPK	19 (AD)	Any	Slowly progressing multisystemic disease leading to muscle wasting, cataracts, heart conduction defects, and myotonia. Endocrine changes reported. Respiratory problems infrequent.	No cure available to date. Symptomatic treatment and physical activity.
DM2	Zinc finger protein 39	(AD)	8–50		
MDC1A = congenital muscular dystrophy with merosin deficiency; MDC1C = congenital muscular dystrophy and abnormal glycosylation of dystroglycan; FCMD = Fukuyama congenital muscular dystrophy; WWS = Walker-Warburg syndrome; X-R = X-linked recessive; AD = autosomal dominant; AR = autosomal recessive.					

**Table 1.** Summary of major muscular dystrophies.

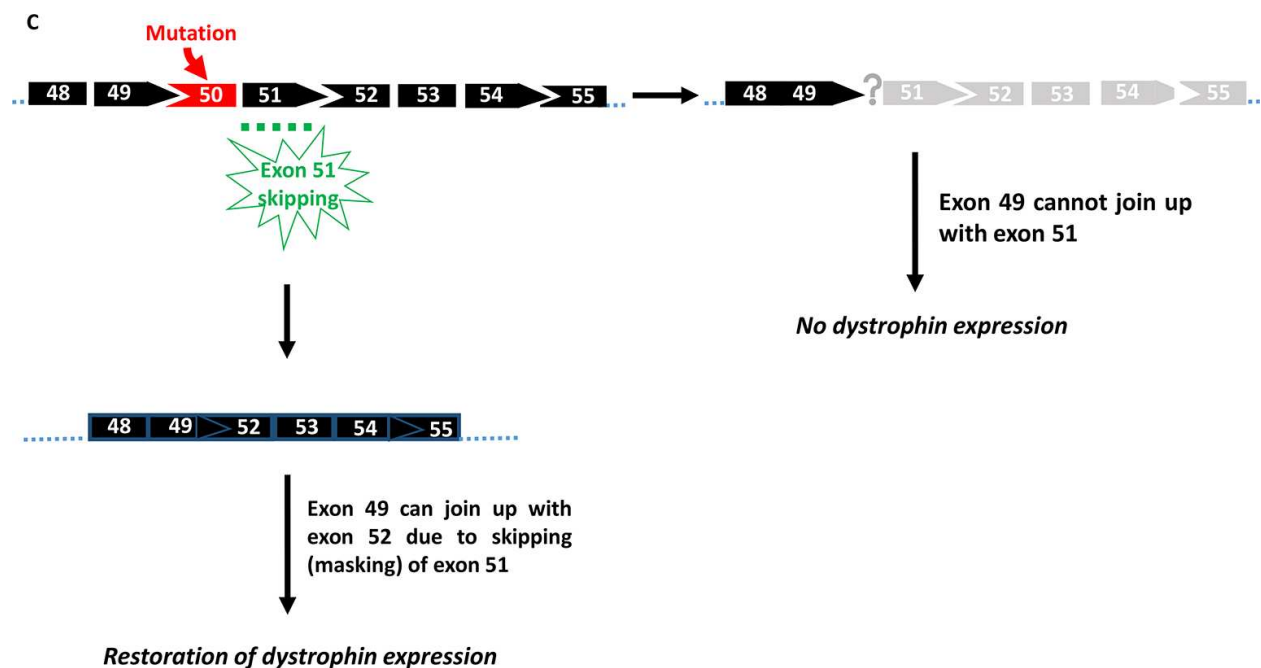
With an estimated incidence of 1 in 3600 to 6000 boys, DMD is one of the most common and severe forms of muscular dystrophies with an early-onset and progressive muscle weakness leading to the loss of ambulation by the second decade. This X-linked recessive condition is caused by mutations in the dystrophin gene (*DMD*, locus Xp21.2), which is expressed in



skeletal, smooth, and cardiac muscles, and hence the pathological involvement of different organs beyond skeletal muscle weakness, including respiratory and cardiac systems. The *DMD* gene comprises 79 exons and encodes a 14-kb mRNA transcript. This gives rise to a large protein product (427 kDa), a crucial cytoskeletal protein that mediates major structural and signalling functions within muscles. Four functional units make up the dystrophin protein. These include the actin-binding domain at the N-terminus, the rod domain consisting of 24 spectrin-like domains with four interspersing hinges, the cysteine-rich domain that mediates binding to  $\beta$ -dystroglycan, and the C-terminal domain mediating binding to syntrophin and dystrobrevin. These binding units confer a principal structural role for dystrophin facilitating its assembly with other proteins to form the dystrophin-associated glycoprotein complex (DAGC) (**Figure 1**) [1, 4, 5].







**Figure 1. The role of dystrophin in muscle physiology and disease.** (A) The Dystrophin-associated Glycoprotein Complex (DAGC) in skeletal muscle; (B) schematic illustration of exons that make up the *DMD* gene; (C) consequences of a frameshift mutation in the *DMD* gene illustrated by exon 50 deletion; and restoration of dystrophin expression with exon skipping therapeutics.

Deletions in one or more of the 79 exons that make up the *DMD* gene are common in DMD and account for approximately two thirds of the reported mutations. Other documented disruptions within the *DMD* gene include duplications (~10%), point mutations (~10%), and smaller rearrangements (15%) [6]. These lead to loss of dystrophin, which in turn destabilises the DAGC, resulting in the weakening of muscle fibre strength, increased susceptibility to stretch-induced damage, and raised intracellular calcium influx. These physiological disturbances account for the underlying histopathological features often observed in skeletal and cardiac muscles from affected patients, including muscle fibre necrosis, inflammation, and substitution with fibroadipose tissue [7, 8].

No curative treatment is currently available for most muscular dystrophies, including DMD. Current approaches involve relieving symptoms, delaying disease progress, and preventing complications [2]. Although these interventions proved beneficial in the short term, none of them can provide a long-term treatment and a permanent correction of the underlying pathological features. From a molecular point of view and based on advances in the identification of genes behind the observed phenotypes, most of these muscle pathologies represent good candidates for treatment with gene-based therapies.

### 3. Gene therapy for DMD

DMD has been the main focus and the proof-of-principle model for most gene therapy strategies targeting neuromuscular disorders, over the past years, with proof-of-concept validated in preclinical and clinical settings. The first clinical trial in the neuromuscular field,

in fact, involved a gene replacement approach to deliver full-length dystrophin via a nonviral method of transfer [9]. The observed low expression levels, however, at local injections sites, highlighted the need for potent gene replacement transfer systems. Of these, adeno-associated viruses (AAVs) are seen as the best available option. The efficiency of functional dystrophin expression in patients with DMD, using AAV as a vector, was assessed in a phase I clinical trial [10]. The approach was shown to be safe with no concerning side effects, although overall efficiency was compromised by the development of dystrophin-specific T-cell-mediated immune responses. This finding was complemented by observations that preexisting T-cell-mediated immune responses to AAV were present, which, together with the dystrophin-specific T-cell responses, could have contributed to the low transgene expression levels detected following intramuscular injection. These early conclusions highlighted the need to circumvent immune destruction of therapeutic transgenes, by delivering unaffected homologs of dystrophin. Of these, a microdystrophin-expressing recombinant AAV2/6 was shown to restore the dystrophin-glycoprotein complex and revert pathology in dystrophin (-/-)/utrophin (-/-) double-knockout mice model [11]. Delivering truncated versions of target proteins or their homologs, however, does not reconstitute a full gene replacement approach for DMD. Microdystrophin and minidystrophin transgenes often lack some crucial rod and hinge domains of full-length dystrophin, including neuronal nitric oxide synthase, syntrophin, and dystrobrevin, hence compromising maximal dystrophin functionality and membrane rigidity. This led to the engineering of triple AAV constructs using trans-splicing and hybrid methods, capable of delivering full-length dystrophin following coinfection of the tri-vectors in affected muscles *in vivo* [12, 13]. In trans-splicing, each vector acts as an independent construct holding sequential exonic sequences of human dystrophin's coding sequence. Coinjection of the vectors cause the constructs to cojoin via their inverted terminal repeats and deliver a full-length therapeutic transgene. These results circumvent the limited packaging capacity of AAVs and offer clinical hopes for boys with severe forms of DMD, for which the reversion of phenotype to a milder disease form [Becker muscular dystrophy (BMD)] using microdystrophin or minidystrophin-expressing AAV is not sufficient.

Although these gene replacement therapies are still in the early clinical phase of research and development, exon skipping-based therapeutics for DMD is progressing faster. Antisense oligonucleotides (AONs) have long been an effective alternative to dystrophin gene replacement therapy. These work by binding to the dystrophin transcript at sites that interfere with normal RNA processing, so that exons containing the mutations are bypassed, giving rise to in-frame transcripts capable of producing shorter yet functional protein products (**Figure 1C**). This was based on observations that BMD-like patients have in-frame transcripts with shorter dystrophin yet still maintain ambulation [14]. Hence, manipulating the splicing pattern of mutant dystrophin in DMD patients with an AON-based approach has the potential to alter disease phenotype from a clinically severe form of DMD to a milder BMD phenotype. For instance, the reading frame of dystrophin could be restored by blocking specific enhancers or splicing regulatory elements responsible for controlling the gene's exon recognitions. The approach was initially demonstrated *in vivo* in the *mdx* mouse model of DMD, bearing a single-point mutation in exon 23 that creates a stop codon with subsequent absence of dystrophin expression. Using a 2'-O-methyl (2'OMe) oligoribonucleotide complementary to the murine

intron 22's 3' splice site, it was possible to restore sarcolemmal expression of dystrophin in transfected myotubes *in vivo* [15]. The proof-of-concept has also been demonstrated in relevant human cell culture models derived from DMD patients bearing an exon 45 deletion, one of the most frequently deleted exons in DMD. The efficient restoration of dystrophin's coding frame was achieved by targeting splicing regulatory elements in exon 46 using a 2'OMe oligonucleotide [16]. Similarly, a successful correction was achieved through exon 51 skipping in a phase II clinical trial, where a successful restoration of both dystrophin and the DAGC was observed in patients with a deletion in exons 45 to 50 or exons 48 to 50 [8, 17], opening up great therapeutic hopes and paving the way towards late-stage clinical trials. A Biologics License Application (BLA) was submitted to the Food and Drug Administration (FDA) for approval of the antisense agent PRO051, 2'OMe (drisapersen) targeting exon 51 for DMD. However, this application was recently rejected, as a phase III study (NCT01803412) of long-term intake failed to meet its primary efficacy endpoint and also showed evidence of significant toxicity to a number of organs. Another similar antisense agent, eteplirsen, is currently under the FDA review for approval.

Although AON-based therapy for DMD has long been the prime attention of scientists and clinicians, this approach does not provide a long-term cure for DMD. Regular administrations of high doses are required to achieve a constant skipping and redirection of gene expression.

#### 4. Gene editing strategies

With advances in human genome and increasing need to provide a simplified long-term curative approach for genetic muscle diseases, new sophisticated technologies based on gene editing have emerged. These aim to permanently correct disease phenotypes in affected individuals using site-directed endonucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR). These corrections occur through the generation of double-stranded DNA breaks that will eventually induce intrinsic cellular DNA repair mechanisms mostly via nonhomologous end joining (NHEJ) [18, 19].

##### *Zinc finger nucleases (ZFNs)*

ZFNs are currently the highly advanced gene editing system. Initially, these were rationally designed taking advantage of key biological properties of zinc finger transcription factors such as their DNA sequence recognition function. Each zinc finger component is composed of ~30 amino acids and is capable of recognising 3 bp of DNA. The overall structure is arranged in tandem repeats of zinc finger motifs, hence allowing the recognition of longer sequences of DNA. The nuclease activity of ZFNs is conferred through fusion to *FokI* endonuclease's catalytic domain. This design ensures that enzymatic and subsequent DNA cleavage activities are only targeted to sites recognised by the binding domains of ZFNs. The latter consist of Cys2His2 zinc finger structures, in which a single zinc atom is surrounded by 30 amino acids and is capable of recognising 3 bp of DNA. More often, however, three to six zinc finger units

are assembled, which allows the recognition of 9- to 18-bp DNA sequences. This is usually regarded as acceptable for a single locus targeting in a human genome. From a structure-activity relationship, however, full functionality is not achieved unless *FokI* is presented in a dimerised form so as two DNA cleavage domains are dimerised around the target DNA sequence [19, 20]. In an attempt to maximise the biosafety profile of ZFNs, several methods have been employed to increase specificity. These include limiting the spacer length between the recognition sites of chimeric ZFN subunits [20]. In fact, ZFNs with shorter interdomain linkers connecting the Cys<sub>2</sub>-His<sub>2</sub> zinc finger and the nuclease domains were shown to have a restricted activity with a 6-amino acid linker exerting the most selective activity at a target DNA site with a 6-bp spacer [21]. Another approach has been to generate obligate heterodimer nuclease domains with decreased off-target effects [22–24]. This is associated with a relatively weaker interaction between the heterodimer cleavage domains, which would necessitate stronger interactions between each monomer and the target site to achieve a site-specific cleavage while minimising cleavage at weakly bound nontarget sites. This low-affinity high-avidity approach has been proposed as a plausible mechanistic approach behind the site-specific cleavage activity of the newly engineered obligate heterodimer *FokI* domains. An alternative to the obligate heterodimer method has been the expression of autonomous ZFN pairs, the combined expression of which was as effective as obligate heterodimer ZFN domains at inducing targeted chromosomal deletion in mammalian cells, with reduced toxic effects that are often thought to be linked with unwanted individual ZFN subunits' cross-reaction [25]. The enhancement of *FokI*'s enzymatic activity has also been reported, whereby an *in vivo* evolution-based method was employed to further increase the cleavage activity of *FokI*. The incorporation of the enhanced domain in heterodimer ZFN structures resulted in a potent product with improved overall cleavage profile [26].

The successful correction of different genetic mutations associated with various diseases, including sickle cell anaemia [27], haemophilia [28],  $\alpha$ 1-antitrypsin deficiency [29], X-linked severe combined immunodeficiency [30], and, more recently, HIV [31, 32], has led to its application in DMD, in which precise gene editing can be achieved by deleting targeted exons from the dystrophin gene. For instance, using extended Modular Assembly (eMA)/Context-Dependent Assembly (CoDA) methods, it was possible to generate several exon 51 targeted ZFNs. Two of which demonstrated a remarkable activity with mild off-target mutagenic effects in myoblast cells from DMD patients, harbouring a deletion of exons 48 to 50. When implanted into the hind limb of immunodeficient mice, the corrected myoblasts were capable of maintaining dystrophin expression *in vivo*, with a correct sarcolemmal localisation [33]. This proof-of-concept study has shown that the ZFN-based approach could potentially be adopted under a cell-based therapy approach for DMD, hence holding a promising faster translation into the clinic, considering that the approach is already in clinical trials for *ex vivo* cell modifications in HIV [32].

### ***Transcription activator-like effector nucleases (TALENs)***

TALENs were originally isolated from *Xanthomonas* bacteria, plant pathogens capable of employing up to 40 effector proteins to circumvent eukaryotic cell defences during a host



infection [34]. These are composed of repeated motifs of 33 to 35 amino acid residues, identical with the exception of the 12<sup>th</sup> and 13<sup>th</sup> residues, which are often known as the repeat-variable di-residues. The latter play a key role in TALEN's DNA-binding specificity, whereby a different pair of amino acids would exhibit a specific binding to a corresponding nucleotide in the target sequence. For instance, the asparagine (Asn; N)-isoleucine (Ile; I) NI, the histidine (His; H)-aspartate (Asp; D) HD, the Asn-Asn NN or the Asn-lysine (Lys; K) NK, and the Asn-glycine (Gly; G) NG pairs preferentially bind to adenine, cytosine, guanine, and thymine, respectively. These constitute TALENs' DNA-specific binding domains, which, like ZFNs, are conjugated to nonspecific *FokI* cleavage domains, hence directing them to the target site for gene editing and subsequent correction of the final protein product. A recognition sequence of 14 to 20 bp, together with an appropriately spaced *FokI* subunits separated by 12 to 19 bp, will ensure good genomic target recognition with maximal cleavage activity owing to efficient *FokI* dimerisation [35, 36].

Although not yet being as clinically advanced as ZFNs, the lower cost and ease of production of TALENs have generated an increasing interest in the technology. Recent work in DMD has shown the feasibility of the approach using optimised exon 51 targeted TALEN-encoding plasmids transfected into myoblast cells isolated from two different patients with deletions in exons 48 to 50. A satisfactory gene correction was observed with up to 12.7% and 6.8% of alleles confirmed to have indels in the two treated patient myoblast cell lines, respectively. This correction was further correlated with a good restoration of dystrophin expression at the expected predicted size of ~412 kDa compared to its expression in the wild-type isolated myoblasts [37]. Whole exome sequencing of the successfully corrected TALEN-treated cells revealed no insertions or deletions, except at the exon 51 target locus. Further analysis using the TALE-NT 2.0 Paired Target Site Prediction web server confirmed the nature of the observed single-nucleotide variants occurring. These were in fact related to expected genomic mutations that normally occur during cell clonal expansion, as none of these showed any similarity to the employed TALEN target site with spacers of 1 to 30 bases [37].

Besides the dystrophin gene as a main target in DMD, other genes have received comparable interest as targets for TALEN-based gene editing. Of these, myostatin (MSTN) has been the centre of attention as a member of the transforming growth factor- $\beta$  family. This is mostly linked to its recognised role in muscle physiology, acting as a negative regulator of skeletal muscle mass. In fact, studies have shown that mutations in the *MSTN* gene cause an increase in skeletal muscle fibre numbers and sizes, which in turn lead to muscle hypertrophy without any alarming consequences [38–40]. These observations were recently translated into therapeutic interventions, whereby inhibiting the MSTN signalling pathway using pharmacological agents has shown real benefits in DMD but also in other muscle wasting pathologies such as sarcopenia and cancer cachexia [41–45]. A phase I study is currently under way to assess the safety of an anti-MSTN monoclonal antibody in advanced cancer patients with cachexia (NCT01524224). The same approach using a different pharmacological agent, BMS-986089 (Bristol-Myers Squibb), will soon be tested in a first clinical trial involving patients with DMD (NCT02515669). From a molecular therapeutic point view, *MSTN* gene editing provides a long-term control to switch off MSTN signalling in the wasted affected muscles. The approach was

successfully demonstrated recently using a pair of TALEN-expressing plasmids targeting the human exon 2 locus, a highly conserved region within the coding sequence of the *MSTN* gene. Consistent with previous ZFN-related toxicity studies, the reported MSTN-TALEN targeted system was engineered using obligate heterodimers of the *FokI* domain to minimise off-target effects often seen with homodimer variants. Initial experiments in the HEK293 cell line revealed that the mutation induced by the TALEN approach was persistent with indels still detected 1 month after transfection using the T7E1 assay, a commonly used enzyme mismatch cleavage method for detecting mutations. A similar finding was reported in four different cell lines from different species including human, bovine, and murine cells. Further investigation in primary myoblast cultures derived from a dysferlin-deficient mouse model as well as from patients with dysferlinopathy confirmed an efficiency of 10.3% to 24.6% of gene editing after treatment [46]. Although most ZFN- and TALEN-based gene editing systems for muscle diseases to date have been engineered based on NHEJ mechanisms, proof-of-concept data are now emerging on TALEN-mediated homology-directed DNA repair (HDR). Targeted integration of dysferlin, a mutation of which is associated with limb-girdle muscular dystrophy (LGMD) type 2B and Miyoshi myopathy, has been shown following cotransfection of HEK293 with MSTN targeted TALEN-expressing plasmid and a donor plasmid expressing dysferlin tagged to an enhanced cyan fluorescent protein under a cytomegalovirus (CMV) promoter [46]. Although this approach is still at its early research and development stage, the results obtained *in vitro* remain promising and could offer the basis for future TALEN-corrected myoblast transplantation therapy for a number of severe muscular dystrophies.

### *Clustered regularly interspaced short palindromic repeats (CRISPR)*

Recent achievement in the field of gene editing has seen the emergence of CRISPR as a novel correction tool in biomedicine. CRISPRs were first described in prokaryotes as part of an RNA-guided adaptive immune system to protect against foreign intrusions such as viruses and plasmids. In response to these, bacteria and archaea are programmed to incorporate short sequences of foreign DNA at one end of a repeat element, which is often referred to as the CRISPR. The integrated inserts between the repeat elements of prokaryotic CRISPR-associated (Cas) loci hence confer a permanent future mechanism of defence against the past invaders [47]. Three types of CRISPR/Cas systems (I–III) have been described, with differing structural, functional, and mechanistic characteristics. Each system, however, shares a similar sequence arrangement of short repeated units of 30 to 40 nucleotides, separated by a unique “target” specific nonrepeated sequence (a spacer) of equal length [48, 49]. A leader sequence incorporating a promoter is often present, which initiates a unidirectional transcription of CRISPR sequences. Whereas types I and III systems are present in both bacteria and archaea, type II systems have been reported in bacteria only [50]. Type II systems that use a unique Cas protein referred to as Cas9 are of particular interest in medical application. The RNA-guided, nuclease-mediated genome editing of a type II CRISPR system is mediated by Cas9, a nuclease that is directed to the target DNA site by a single-guide RNA recognising a specific locus next to the protospacer adjacent motif. This subsequently creates a double-stranded break, which could be repaired by NHEJ or by a homology-directed mechanism of repair provided that an exogenous plasmid donor is codelivered [51].

In muscular dystrophy, the CRISPR/Cas9-based technology could theoretically be employed to correct germ-line DNA prenatally in one-cell zygotes or as a postnatal treatment. The first approach was recently tested *in vivo* in *mdx* zygotes. By injecting a single-guide RNA-guided CRISPR/Cas9 together with an appropriate HDR template, Long et al. demonstrated for the first time the feasibility of the approach to correct a nonsense mutation in exon 23 of the *DMD* gene [52]. Although the CRISPR/Cas9-treated *mdx* zygotes produced mice with varying degrees of gene correction, those showing 41% of gene correction by HDR and 83% correction by NHEJ repair mechanisms demonstrated a complete disease-free phenotype with normalisation of dystrophin expression at the histological level [52]. Despite showing some promising results in DMD, a successful germ-line DNA gene editing strategy would (if ever ethically permissible) require a good knowledge of the nature of mutation affecting the maternal disease carrier to allow a site-specific targeted correction. This prior knowledge, however, could be problematic in X-linked DMD in which it is estimated that one third of all mutations in the *DMD* gene arise spontaneously [53] and hence would not qualify for a Cas9 gene editing-based treatment. This would restrict the approach to the correction of well-characterised known point mutations, which only make 15% of DMD mutations. One alternative approach to consider would be a postnatal *DMD* gene correction. This was recently demonstrated using multiplexed single-guided RNAs to direct the Cas9 nuclease to mutations at exons 45 to 55 of the dystrophin gene [54], an approach that has the potential to correct more than 60% of mutations in DMD patients owing to the large hotspot mutation deletion achieved. Despite a good restoration of dystrophin expression both *in vitro* and *in vivo* following transplantation of the CRISPR/Cas9-treated DMD patient myoblasts into immunodeficient mice, the overall deletion efficiency, however, was less than that obtained following exon 51 deletion [54]. This suggests that the size of the targeted sequence is crucial in dictating Cas9-associated gene editing capacity to mediate an efficient repair by NHEJ or HDR, where a size-dependent decrease in nuclease-mediated gene deletion was previously noted [55]. Although proof-of-concept has eloquently been shown in research settings, CRISPR/Cas9-based gene editing in DMD would only be envisioned in the clinic should appropriate gene delivery methods be employed. Gene transfer systems capable of directing the elements of the CRISPR/Cas9 cassette to the diseased muscles *in vivo* are needed to ensure a satisfactory *DMD* gene editing and a full therapeutic outcome. For instance, AAV has previously been shown to be a safe and an effective gene transfer system in some clinical trials for gene replacement therapy [56]. In this regard, the AAV8 and AAV9 serotypes would be useful delivery tools for CRISPR/Cas9-mediated gene editing for DMD, owing to their reported efficient gene transfer to skeletal muscles and the heart following systemic administration [57, 58], which therefore could potentially result in a robust gene correction at the key target tissues harbouring the mutation in DMD patients. Recent studies have demonstrated the efficiency of AAV as a good platform tool for CRISPR/Cas9-mediated gene editing transfer to dystrophic muscles in DMD disease models *in vivo*. The approach was recently tested following administration by three different systemic routes, including intraperitoneal, intramuscular, and retro-orbital injections in postnatal *mdx* mice, and proved to be successful with an increasing dystrophin expression reported from weeks 3 to 12 after virus injection. This correlated with an overall improvement in skeletal muscle function [59]. Mutated exon 23 targeted deletion was also demonstrated to restore disease



phenotype in both neonatal and adult DMD models *in vivo* by systemic and local delivery of a CRISPR/Cas9-expressing AAV [60]. A similar viral-mediated transfer of a CRISPR/Cas9 system coupled with paired guide RNAs flanking the mutated exon 23 in DMD proved that AAV is a good gene editing transfer system for local and systemic restoration of dystrophin expression in muscle cells but also myogenic stem cells in diseased muscle *in vivo* [61].

## 5. Application in trinucleotide repeat expansion muscular dystrophies

Proof-of-concept studies in DMD have opened new horizons in the application of gene editing in other muscular pathologies for which the correction of toxic genes is believed to address the underlying dynamic mutations often associated with triplet repeat expansion and some locus contractions disorders.

### *Oculopharyngeal muscular dystrophy*

OPMD is an inherited autosomal dominant, slow-progressing, late-onset degenerative muscle disorder characterised by progressive eyelid drooping (ptosis), swallowing difficulties (dysphagia), and proximal limb weakness. Whereas the incidence in Europe is 1/100,000, the disease has been largely reported in the Bukhara Jew population in Israel (1/700) and the French Canadian population in Quebec (1/000) [62].

The underlying genetic defect behind OPMD is an abnormal expansion of a (GCG)<sub>n</sub> trinucleotide repeat in exon 1 of the PABPN1 gene, which leads to an expanded polyalanine tract at the N-terminal of the PABPN1 protein (12–17 repeats are often detected in mutant PABPN1 compared with only 10 repeats in the wild-type protein) [62].

From a molecular therapeutic point of view, OPMD is a good candidate for gene editing-based treatment. Previous studies have shown the importance of abrogating intranuclear inclusions (INIs) of mutant PABPN1, the main pathological hallmark of the disease, with neutralising agents capable of binding aggregated mutant proteins. Of these, intrabodies were shown to be effective in reducing mutant PABPN1-associated INIs and restoring a normal pattern of gene expression in a *Drosophila* OPMD model [63]. Although these show antiaggregate properties by tackling the disease phenotype at the protein level, they do not address the underlying molecular pathology behind it, hence necessitating a repeated administration approach to keep the disease under control. In this regard, gene editing could offer a long-term permanent therapeutic advantage. Allele-specific correction of expPABPN1 can be achieved by CRISPR/Cas9-mediated editing targeting the expanded GCG moiety. Unlike recently tested short hairpin RNA (shRNA)-based knockdown of PABPN1 [64], this gene level of correction could result in a permanent production of functional PABPN1 protein that is capable of correcting the OPMD phenotype at both histological (reduction of pathological aggregates) and molecular (abrogation of mutant PABPN1) levels. The need for an adjunct gene replacement therapy as is often the case in knockdown approaches [62] could no longer be necessary. However, thorough characterisation and mechanistic studies would need to be conducted both *in vitro* and *in vivo* to confirm CRISPR/Cas9 editing specificity towards the expanded mutant

PABPN1 with no obvious off-target effects on the wild-type PABPN1 gene or indeed other genes within the treated tissues.

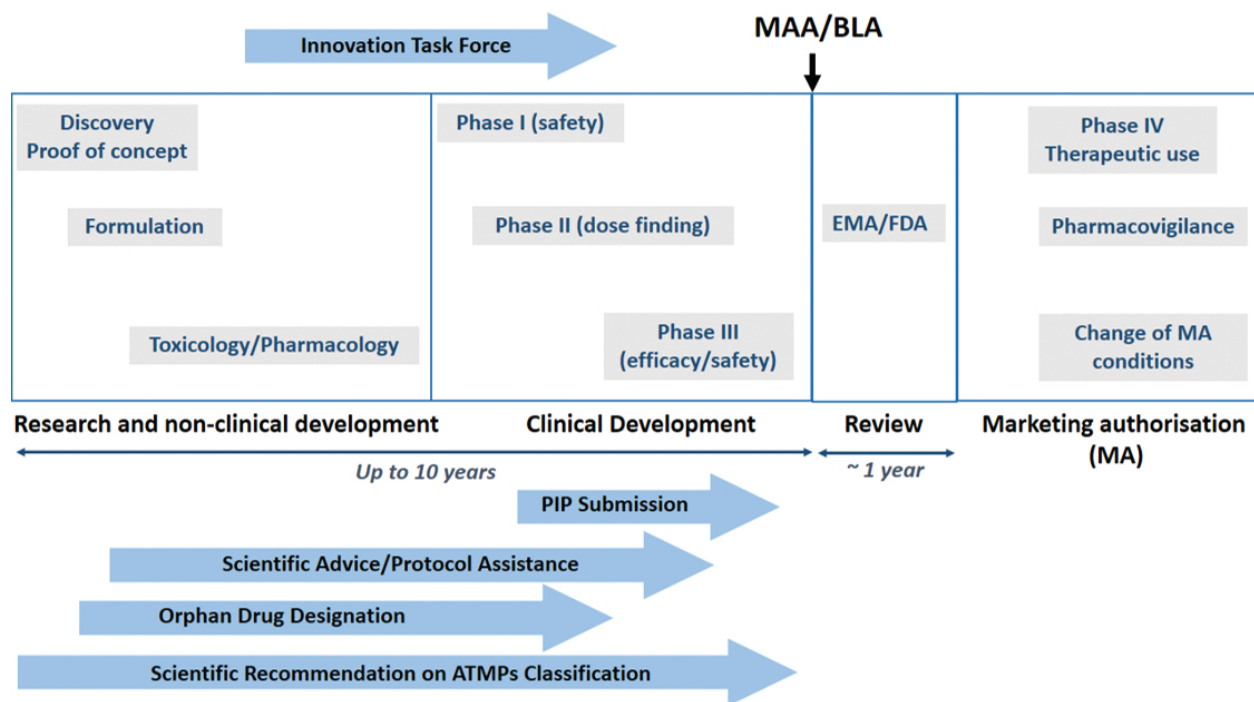
### *Myotonic dystrophies*

DM is an autosomal dominant, slow-progressing inherited multisystem genetic disorder affecting the muscles (causing wasting), the eyes (leading to cataracts), and the heart (causing conduction defects). The disease is also characterised by metabolic disturbances (endocrine changes) and prolonged contraction of skeletal muscles (myotonia).

Two types of DM have been defined to date. These include DM type 1 (DM1) and DM type 2 (DM2), with DM1 being the most severe and most common form of DM. Overall incidence has been estimated at 3 to 15 per 100,000 in Europe with a higher prevalence in Iceland (1:10,000) and a reported incidence of as high as 1:500 in Quebec [65]. The disease is caused by a CTG trinucleotide expansion in the 3'-untranslated region of the DMPK gene. CTG repeats exceeding 37 are considered abnormal with larger repeats (below 400 repeats) leading to a more severe disease [66, 67]. Like most debilitating muscular pathologies, no curative treatment currently exists for DM. The use of pharmacological agents [68, 69] and antisense RNA-based therapies [70–72] have shown improvements in disease phenotype, but overall benefits on the long-term remain, in some cases, limited and full therapeutic efficacy is yet to be demonstrated in the clinic. Although still in its early infancy, gene editing for DM treatment is an appealing alternative to most pharmacological and gene therapy approaches reported to date. Correction of mutant DMPK at the gene level offers the possibility for a permanent modulation of pathological phenotypes and long-term disease control. A recent study has shown the feasibility of editing intron 9 in the DMPK gene in DM1 neural stem cells derived from human DM1 induced pluripotent stem cells (iPSCs) using a TALEN-mediated homologous recombination-expressing cassette integrated upstream of the CTG repeats [73]. A significant reduction in nuclear RNA foci, together with restoration of normal microtubule-associated protein  $\tau$  (MAPT) and muscleblind-like (MBNL) splicing patterns, were observed [73]. Although transition into clinical use may be long and difficult, this proof-of-concept *ex vivo* study offers a rationale for the genetic correction of DM1-derived stem cells as a potential autologous cell therapy for DM1 patients in the future.

## **6. Regulatory challenges and pathways into the clinic**

In October 29, 2015, the United Kingdom became the first country in the world to legally approve one type of a human germ-line gene modification based on mitochondrial replacement. This approach is thought to save at least 10 children each year from mitochondrial diseases by preventing maternal transfer of mutations in mitochondrial DNA to offspring (Department of Health, 2014). Human germ-line genetic engineering, however, is currently not permitted in the United Kingdom and in other European countries. Although a somatic gene editing-based approach would be less questionable from an ethical point of view, a number of safety concerns would need to be addressed from a regulatory perspective. In



**Figure 2. Regulatory pathway for advanced therapy medicinal products.** MAA = Marketing Authorisation Application; BLA = Biologics License Application; EMA = European Medicines Agency; FDA = U.S. Food and Drug Administration; PIP = Paediatric Investigation Plan; ATMPs = Advanced Therapy Medicinal Products.

accordance with Article 2(1)(a) of Regulation (EC) No. 1394/2007 and as per the European Medicines Agency (EMA) classification, gene editing-based products, including cells modified *ex vivo*, will be regulated as advanced therapy medicinal products (ATMPs) for which a central authorisation procedure governed by the EMA would apply (**Figure 2**). This would eventually lead to a single marketing authorisation that is valid across the entire EU and the European Economic Area (EEA) countries. A number of guidelines on the requirements for product quality and preclinical and clinical studies have been issued by the EMA over the past years to facilitate the transition of promising experimental advanced therapies into the clinic.

### *Safety considerations for gene editing*

The risk of inducing modifications at off-target genes, leading to unwanted side effects, is a major safety concern. The degree of off-target events and their clinical implications are crucial questions that need to be carefully addressed as part of a regulatory new investigational drug development plan. The primary concern is that modifications could be carcinogenic. It must also be remembered that (unless the therapy is based on a cell type that is selected and clonally expanded after modification) the off-target effects will be heterogeneous from cell to cell, so the analysis must encompass a suitably large (and potentially diverse) population of cells exposed to the modifying agent to evaluate if any cells may be adversely transformed even at low frequency. There may also be risks associated with changes that have cytotoxic effects,

and if such effects are frequent in the same cells that undergo correct gene editing, the consequences could include loss of efficacy.

In this regard, rigorous quality assurance tests would need to be conducted to identify any insertions or deletions that could result from a gene editing NHEJ-based treatment. These include gene sequencing, mismatch cleavage assays based on Cell or T7 endonuclease I enzymes, and the tracking of indels by decomposition (TIDE) method. These tests could be used as quality assurance tools to determine the number and location of indels or substitution events occurring after gene editing treatment, more often based on *in silico* predictions. However, as previously noted with ZFNs, some mutations could occur near cryptic off-target sites that are not predictable *in silico* [74]. Care should also be taken when validating the assays employed for product characterisation purposes. This follows from previous observations whereby initial analysis by whole genome sequencing (WGS) in gene-corrected human iPSCs (hiPSCs) revealed a large number of indels, of which some were confirmed to be false positive [75]. This highlights some limitations of WGS and calls for a multitesting approach employing different analytical methodologies.

Although current assays would be valuable in identifying the location of suspected off-target mutations that could arise from NHEJ or HDR-based gene editing treatments, they do not, however, provide precise information on the ability of these mutations to cause carcinogenicity in the long term. Hence, additional functional studies are required to assess the significance of these off-target events at the molecular and cellular levels. For instance, an unwanted insertion in the middle of an essential gene sequence could have dramatic consequences. One should have enough knowledge on the biological and physiological functions of the affected gene to draw an informed decision on the overall safety of the proposed approach. Similarly, an off-target insertion into an enhancer or a repressor region would disturb genetic homeostasis resulting in an unwanted down-regulation or up-regulation of genes that come under the affected promoter. This could subsequently interrupt normal cellular activity with potential alteration of phenotype at histological and physiological levels. Although not all mutations could affect cellular proliferation, an unwanted “indel” mutation occurring within genes known to regulate cell replication or involved in programmed cell death raise an alarming concern from a safety point of view. This is due to potential risks arising from a compromised cellular viability leading to severe toxicity in healthy tissues or an uncontrolled replication that could eventually lead to tumour formation. These potentially serious consequences warrant careful considerations during early product development stages; hence, basic biological studies to further characterise the sites at which these mutations have occurred should be conducted as ad hoc validation studies to rule out any unwanted insertional mutagenesis and/or tumourigenic consequences following a gene editing-based treatment.

Although clinical safety experience with biological therapies based on gene editing is lacking, current product development and regulatory strategies should also draw from past and available human clinical trial data on retroviral and lentiviral vectors’ integration sites. A key safety parameter would be to satisfy the regulatory bodies that those detected off-target effects have been thoroughly characterised and occur at “low risk” sites unlikely to cause insertional oncogenesis following integration of foreign DNA. In fact, not all events at off-target genomic



sites would result in serious side effects. For instance, those occurring at “extragenic” sites distant from essential gene regulatory sequences are less likely to cause harm than those affecting “intragenic” sites. Modifications to sites that do not fall within a gene transcription unit as well as those situated more than 50 kb away from the 5′ end of any gene and more than 300 kb from genes linked with cancer or microRNA sites are generally considered safer. Genomic sites not within ultraconserved regions and outside long noncoding RNAs would also represent low-risk sites in the event of off-target DNA integration [19, 76].

Furthermore, validation studies should not only focus on the affected off-target sites but also neighbouring sites by measuring the effect of these mutations on neighbouring gene expression. These studies should ideally be validated *in vivo*. The choice of appropriate animal models, however, remains a challenge and care should be taken when interpreting data [19, 76, 77].

### ***Validation and choice of functional studies for assessment of toxicity***

Although basic biological studies are valuable in assessing the extent of genome modification as a whole and increasing our understanding of different alterations taking place including those that are unlikely to result in clinical toxicity, they do not fill all the regulatory requirements gaps. Functional toxicity studies would, therefore, need to be conducted in parallel early during the preclinical stage of product development. Two paramount questions would need to be carefully addressed to satisfy regulators’ concerns on serious toxicity issues that are specific to genome editing-based medicines. These include cytotoxicity and genotoxicity. Tumourigenicity is the most important safety consequence to consider, but germ-line effects are also not expected to be permissible. Cytotoxicity may be caused by (i) the vector itself (including expression of viral vector antigens and potential persistence of the vector in cells), (ii) gene editing machinery (and any persistence thereof, especially DNase), (iii) off-target gene editing, or (iv) on target gene editing (possible if the editing is either not accurately restoring the wild-type or is generating new antigenicity and/or genetic instability).

Eloquent studies and approaches would have to be designed to address these issues. At preclinical stage, this could include viability assays based on GFP-positive (GFP<sup>+</sup>) cells, whereby the treated cells are cotransfected with the tested nuclease-expressing construct and a GFP-expressing plasmid. This would allow investigators to track and quantify any observed decline in the GFP<sup>+</sup> cell population as a result of a nuclease-related toxicity. The approach would also be valuable in dose escalation studies when deciding on optimal dosage administration for subsequent first-in-human clinical studies. Monitoring of clonal changes has also been used as an informative way to assess genotoxicity *in vitro* in cells pretagged with unique short sequence identifiers to allow tracking of changes in starting clone dynamics over a period of time [78]. Similarly, other approaches have relied on fluorescently tagged cell cycle indicators in cell lines such as HeLa FUCCI cells as a complementary *in vitro* validation method to assess the genotoxicity effects of genome editing at the cell cycle level [79].

However, it is important to bear in mind that measuring cytotoxicity and genotoxicity using approaches based on reporter genes and tagging systems is restricted to preclinical assessments and cannot be employed at later stages of product development when assessing

potential toxicity in human clinical trials. Hence, the need for clinical toxicity assays that are fit for purpose should not be neglected. Furthermore, correlation of these assays with clinical outcomes is yet to be demonstrated. For this reason, all functional toxicity studies conducted *in vitro* should use cellular models and gene transfer methods that are similar to those intended to be applied in clinical settings to fulfil some of the safety regulatory requirements.

### ***Vector considerations for in vivo and ex vivo gene editing***

Potential cytotoxic effects do arise not only from on-target and off-target effects but also from the employed gene transfer system itself. In this regard, the type of vector (viral or nonviral) and the gene (nuclease) transfer approach (direct *in vivo* delivery versus *ex vivo*) adopted will have to be implemented earlier during product development and considered as part of the constructed regulatory strategy for the nuclease-based medicine under consideration.

For severe neuromuscular disorders in which there is a widespread distribution of affected muscles, AAV vectors and in particular serotypes 8 and 9 are seen as ideal systems for gene editing-based nuclease transfer to diseased muscle tissues owing to their relatively high tropism for skeletal muscle cells and the heart [57, 58] as well as their documented safety profile in the clinic following recent approval of Glybera® in Europe [80]. From a product development point of view, this can be achieved through an HDR-based gene editing construct packaged within a recombinant AAV. Although technically this is achievable with ZFNs due to a relatively small monomer insert size of ~1.2 kb, this might not be the case for Cas9 nucleases. With an insert size of ~4.1 kb, this could be an issue when it comes to packaging a bicistronic construct harbouring a donor DNA template [81], considering the limited packaging capacity of rAAVs. Taking into account the EMA's guidelines on quality, nonclinical and clinical issues related to the development of recombinant adeno-associated viral vectors (EMA/CHMP/GTWP/587488/2007 Rev. 1) [82], caution should be taken during the manufacturing stage of the vector to avoid the possibility of producing AAV particles whose packaged DNA is greater than that of wild type virus. Technically, this could be overcome by splitting the Cas9 gene between two vectors. However, this markedly increases the developmental and regulatory complexity, with each product warranting a full characterisation and a thorough assessment of approaches employed during manufacturing, quality control, and preclinical evaluation (including choice of animal models for testing, vector persistence and tropism, reactivation of virus infection, and germ-line transmission) in addition to clinical studies. The latter would have to be based on a data-driven dose selection for each vector used and show a comprehensive picture of virus biodistribution and shedding, immunogenicity profile, and germ-line transmission considering the permanent gene modification achieved with gene editing-based nucleases and the risks associated if the virus expressing these genes accidentally infect germ-line cells. A long-term follow-up is therefore highly recommended and should not be neglected as part of a complete regulatory plan for a gene editing-expressing AAV vector.

Similarly, the use of genetically modified and nonmodified cells including myoblast cells and myogenic stem cells for the treatment of monogenic inherited neuromuscular diseases has been well validated in preclinical and clinical studies [83–86] and it is anticipated that these therapies would become normal treatment modalities in the clinic in the future.

Although gene edited-based cell therapies for HIV and leukaemia are seeing a rapid positive progress in clinical trials and are rapidly extending to other debilitating diseases, a smooth and safe progress of these life-changing advanced therapies in the market would require fulfilments of a number of criteria from a regulatory point of view. The ultimate goal is to ensure that these therapies would reach the severely affected individuals at minimal health and safety risks with regards to the treated patients themselves as well as third parties and the environment in large. According to the EMA's guidelines, a multifactorial risk assessment approach needs to be considered taking into account the origin of cells involved, the type of vector employed during the genetic modification procedure, the manufacturing process, the noncellular components used as part of the formulated product, and the intended specific therapeutic use of the final product.

Lentiviral vectors have long been the vector of choice for the *ex vivo* engineering of stem cells and muscle progenitor cells for the treatment of muscular dystrophies [87–89]. This adds an additional layer of regulatory requirements from quality, nonclinical and clinical perspectives. The manufacture of viral vectors to the GMP quality standards required for an approvable medicinal product is expensive and often inefficient, but developers are helped by the guidance issued in these areas by the EMA (for example, “Guideline on the quality, nonclinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)”) [90], as well as the monograph of the European Pharmacopoeia (Ph. Eur. 5.14). A thorough product characterisation is required as part of this process that would include different but complementary methods based on molecular, biological, and immunological assays with the aim of assuring the identity, purity, and potency of the produced genetically modified cells as a final product. Major considerations for viral vectors used *in vivo* are the biodistribution, potential for generation and/or shedding of infective virus, potential for germ-line modification, and the impact of interaction with the recipient's immune status. Clinical use of viral vectors and genetically modified cells should also comply with applicable regulations for genetically modified organisms.

Complexities arise from the genetic manipulation (gene correction) as well as the differentiation status and capacity of the modified cells, which could result in a mosaic cell population. Furthermore, intrinsic variations between cells as a result of donor differences give rise to massive batch variations in the final product. From a regulatory perspective and in line with the EMA's guidelines, nonclinical and clinical studies need to be conducted with cell medicinal products that are well characterised. The manufacturing process needs to be robust and quality control focused capable of maintaining consistency and reproducibility of the final cell-based product. For this, all starting materials need to be well defined and carefully documented. For treatment of muscle diseases, it is often reasonable to administer cell-based products that are in a differentiated state, which may pose less tumourigenic risks. However, one cannot exclude the existence of a subpopulation of cells in an undifferentiated proliferative state. For iPSCs, for example, it is empirical to conduct additional testing of cell transformation and tumour formation during the early manufacturing stage of the product as a precaution measure. This would often need to be combined with the selection of appropriate markers during critical



manufacturing steps for assuring a defined stage of differentiation that is intended for therapeutic use.

For any cell-based product that has undergone a substantial *ex vivo* manipulation, a robust process validation process is therefore paramount and should include a combination of genetic stability, tumourigenicity, and phenotypic profile assessments of both wanted and unwanted cell populations at all critical stages of manufacturing to ensure safety requirements are met. The mosaic nature of cell-based medicinal products often complicate their identity and purity-related characterisation. Most often, it is reasonable to accept that purity does not always equate homogeneity. Truly selective markers that could accurately map and distinguish different cell types and differentiation stages are yet to be identified, which often render product characterisation a challenging task for most developers. Nevertheless, one should not underestimate the importance of a thorough demonstration of product consistency as a minimum requirement for characterisation purposes.

The ability to track any cell-based therapy following administration in patients is crucial for clinical monitoring purposes. However, limitations in current medical methodologies do not allow a full biodistribution profile to be drawn from human studies. Hence, the importance of thoroughly designed biodistribution studies in nonclinical models should take into account the multistep biodistribution characteristics of cell products, including migration, niche, engraftment, differentiation, and persistence, together with reliable *in vivo* tracking methods such as the use of marker genes or labelled cells. Current European regulatory requirements do not give exemptions when the risk profile of the cell-based product under investigation is subject to a safety concern or when its route of administration (such as intravenous delivery) warrants a special attention. For this, noninvasive methods based on clinically accepted tracers should be considered and their use should be justified when conducting biodistribution studies in human trials.

## 7. Conclusion

For diseases caused by genetic defects, means to correct the defect are attractive routes to cure the disease at its source. Medical scientists working in the field of muscular dystrophies are therefore excited by the opportunities that recent developments in gene therapeutics bring to the field. Technologies do now exist for manipulating the human genome in *ex vivo* cultured cells with considerable specificity. However, there are further technical challenges to solve before such technologies translate into viable medical treatments for affected individuals. The medical need is evident from the severity and incidence of these conditions, which are frequently severely debilitating. It should also be noted that muscular dystrophies are not a single disease but cover a range of conditions caused by different mutations, and it is to be expected that, for specific gene-based therapeutics, a different medicinal product will generally be required according to the different mutation that has caused the disease.

The increasing number of investigational medicinal products entering clinical trials involving patients with DMD and BMD has prompted the EMA to publish its guidelines on the clinical

investigation of medicinal products for the treatment of DMD and BMD (EMA/CHMP/236981/2011, Corr. 1) [91], expected to come into effect in July 2016. This gives a general guidance to be taken into account during the clinical development and evaluation of currently investigated therapies for DMD and BMD [91].

As described in this chapter, major recent advances for the enablement of gene-based treatments of muscular dystrophies include the following:

- The technologies for the manipulation of the human genome have been refined to levels that achieve the high precision of gene editing that would be expected for a medicinal use. The CRISPR/Cas9-based technique has undoubtedly revolutionised the field that was pioneered through sequence-specific DNA recognition proteins based on zinc finger motifs or TALENS. It is clear that the technology now exists to make desired changes to a genome and therefore in theory to correct genetic defects. However, the remaining molecular challenges to address include (i) achieving sufficient efficiency of the modification for therapeutic benefit, (ii) controlling and/or characterising unwanted off-target genetic modifications to acceptable levels, and (iii) delivering the gene correction throughout the affected tissues of the body.
- Much of the recent renaissance of optimism in the potential of gene therapy is driven by a new emphasis on *ex vivo* treatments, especially for autologous cells. Vectors, such as lentivirus, which generally yield rather low efficiency of genetic transduction *in vivo*, become therapeutically viable when used *ex vivo* on cell populations that can also be selected and expanded before readministration to the patient. Cell production technologies have also been adapted to a paradigm based on GMP-compliant enclosed system manufacture of multiple single-patient batches in place of large-scale multipatient batches. However, although this strategy is well suited to cell types such as circulating lymphocytes (for example, for CAR-T therapies), the applicability to differentiated muscle cells is much less clear. The solution may lie in the further development of treatments using stem cells that have potential to at least partially repopulate muscle tissues with corrected cells. Stem cell-based products do have their own safety concerns to be considered, although recent experience, notably the EU approval of the limbal stem cell-based product Holoclar<sup>®</sup>, indicates that these can be addressed.
- Developers of gene-based therapies have also long been concerned that the standards required by medicine regulatory authorities would be very difficult to meet with these complex biological products, but the EMA has demonstrated that its standards are pragmatically science-based and achievable. At the time of writing, the gene therapy and oncolytic viral product Imlygic<sup>®</sup> has been recommended for approval to add to the previously approved Glybera<sup>®</sup>. Several autologous cell-based tissue engineered therapeutics have been approved, namely, ChondroCelect<sup>®</sup>, MACI<sup>®</sup>, and Holoclar<sup>®</sup>, and *ex vivo* genetically modified cell-based therapies are either under review or being readied for submission. It can therefore be concluded that there is no fundamental obstacle to approval of either *in vivo* or *ex vivo* gene therapies.

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