Molecular targets to treat muscular dystrophies

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Summary

Muscular dystrophies are classically subdivided according to their clinical phenotype, and were historically defined as progressive myopathies in which muscle biopsies demonstrate muscle fibre necrosis and regeneration, as well as replacement of muscle fibres by adipose and connective tissue. In recent years, great progress has been made in identifying the genetic basis of many myopathies, thereby presenting opportunities to develop therapeutic strategies that act on specific molecular pathomechanisms. The different therapeutic strategies and their molecular targets will be reviewed.

Key words: myopathies; dystrophinopathy; limb-girdle muscular dystrophy; dysferlinopathy; exon-skipping

Introduction

The muscular dystrophies are caused by mutations in a large variety of genes with different functions, encoding proteins of the contractile apparatus, structural proteins, enzymes or nuclear proteins (fig. 1). The disease-causing mutations lead in most cases to a loss of function, but in some cases mutations can also cause a toxic gain of function. Although most genomic mutations in muscular dystrophies encode altered proteins, in some instances the genetic alterations exert their pathogenicity at the level of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The obvious primary molecular targets in muscular dystrophy are, therefore, the affected genes and/or their respective products. Theoretically, the most effective strategy would be to correct the mutated DNA by genetic engineering. Although recent developments in genetic engineering are promising, this approach is still technically challenging and poses ethical problems. Currently, most of the therapeutic strategies are focused on the mutated gene products or their downstream targets.

Despite great efforts, a cure for muscular dystrophies is unfortunately still unavailable; however, some therapeutic strategies are currently being tested in clinical trials. The most commonly encountered forms of muscular dystrophy in adults are myotonic dystrophy (MD), facioscapulohumeral muscular dystrophy (FSHD) and the large group of limb girdle muscular dystrophies (LGMDs). The most common childhood form of muscular dystrophy is Duchenne muscular dystrophy (DMD). Experimental therapeutic efforts have therefore primarily focused on these disorders, which will be reviewed here.

Dystrophinopathy

Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy caused by mutations in the X-linked dystrophin gene. The disease affects about 1 in 3,500 live born boys, who demonstrate delayed developmental motor milestones. Gower's manoeuvre when rising from the floor and difficulty climbing stairs in young boys raises the suspicion of DMD. Hypertrophy of the calves is present and may also affect other muscle groups. Wasting and weakness predominantly affect limb girdle muscles of the upper and lower extremities. With disease progression

Figure 1

Illustration of proteins and potential targets involved in muscular dystrophies.

Schematic representation of the localisation and interaction of selected proteins involved in muscular dystrophies. Dystrophin links the extracellular matrix to the actin cytoskeleton via the dystrophin-associated glycoprotein complex (DGC). Absence of dystrophin results in destabilisation of the DGC. Some genes mutated in muscular dystrophies encode proteins responsible for the correct glycosylation of dystroglycan, necessary for interaction with the extracellular matrix. The tail anchored membrane protein dysferlin is involved in membrane repair.

FKRP = fukutin-related protein; POMT = protein O-mannosyl transferase

the child looses ambulation at around 12 years of age and becomes wheelchair-bound [1]. Dystrophinopathy patients who remain ambulant beyond 16 years of age are considered to suffer from the Becker-type of the disease [2]. With progression of muscle weakness, contractures develop and respiratory problems are aggravated by thoracic deformities and weakness of intercostal and diaphragmatic muscles. Cardiac problems may become apparent at a stage when muscle weakness is already pronounced.

Becker muscular dystrophy (BMD) is a milder allelic variant of DMD, with later age of onset (in very rare instances up to 60 years) and slower disease progression involving the same muscle groups as DMD, and affecting 1 in about 17,000 males [2]. Clinical cardiac involvement is more frequent in BMD since skeletal muscle strength still allows ambulation and strain on the heart muscle is greater than in DMD [3, 4]. Cognitive dysfunction is less pronounced than in DMD patients.

X-linked dilated cardiomyopathy (XDLC) is the clinical presentation of a cardiomyopathy caused by dystrophin mutations without symptoms of skeletal muscle involvement [5]. However, on laboratory testing serum creatine kinase levels are elevated and skeletal muscle biopsy shows myopathic changes. Typically, 20-year-old male patients present with congestive heart failure that is lethal within 1 year if not transplanted. Women may also be affected, but as a result of their mosaicism may have a slower progression of the disease.

Manifest carriers are women who carry one copy of a mutated dystrophin gene and may present with asymmetric muscle weakness or muscle hypertrophy with or without cardiomyopathy [6–9].

The dystrophin gene spans a region of about 3Mb of DNA and is composed of 79 exons [10]. It is the largest gene known to date. The transcribed messenger RNA (mRNA) is 14kb in size and the mature protein 427kD in weight [11]. The dystrophin molecule has an N-terminal actin-binding domain followed by 24 spectrin-like triple helical repeats that form the rod domain, which includes four non-helical "hinge regions". The C-terminal part contains a cysteine-rich domain that binds to beta-dystroglycan, followed by a region binding to syntrophins and dystrobrevins [12]. Thus dystrophin provides a link between the actin cytoskeleton and the extracellular matrix (figs 1 and 2) [13].

The majority of mutations in dystrophin are exonic or multiexonic deletions (about 65% in DMD), less frequent are duplications (around 7%) and the remainder of the mutations are single nucleotide changes generating nonsense (DMD) or missense mutations (BMD). There are two mutational hotspots: a "major hotspot" region, which spans exons 45 to 53 and a "minor hotspot", which spans exons 2 to 20. Mutations in DMD usually disrupt the open reading frame, whereas mutations in BMD retain the open reading frame, generating an internally truncated dystrophin molecule with sufficient biological activity to account for the milder phenotype (fig. 2) [14].

Several clinical trials suggest that there is a significant increase in strength, timed muscle function tests and pulmonary function in DMD patients receiving steroid treatment [15]. Daily treatment with prednisone at a starting dosage of 0.75 mg/kg/day or deflazacort at a starting dosage of 0.9 mg/kg/day offers an effective initial treatment. This opinion is shared by a Cochrane review on glucocorticoid corticosteroid therapy in DMD [16] and by the report of the Quality Standards Subcommittee of the American Academy of Neurology and the Practice Committee of the

Figure 2

Exon skipping as a therapeutic strategy for the treatment of Duchenne muscular dystrophy.

During splicing of the dystrophin pre-mRNA the introns are removed and exons are joined to build mRNA with a reading frame to allow synthesis of functional dystrophin proteins. In the case of exonic deletions, "out of frame" mRNA can be produced, inducing premature termination of protein translation. This leads to a nonfunctional dystrophin protein, rapidly removed by the cellular degradation machinery. An exon skipping strategy is applied to restore the reading frame. By use of antisense oligonucleotides targeting splice sites of the pre-mRNA, one or more exons are removed to correct the reading frame. This results in a shortened but partially functional dystrophin protein.

Figure 3

Different gene therapy strategies used in muscular dystrophies.

In muscular dystrophies, gene therapy is used to deliver genetic material into the host and finally target the diseased muscle tissue to correct the genetic defect. In most cases, the transferred DNA encodes for the protein missing due to a mutation. Gene transfer can be mediated by different strategies and vectors. The methods differ in their transduction efficiency, packaging capacity, risk of immunogenicity or oncogene activation by genome insertion.

Child Neurology Society [17]. In a recent prospective longitudinal observational study, the long-term effects of intermittent and daily glucocorticoids treatment in DMD patients were compared and analysed [18]. This study demonstrated that daily treatment was associated with longer ambulation expectancy and slower decline of motor function than the intermittent course, but the intermittent therapy was overall better tolerated with fewer adverse effects.

In recent years, however, more specific therapeutic strategies have been developed for dystrophinopathies, which include cell replacement therapies, gene therapy, the use of aminoglycosides or similar compounds allowing stop-codon read-through and the upregulation of utrophin, as well as the clinically most advanced strategy using antisense oligonucleotides for exon-skipping.

Cell replacement therapy

Experimental cell replacement therapy approaches for muscular dystrophy have been pursued for a long time and included experiments whereby normal muscle was transplanted into a dystrophic animal [19, 20]. Ethical issues, in particular nonavailability of newborn muscle that would more easily be reinnervated and revascularised, made it difficult to pursue this avenue further. A different experimental procedure consists of injecting donor muscle precursor cells (myoblasts) into a dystrophic host, where donor myoblasts, which express dystrophin, would fuse with host myotubes, originally dystrophin deficient [21, 22]. For this strategy, immune reactions against the donor cells have to be overcome [23, 24]. Other experimental strategies involve the genetic manipulation of autologous donor myoblasts *ex vivo* [25]: the introduction of a dystrophin transgene restores functional dystrophin expression in injected myoblasts [26–28]. Additional cell-based experiments included systemic delivery of multipotent precursor cells, which can be isolated from nonmuscle tissue such as blood, blood vessels, bone marrow and adipose tissue [29]. Another approach is to use induced pluripotent stem cells, which can be generated from different cell types such as fibroblasts of the skin or muscle. These pluripotent stem cells can be differentiated *in vitro* into muscle precursor cells before being injected systematically into the recipient host [30, 31].

Myoblasts or satellite cells were most often used in human clinical trials for dystrophinopathies. Most of these clinical trials were not successful in improving muscle strength, despite multiple injections of a large number of donor myoblasts [32–34]. The disadvantage of satellite cells is their inability to effectively cross the vessel wall and that they cannot be administered systemically but have to be injected intramuscularly.

Therefore, the systemic administration of haematopoetic stem cells (CD133+) has been evaluated in a clinical phase I trial and was shown to be safe [25]. Dystrophin-expressing muscle fibres could be obtained in experiments applying this strategy to mdx mice (an animal model harbouring a stop codon in exon 23 of the dystrophin gene) [35]. Experiments in animal models using mesoangioblasts demonstrated their capacity to ameliorate the phenotype in the mouse [36] and dog [37], and subsequently led to clinical trials with human allogeneic human leucocyte an-

tigen (HLA) identical mesoangioblasts for the treatment of patients affected by DMD (https://www.clinicaltrialsregister.eu; EudraCT Nummer: 2011-000176-33), which are currently ongoing.

Gene replacement therapy

Diseases caused by deficiency of a product from a mutated gene (rather than a toxic gain of function) are, in theory, amenable to gene replacement approaches.

Experimental methods aiming at the introduction of a foreign gene into the host organism include administration of plasmid DNA [38] or vector-mediated gene transfer [39, 40] (fig. 3).

Several plasmid-mediated gene transfer studies have been performed in the mdx mouse, through direct injection of plasmids into skeletal muscle [41–45]. Direct plasmid DNA injection into skeletal muscle is usually less efficient than virus-mediated gene transfer, but is not associated with an immune response to the viral vector. However, the generation of immune responses to new epitopes encoded by the transgene is still a possibility [45]. In mice, intravascular injections of dystrophin-encoding plasmids have resulted in dystrophin expression in up to 10% of muscle fibres, which lasted for 6 months [45]. A phase I clinical trial of plasmid-mediated dystrophin complementary DNA (cDNA) delivery was conducted in Duchenne boys. A forearm muscle was injected and no adverse effects were noted. Six of the nine patients had low levels of dystrophin containing fibres after 3 weeks [46].

Viral vectors can enhance transgene uptake, but have other disadvantages. Commonly used viral vectors for gene transfer into muscle cells are retroviral, adenoviral, and adeno-associated viral vectors (fig. 3).

Retroviral vectors stably integrate the desired transgene into the host genome [47]. The integration is mainly a random process and can, therefore, depending on the integration site, activate oncogenes [48]. The development of self-inactivating lentiviral vectors, which integrate without their viral promoters and enhancing sequences, may solve this problem [49]. The major advantage of retroviral vectors is their stable integration into the genome, resulting in a permanent expression of the transgene. This approach, in combination with cell replacement therapy, has been used to repair dystrophin deficient cells *ex vivo* [50, 51].

The commonly used adenoviral vectors have the advantage of a large insert capacity and the ability to infect postmitotic cells. Adenoviral vectors have been used as a therapeutic strategy for dystrophinopathies [39, 52–54]. Adenoviral vectors have an insert capacity of about 8kb. Deletion of genes from the viral genome can further increase the insert capacity of the adenovirus up to 36kb. These "gutted" adenoviruses need to be grown in the presence of helper viruses, which provide the viral gene products originally encoded by the deleted genome. Studies in mdx mice with viruses delivering dystrophin have shown restoration of the dystrophin-glycoprotein complex at the sarcolemma, long-term expression of dystrophin and correction of histological and physiological indices of muscle disease, especially in young mice [39, 55–57].

Adenovirus-associated viruses (AAVs) have a higher muscle tropism, are less immunogenic, and have not been

reported to be associated with any human disease. AAV vectors, however, have a considerably smaller insert capacity of about 4kb; insertion of full length dystrophin cDNA is therefore not possible. However, small, functional "minidystrophin" molecules can be incorporated into these viral vectors and have been used in gene transfer experiments in dystrophinopathy mouse models (fig. 2) [40, 58, 59]. Stable gene expression could be observed up to 7 years after injection of AAV vectors in dogs and rhesus monkeys [60, 61]. In a human clinical trial, AAV vectors coding for minidystrophin have been injected intramuscularly into DMD patients [62, 63]. Although the viral genome could be detected in all biopsies of injected muscles, in only a few patients a small number of dystrophin-expressing muscle fibres could be identified. The observed T-cell-mediated immune response against minidystrophin in these patients could be an explanation for the low number of muscle fibres expressing dystrophin [62].

Mutation suppression therapy

Stop-codon read-through has been reported to be achievable with aminoglycoside antibiotics in cell culture and *in vivo* [64]. As about 10% of mutations in the dystrophin gene are point mutations leading to premature termination of translation, such "read-through therapy" would be of potential therapeutic interest. Therapeutic applications could be expanded to include other muscular dystrophies or other genetic diseases arising from premature termination of translation. Mdx mice harbouring a stop codon in exon 23 treated with gentamicin showed increased dystrophin expression and partial restoration of the dystrophin glycoprotein complex (DGC), as well as protection from contraction-induced muscle fibre damage and reduction in serum creatine kinase levels [64]. This observation, however, could not be repeated in another animal study [65]. Clinical trials did not show dystrophin staining after 2 weeks of daily gentamicin treatment (7.5 mg/kg) in four patients [66]. In a second study, 12 patients with either dystrophinopathy or sarcoglycanopathy were treated for 2 weeks with daily gentamicin (7.5 mg/kg) [67]. The latter study reported a decrease of serum creatine kinase levels, but without improvement in strength, functional testing or dystrophin expression. A more recent clinical trial demonstrated that 6 months of gentamicin treatment increased dystrophin levels with reduced creatine kinase levels, suggesting that the administration of gentamicin successfully induced read-through of stop codons [68]. However, the toxicity of gentamicin remains a major problem.

Ataluren (PTC-124, a 1,2,4-oxadiazole compound) is a small molecule that can override nonsense stop translation signals to produce full-length proteins. PTC-124 was effective in restoring the production of full-length proteins in animal models of cystic fibrosis and DMD [69]. In a large multicentre placebo-controlled double-blind study, patients received either one of two different doses of ataluren or placebo. In a 6-minute walk test, patients with the lower dose of ataluren showed an improvement in walking distance compared with the placebo group. However, this difference was not statistically significant and the group receiving the high dose of ataluren did not show any difference compared with the placebo group [70, 71].

Exon skipping therapy

The dystrophin gene includes 79 exons, which must be spliced together to produce the mature muscle, brain and cardiac transcripts. The splicing process is made possible by the splicing machinery and specific nucleotide motifs present on the prespliced RNA that defines exon-intron boundaries. The masking of such domains does not allow exon definition and leads to exclusion of the exon with the adjacent introns from the mature messenger RNA (mRNA) transcript, thus leading to exon skipping. The dystrophin gene sequence is known and elements involved in exon definition can be predicted. Thus it is possible to design specifically antisense nucleotides that would mask such domains important for splicing and induce exon skipping. Hence, exons containing stop codons could, theoretically, be skipped with this technique. It could be envisaged that several exons could be skipped until an open reading frame is reinstalled, in the case where skipping of one particular exon leads to out-of-frame splicing. This technique could, in theory, be of use for all dystrophin mutations that do not reside in essential protein domains, which make up about 80% of mutations in Duchenne patients.

In-vitro and *in-vivo* experiments in the mdx mouse and in dogs have demonstrated the feasibility of the exon skipping strategy for dystrophinopathies [72–74]. Systemic delivery of antisense nucleotides (AOs) into the mdx mouse [73] led to low levels of dystrophin devoid of exon 23, and AOs led to expression of dystrophin in the golden retriever dog model [74]. Exon skipping could be achieved in humans after intramuscular, as well as systemic, administration of AOs [75, 76].

In ongoing clinical trials, two different antisense molecules are being used: a 2'O-methyl phosphorothionate oligonucleotide (PRO51/GSK2402968 Prosensa Therapeutics/GSK) and a phosphorodiamidate morpholino (AVI-4658/Eteplirsen; Sarepta Therapeutics). Currently, clinical studies to demonstrate efficacy of these two molecules in DMD patients are being conducted [77].

Utrophin upregulation

Newly expressed dystrophin epitopes can trigger an immune response in a dystrophin deficient host. Upregulation of an endogenous protein with functions similar to those of dystrophin could circumvent this undesired side effect. Utrophin contains C' and N' terminal domains that are highly homologous to dystrophin, with less homology in the central rod domain. Utrophin preferentially accumulates at the neuromuscular and myotendinous junctions [78, 79] in intact muscle, where it has similar functions to dystrophin, whereas dystrophin is found along the entire length of the sarcolemma. Utrophin can serve as a link between the actin cytoskeleton and the extracellular matrix through association with the dystrophin glycoprotein complex [80]. Therefore, it can be hypothesised that utrophin upregulation might correct dystrophin deficiency. Indeed, in muscle from dystrophin deficient patients, utrophin is already upregulated [32], possibly in an attempt by the muscle cell to compensate for dystrophin deficiency. Experimental overexpression of utrophin can lead to structural and functional improvements in mdx mice [81, 82]. Enhancement of utrophin expression through upregulation of the endogen-

ous gene [83–85] could be of benefit without causing an immune response [86, 87].

Limb girdle muscular dystophies

The LGMDs are a heterogeneous group of muscle disorders characterised clinically by weakness and wasting in the pelvic and shoulder girdle muscles, and pathologically by muscle necrosis and regeneration. This group of disorders can be divided into two types on the basis of their pattern of inheritance: type 1 – autosomal dominant and type 2 – autosomal recessive. The dominantly inherited LGMDs are rare, representing <10% of all LGMDs. The more common autosomal recessive forms have a prevalence of about 1:15,000 to 1:100,000. LGMDs are further subdivided according to their different genetic loci (table 1).

Mutations causing LGMD are found in genes encoding proteins that are membrane-associated, cytosolic, sarcomeric or constituents of the nuclear envelope (fig. 1).

Clinically, age at onset and rate of disease progression are variable in the LGMDs, both within the same disease group and even within members of the same family. Moreover, identical mutations may cause different disease phenotypes, even among family members. These findings point to the existence of putative modifying factors. Such phenotypic variability makes prediction about disease course difficult and should be considered when counselling patients. Currently, immunohistochemistry of histological sections and Western blot analysis on muscle homogenates are performed as diagnostic steps to determine the putative protein defects in LGMD. However, in many instances, reduction of a given protein may be secondary to the absence of another. This points to shared pathophysiological pathways, but also poses diagnostic difficulties. Therefore, whenever possible, mutational analysis should be performed to confirm the biochemical findings.

Dysferlinopathies

Dysferlin is a membrane protein of about 230kD that contains several C2 motifs. These motifs are present in many membrane-associated proteins, and bind calcium and anionic phospholipids [88–90]. Dysferlin is necessary for the repair of membrane tears in muscle cells, which can arise during muscle exercise [91]. Loss of dysferlin leads to a defective repair mechanism and the integrity of the plasma membrane cannot be restored following injury, leading to muscle fibre necrosis (fig. 4B).

Dysferlin mutations can result in two distinct phenotypes that are characterised by either proximal or distal weakness and wasting; the distal form is known as Miyoshi myopathy and the proximal form as LGMD2B. Both phenotypes can be present within the same family [92, 93]. Dysferlin mutations account for about 20% of patients with recessively inherited LGMDs. Facial and pharyngeal muscles remain unaffected. Cardiac, respiratory or cognitive impairment is not part of the disease, although some reports mention cardiac involvement [94]. Serum creatine kinase levels can be very high (up to 150 times normal) and, occasionally, inflammatory infiltrates are seen on skeletal muscle biopsy, which may be mistaken for a primary inflammatory myopathy. Age of onset can be variable, but is mostly in the teenage years. Patients can be engaged in athletic activities prior to disease onset.

Several therapeutic strategies similar to the approaches described for DMD have been applied in experiments: exon skipping strategies [23, 95, 96], stop codon read through [97] and somatic gene therapy using minidysferlins [98,

Table 1: Limb girdle muscular dystrophies (LGMDs).				
type	Inheritance AD = autosomal dominant AR = autosomal recessive	Gene	Chromosome	Reference
LGMD 1A	AD	Myotilin	5q31	[105]
LGMD 1B	AD	Lamin A/C	1q22	[106]
LGMD 1C	AD	Caveolin	3p25	[107]
LGMD 1D	AD	DNAJB6	7q36	[108]
LGMD 1E	AD	Desmin	2q35	[109]
LGMD 2A	AR	Calpain-3	15q15.1-q21-1	[110]
LGMD 2B	AR	Dysferlin	2p12-14	[111]
LGMD 2C	AR	Sarcoglycan, gamma	13q12	[112]
LGMD 2D	AR	Sarcoglycan, alpha	17q21	[113]
LGMD 2E	AR	Sarcoglycan, beta	4q12	[114]
LGMD 2F	AR	Sarcoglycan, delta	5q33-q34	[115]
LGMD 2G	AR	Telethonin	17q12	[116]
LGMD 2H	AR	TRIM32	9q33.2	[117]
LGMD 2I	AR	FKRP	19q13.32	[118]
LGMD 2J	AR	Titin	2q31	[119]
LGMD 2K	AR	POMT1	9q34.1	[120]
LGMD 2L	AR	Anoctamin 5	11p14-12	[121]
LGMD 2M	AR	Fukutin	9q31-33	[122]
LGMD 2N	AR	POMT2	14q24.3	[123]
LGMD 2O	AR	POMGNT1	1p34.1	[124]
LGMD 2Q	AR	Plectin 1	8q24	[125]
DNAJB6 = DnaJ (Hsp40) homolog, subfamily B, member 6; FKRP = fukutin-related protein; POMGNT1 = protein O-linked mannose N-acetylglucosaminyltransferase 1; POMT1 = protein O-mannosyl transferase 1; POMT2 = protein O-mannosyl transferase 2; TRIM32 = tripartite motif containing 32				

99]. Another strategy is the inhibition of degradation of missense-mutated dysferlin [100, 101].

The elimination of mutated and misfolded proteins is conducted by the cellular quality control system, which recognises misfolded proteins and initiates their degradation to prevent aggregation within the cell. *In-vitro* experiments on muscle cells from dysferlinopathy patients demonstrated that missense mutations destabilise the dysferlin protein and lead to its rapid degradation. Treatment of patient-derived myoblasts with inhibitors of the proteasome prevented the degradation of the mutated protein and restored the membrane repair function of the cultured muscle cells. Currently, there is an ongoing clinical trial to validate this strategy in dysferlinopathy patients (NCT01863004 / clinicaltrials.gov)

Myotonic dystrophy

Figure 4

Molecular pathomechanisms in muscular dystrophies.

Illustration of the molecular pathomechanisms of myotonic dystrophy type 1 (A), dysferlionpathy (B), and facioscapulohumeral muscular dystrophy (C).

(A) Missplicing in myotonic dystrophy type 1 (DM1). The molecular basis of DM1 is an expansion of an unstable repeat sequence in the noncoding part of the *DMPK* gene. Severity of disease is correlated with repeat expansion size. In DM1 the mutation is located in a noncoding region and does not alter the protein sequence, but leads to toxic RNA. The sequestration of the alternative splicing factor MBNL1 by toxic RNA leads to altered splicing of target mRNAs like the muscle-specific CLCN1 chloride channel. The missplicing of the chloride channel leads to a loss of protein and finally to symptoms of myotonia.

(B) Defective membrane repair in dysferlinopathy. Normal muscle cells respond to membrane damage by the recruitment of vesicles. In a dysferlin dependent mechanism the vesicle fuse to form a membrane patch sealing the damaged membrane. In dysferlinopathy patients, mutations in the dysferlin gene leading to a loss of dysferlin and the muscle cells are not capable to repair the membrane.

(C) Toxic expression of DUX4 in facioscapulohumeral muscular dystrophy (FSHD). The FSHD locus maps to chromosome 4q35 and consists of a variable number of tandem repeats (D4Z4), which in normal individuals usually consist in multiple copies (>11).

In FSHD patients, DUX4 protein is expressed upon epigenetic activation of the locus and stabilisation of the DUX4 mRNA by a polyadenylation site (PAS). The activation of DUX4 transcription is a consequence of a D4Z4 repeat deletion (<11 repeats) or of a mutation in the SMCHD1 gene. The expression of the DUX4 protein, normally not present in muscle cells, leads to activation of a set of genes inducing muscle cell death.

Myotonic dystrophy is an autosomal dominant multisystem disease affecting skeletal muscle, heart, brain, lens and endocrine organs. On the basis of genetic loci and clinical characteristics, two myotonic dystrophies can be distinguished: type 1 (DM1, Curschmann-Steinert) and type 2 (proximal myotonic myopathy, PROMM, DM2). DM1 is caused by a CTG expansion in the 3' untranslated region of the *DMPK* gene [102], and DM2 by a CCTG expansion in intron 1 of the *ZNF9* gene [103].

The clinical findings in DM1 have been subdivided according to severity into three overlapping phenotypes: mild, classic and congenital. These phenotypes roughly correlate with CTG expansion size. Individuals with the mild form of DM1 usually have a CTG repeat size between 50 and 150, age at onset is between 20 and 60 years, life-span is unaffected and disease may manifest itself with cataracts, diabetes and mild myotonia.

Patients affected by the classical form of DM1 have a CTG repeat size of up to 1,000 and develop muscle weakness and wasting, myotonia, cataracts, diabetes and heart conduction abnormalities. A myopathic facial expression can develop as a result of weakness of the facial muscles. Muscle weakness predominantly affects distal muscles in the lower and upper extremities. Smooth muscle involvement can result in swallowing difficulties and difficulties with bowel movements.

Life-span is mildly reduced and the most common cause of death is respiratory insufficiency due to diaphragmatic, intercostal and orpharyngeal involvement [104]. Cardiac sudden death caused by heart block and ventricular arrhythmia is the second most common cause of death in DM1 patients.

Central nervous system manifestations of the disease may include hypersomnia, personality disorders, altered regulation of respiration and cognitive impairment. Other manifestations of the disease include cataracts of the posterior subcapsular zone, testicular atrophy, balding, and diabetes due to insulin resistance. Women affected by DM1 may present problems during pregnancy and labour, such as prolonged labour, retained placenta and postpartum haemorrhage, owing to uterine smooth muscle involvement. The spontaneous abortion rate is higher in women with DM1, and if the foetus is affected by a congenital form of DM1, further complications include polyhydramnions and reduced foetal movements.

Children with the congenital form of DM1 have usually over 2,000 CTG repeats and they may present prenatally with polyhydramnions. Neonatal hyptonia, feeding difficulty and respiratory compromise may be present [105]. Ensuing from facial muscle weakness *in utero* is the typical V-shaped (tented) mouth. Mental retardation is present in about half of the patients with congenital DM1 and some patients have a certain degree of cerebral atrophy and ventricular dilatation. About a quarter of children with DM1 die from failure to develop adequate respiratory function, and surviving patients have a reduced life-span [106]. Current treatment is symptomatic, and involves vigilant cardiac monitoring and implantation of a pacemaker when indicated, cataract removal, and treatment of diabetes and of pain. Adaptation of assistive devices as disease pro-

gresses is helpful. Daytime sleepiness can be treated with modafinil [107, 108].

DM1 is caused by an unstable CTG repeat expansion in the 3' untranslated region of the myotonic dystrophy protein kinase gene (*DMPK*) (fig. 4A) [102]. As the unstable repeats enlarge when transmitted from one generation to the next, subsequent generations are expected to be more affected than their parents. Repeats smaller than 100 tend to be more unstable in sperm than in ova [109] and the transition from mild to classical DM1 is usually transmitted by the father. However, congenital DM1 is usually transmitted by the mother, suggesting that sperm probably are not functional when harbouring very large repeats [110].

Apart from germline instability, there is also somatic instability, with over a 10-fold increase of repeat length in skeletal muscle compared with DNA derived from blood leucocytes. Prolongation of nucleotide repeats probably occurs during DNA repair [111].

The triplet repeat in DM1 is located in the 3' untranslated region of the *DMPK* gene. Although many studies suggest that DMPK levels are reduced in DM1 [112], DMPK-deficient mice show only a subtle phenotype with first degree atrioventricular (AV) cardiac conduction block without myotonia in the heterozygous state [113, 114]. These experiments suggest that DMPK is dispensable for muscle development and that a reduction of DMPK may account at most for only a few of the features seen in DM1. The finding that an intronic nucleotide repeat expansion on a different chromosome causes DM2 with very similar features to DM1 further questions whether haploinsufficiency of DMPK can account for the disease phenotype of DM.

Transgenic mice with expanded CTG repeats introduced into the 3' untranslated region of a human skeletal actin gene develop myotonia and muscle pathology similar to patients with DM1 [115]. Transgenic mRNA accumulates in the nucleus and forms aggregates similar to DMPK mRNA in DM1 patients. These elongated CUG repeats form hairpin structures and can sequester RNA-binding proteins implicated in splicing, such as the protein muscleblind-like-1 (MBNL1) [116-118]. Human muscleblind-like genes *MBNL1*, *MBNL2*, and *MBNL3* are homologous with the Drosophila gene muscleblind, which is essential for muscle and eye differentiation [119].

Muscleblind is implicated in splicing the chloride channel CLCN-1, which is developmentally regulated. Mutations in *CLCN-1* can lead to the dominantly (Thompson) and recessively (Becker) inherited myotonias. A broad range of splicing alterations in CLCN-1 was observed in muscle tissue from DM1 and DM2 patients [120]. Thus, alteration of CLCN-1 splicing can explain myotonia in DM. Along these lines, MBNL1 deficient mice show reduced expression of CLCN-1 owing to aberrant splicing, and exhibit myotonia. Furthermore, *MBNL1* knock-out mice recapitulate many features of the DM phenotype [121]. Other genes are incorrectly spliced in DM, such as the insulin receptor, leading to expression of the nonmuscle insulin receptor in muscle tissue. The nonmuscle insulin receptor has a decreased metabolic response to insulin. MBNL deficient mice also develop cataracts [121]. The CUGBP/Elav like family member 1 (CELF1) is another splicing factor, which is misregulated owing to the trinucleotide expansions and

seems to be involved in the missplicing phenotype. CELF1 levels are elevated and the protein is hyperphosphorylated, leading to a missplicing of certain gene transcripts, in DM1 [122, 123].

The concept of a "splice-opathy" as the pathogenic mechanism raises very interesting potential therapeutic possibilities. First, muscle damage is not well advanced early in the disease in DM and is only slowly progressive, suggesting that restoration of the splicing abnormality may allow "recovery" of muscle tissue. The hairpin loops formed by expanded triplet repeats that bind and sequester MBLN proteins could be targeted by low molecular weight compounds with the idea of disrupting the hairpin structure, and thus interfering with MBLN binding (fig. 4A). Different therapeutic strategies have been tested in *in-vitro* or *in-vivo* experiments using animal models: antisense technologies [124–127], RNA interference [128, 129] and ribozyme technology [130], as well as peptides [131] and small molecular compounds [132–134].

Facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD, MIM 158900) is an autosomal dominantly inherited neuromuscular disorder with an incidence of approximately 1 in 20,000 [135]. Typical age at diagnosis is in the second decade of life. Symptoms can range from severe upper and lower limb involvement and wheelchair use in early childhood, to very mild facial weakness in the seventh decade [136]. Scapula-fixators are usually first affected; rarely the disease begins with weakness of facial, foot-dorsiflexor or pelvic girdle muscles. Shoulder girdle weakness leads to asymmetrical scapula alata and pectoralis muscle atrophy. An initially fairly normal deltoid muscle contributes to the distinctive high rise of the scapula on elevation of the arms. Another very typical feature is the asymmetrical facial weakness. Muscle pain and fatigue are frequently reported; dysphagia is rare, as is respiratory insufficiency [137, 138]. Occasionally, cardiac conduction defects have been observed, although most authors claim no cardiac involvement. A subclinical high-tone hearing loss and a subclinical retinal vasculopathy have been described as part of the disease [139, 140].

The FSHD locus maps to chromosome 4q35 adjacent to the 4qter subtelomeric region [141]. A variable number of tandem repeats, designated D4Z4, has been identified in this region, which in normal individuals usually contains multiple integral copies (11–>100) of a 3.3kb tandem repeat sequence [142]. The number of the repeats is reduced in the vast majority of FSHD patients to less than 11.

Recent studies have uncovered the pathomechanism of FSHD. Experiments demonstrated that induced expression of DUX4, a transcriptional factor within the DZ4Z repeats, initiates a gene expression programme toxic for muscle cells. The *DUX4* gene is normally epigenetically repressed. There are two requirements for developing the disease: (1.) induction of *DUX4* transcription by epigenetic activation and (2.) stabilisation of the *DUX4* mRNA transcript allowing translation of the DUX4 protein (fig. 4C). In FSHD1, the epigenetic activation is induced by the reduction of the

DZ4Z repeat number to below 11. The deletions lead to hypomethylation and finally transcription of the *DUX4* gene locus. FSHD2 patients have normal DZ4Z repeat numbers, but show hypomethylation and active transcription of the *DUX4* gene. A recent study showed that in a subset of FSHD2 patients the epigenetic activation is a consequence of a mutation in the *SMCHD1* gene.

A second genetic variant is necessary to allow translation of the DUX4 protein: Two distinct 4qter subtelomeres, designated 4qA and 4qB, have been recognised [143, 144]. The 4qA- and 4qB-defined 4qter subtelomeres are found to occur with almost equal frequency in the population [144]. However, in essentially all FSHD patients, disease expression is not only associated with large D4Z4 contractions, but these contractions must be specifically located on a 4qA-defined 4qter subtelomere [143, 144]. The 4qA variant has a polyadenylation site stabilising the *DUX4* mRNA and allowing translation of DUX4 protein. Thus, induction of *DUX4* transcription by epigenetic activation and stable *DUX4* mRNA transcripts need to be present for disease development (fig. 4C).

Possible therapeutic strategies could target the DUX4 mRNA for degradation to prevent DUX4 protein production within muscle cells or directly target the DUX4 protein to inhibit its function as a transcription factor. Recent insights into the molecular pathomechanism of FSHD will allow the development of specific drugs targeting either DUX4 mRNA or protein.

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Figures (large format)

Figure1

Illustration of proteins and potential targets involved in muscular dystrophies.

Schematic representation of the localisation and interaction of selected proteins involved in muscular dystrophies. Dystrophin links the extracellular matrix to the actin cytoskeleton via the dystrophin-associated glycoprotein complex (DGC). Absence of dystrophin results in destabilisation of the DGC. Some genes mutated in muscular dystrophies encode proteins responsible for the correct glycosylation of dystroglycan, necessary for interaction with the extracellular matrix. The tail anchored membrane protein dysferlin is involved in membrane repair.

FKRP = fukutin-related protein; POMT = protein O-mannosyl transferase

Figure 2

Exon skipping as a therapeutic strategy for the treatment of Duchenne muscular dystrophy.

During splicing of the dystrophin pre-mRNA the introns are removed and exons are joined to build mRNA with a reading frame to allow synthesis of functional dystrophin proteins. In the case of exonic deletions, "out of frame" mRNA can be produced, inducing premature termination of protein translation. This leads to a nonfunctional dystrophin protein, rapidly removed by the cellular degradation machinery. An exon skipping strategy is applied to restore the reading frame. By use of antisense oligonucleotides targeting splice sites of the pre-mRNA, one or more exons are removed to correct the reading frame. This results in a shortened but partially functional dystrophin protein.

Figure 3

Different gene therapy strategies used in muscular dystrophies.

In muscular dystrophies, gene therapy is used to deliver genetic material into the host and finally target the diseased muscle tissue to correct the genetic defect. In most cases, the transferred DNA encodes for the protein missing due to a mutation. Gene transfer can be mediated by different strategies and vectors. The methods differ in their transduction efficiency, packaging capacity, risk of immunogenicity or oncogene activation by genome insertion.

Figure 4

Molecular pathomechanisms in muscular dystrophies.

Illustration of the molecular pathomechanisms of myotonic dystrophy type 1 (A), dysferlionpathy (B), and facioscapulohumeral muscular dystrophy (C).

(A) Missplicing in myotonic dystrophy type 1 (DM1). The molecular basis of DM1 is an expansion of an unstable repeat sequence in the noncoding part of the *DMPK* gene. Severity of disease is correlated with repeat expansion size. In DM1 the mutation is located in a noncoding region and does not alter the protein sequence, but leads to toxic RNA. The sequestration of the alternative splicing factor MBNL1 by toxic RNA leads to altered splicing of target mRNAs like the muscle-specific CLCN1 chloride channel. The missplicing of the chloride channel leads to a loss of protein and finally to symptoms of myotonia.

(B) Defective membrane repair in dysferlinopathy. Normal muscle cells respond to membrane damage by the recruitment of vesicles. In a dysferlin dependent mechanism the vesicle fuse to form a membrane patch sealing the damaged membrane. In dysferlinopathy patients, mutations in the dysferlin gene leading to a loss of dysferlin and the muscle cells are not capable to repair the membrane.

(C) Toxic expression of DUX4 in facioscapulohumeral muscular dystrophy (FSHD). The FSHD locus maps to chromosome 4q35 and consists of a variable number of tandem repeats (D4Z4), which in normal individuals usually consist in multiple copies (>11). In FSHD patients, DUX4 protein is expressed upon epigenetic activation of the locus and stabilisation of the DUX4 mRNA by a polyadenylation site (PAS). The activation of DUX4 transcription is a consequence of a D4Z4 repeat deletion (<11 repeats) or of a mutation in the SMCHD1 gene. The expression of the DUX4 protein, normally not present in muscle cells, leads to activation of a set of genes inducing muscle cell death.