1. Gene and Cell Therapies for Muscular Dystrophies

Sara Benedetti[1], Giulio Cossu[1,2] and Francesco Saverio Tedesco[1]*

[1]Department of Cell and Developmental Biology, University College London, UK.
[2]Institute of Inflammation and Repair, University of Manchester, UK.

*Corresponding author (F.S.T.):
Department of Cell and Developmental Biology, University College London, WC1E 6DE London, UK.
Tel: +44 (0) 2031082383.
E-mail: f.s.tedesco@ucl.ac.uk

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1.1 INTRODUCTION

The main goal of gene and/or cell therapy is to repair and/or replace the mutated gene responsible for a genetic disease, thus reverting once and (ideally) forever the pathological phenotype. This is an exceptionally challenging aim to achieve in the case of muscular dystrophies, hereditary disorders that severely affect skeletal muscle tissue. Skeletal muscle is indeed the most abundant tissue of the human body and it must support mechanical/physiological stress due to continuous contractions. In order to ameliorate the dystrophic phenotype the mutated gene has to be repaired/replaced into an incredibly large number of muscle fibers, whose nuclei are post-mitotic. Moreover, due to the progressive deterioration of the tissue that accompanies the progression of the disease, repairing/replacing the gene in the late phase of the disease would be very challenging and with limited efficacy outcome.

As detailed later in the chapter, in the last two decades the identification of genes and mutations responsible for the majority of muscular dystrophies tremendously increased the hope of developing suitable and focused therapeutic strategies (reviewed in [1]). Despite the fact that in a reasonable number of muscular dystrophies the skeletal muscle phenotype is associated with cardiac involvement [2-4] and/or mental impairment [5], in this book chapter we will analyze the current status of gene and cell therapy strategies focusing only on skeletal muscle. Similarly we will not address the status of pharmacological approaches as well as up-regulation and/or over-expression of dystrophin-related proteins that have been extensively reviewed elsewhere [6-9].

1.2 MUSCULAR DYSTROPHIES

Muscular dystrophies are a miscellaneous group of neuromuscular genetic disorders mainly characterized by progressive muscle weakness, which in the most severe forms lead to an independent-ambulation loss and to a premature death caused by a cardiac or respiratory attack [10]. They are characterized by changes of muscle histological features such as fiber size heterogeneity, inflammatory cell infiltration, continuous and repeated cycle of skeletal myofibers degeneration and regeneration [10, 11].

Despite these shared features, muscular dystrophies differently affect different muscles, are caused by mutations in a large number of genes and show a great heterogeneity in clinical onset as well as in the rate of progression and severity. On the basis of these parameters, major groups have been identified and described as Duchenne and Becker, Limb-girdle, Congenital, Myotonic, Facioscapulo-humeral, and Oculopharyngeal muscular dystrophies [10]. Some groups that show a greater heterogeneity such as Limb-girdle or Congenital muscular dystrophies have been further sub classified according to the genetic mutation they origin from and/or their autosomal or recessive/dominant inheritance. Indeed, in parallel with the traditional classification based on clinical features/symptoms we described above, the recent large-scale genotyping of muscular dystrophies affected patients allowed even a more precise sub classification based on their molecular deficits (i.e. dystroglycanopathies) [12].

Duchenne and Becker muscular dystrophy

Duchenne muscular dystrophy (DMD) is one of the most severe and common forms of muscular dystrophy caused by mutations in the largest gene in the human genome, the dystrophin gene (2.4Mb), which is the located on the X-chromosome. It codifies for dystrophin, a rod-shaped protein belonging to the dystrophin-glycoprotein complex (DGC) [13] that connects the cytoskeletal actin with the surrounding extracellular matrix [14]. Indeed dystrophin and in general DGC play a crucial role in stabilizing the sarcolemma and in preserving myofibers integrity from long-term contraction-induced damage [15-18]. As a consequence, mutations in the dystrophin gene generate sarcolemma instability and skeletal myofibers damage/degeneration. Indeed, DMD presents an early onset with difficulty in ambulation and proximal muscle weakness. Sadly, the majority of DMD patients died within the third decade of life by respiratory or cardiac failure, though better cardiac and respiratory assistance have recently prolonged their life but unfortunately not its quality.

Becker muscular dystrophy (BMD) is also caused by mutations in the dystrophin gene but in contrast with DMD, BMD patients show a milder phenotype and a late onset [19]. The “reading frame” theory tried to explain the phenotypic differences for mutations occurring on the very same gene: DMD is caused by out-of-frame mutations with no protein production as a consequence, whereas BMD is the consequence of in-frame mutations resulting in the production of a shorter but partially functional dystrophin protein. Nevertheless, exceptions to the “reading frame” theory for both DMD and BMD patients make less obvious a clear genotype-phenotype correlation [20]

Limb-girdle muscular dystrophies

Limb-girdle muscular dystrophies (LGMDs) are considered one of the most heterogeneous groups among muscular dystrophies. Some forms of LGMDs show a dominant inheritance (LGMD1) whereas some others have a recessive inheritance (LGMD2) [21]. Similarly, there is a great variability concerning onset, severity of the progression and survival. Even if LGMDs are all characterized by weakness of the proximal-girdle muscles, they are caused by genetic mutations in distinct genes, some of which encode for DGC-associated proteins while other mutations have yet to be identified [22, 23]. Recently, thanks to the use of new technologies (i.e. next generation sequencing) new LGMDs causative genes have been identified increasing the possibility of designing appropriate and specific therapeutic strategies [24-28].

Congenital muscular dystrophies

A greater clinical and genetic heterogeneity is associated with congenital muscular dystrophies (CMD), autosomal recessively inherited myopathies with a very early onset and a generally slow muscle weakness progression [29, 30]. As for LGMDs, mutations in distinct genes are responsible for individual forms of CMDs, the majority of which are still unknown. However, very recent work describes pathogenesis and possible molecular mechanisms underlying some forms of CMDs [27, 28, 31-34].

Myotonic dystrophies

Myotonic dystrophies (DMs) are autosomal dominant dystrophies characterized by myotonia and progressive muscle wasting in association with

secondary multisystemic clinical features [35, 36]. There are two types of DMs caused by mutations in two distinct genetic loci: type 1 (DM1), with mutations in the DMPK gene, and type 2 (DM2), due to mutations in the ZNF9 gene. In both cases mutations caused an expansion of untranslated DNA sequences. The result is an aberrant alternative splicing of a number of genes and an altered cellular function [37-41].

Facioscapulo-humeral and oculopharyngeal muscular dystrophies

Facioscapulo-humeral dystrophy (FSHD) and oculopharyngeal (OPMD) muscular dystrophies are autosomal dominant myopathies that affect a defined group of muscles [42]. FSHD is the third common form of muscular dystrophy that primarily involves facial, upper arm, and shoulder girdle muscles, and then progressively extends to all body muscles. It is caused by a reduction in the copy number of the D4Z4 macro-satellite repeats at the subtelomeric region of chromosome 4q35, which turn into epigenetic changes as the de-repression of some genes transcription [43]. In addition, in FSHD patients a polyadenylation stabilizes DUX4 transcripts that are instead degraded in normal individuals [44]. DUX4 aberrant expression triggered a gene deregulation cascade that turn into a toxic effect and indeed lead to muscle cells apoptosis.

In OPMD extra-ocular and upper facial muscles are severely affected leading to a progressive neck and proximal upper (and even lower) limb musculature weakness. At variance with FSHD, where the pathology is caused by a reduction in DNA sequence repeats, OPMD is the results of a short guanine-cytosine-guanine (GCG) expansion in the poly adenylate

binding nuclear I protein (PABN1) gene, whose protein product is a regulator of mRNA stability [45, 46]. Recently genome-wide RNA expression profiles and transcriptome analysis revealed similarity among OPMD and elderly muscles, suggesting so that PABPN1 protein have a key role in muscle aging [47].

1.3 TRANSCRIPT AND GENE REPAIR

1.3.1 ANTISENSE OLIGONUCLEOTIDES AND EXON SKIPPING

Antisense oligonucleotides (AONs) were first discovered in late 1970s for their role in inhibiting viral replication [48, 49]. Less than two decades later, the use of new generation AONs in re-directing and modulating pre-mRNA splicing was strongly supported by studies in beta-thalassemia [50]. In particular, it was demonstrated that AONs could target specific exons preventing their recognition by the splicing apparatus.

Based on the observation made in the late 80' that BMD is caused by in-frame mutations leading to a shorter protein, while DMD is caused by a frame shift with no protein made [51], AON appeared as a strategy to turn DMD into BMD. Few years later, the first proof-of-concept of AONs as an effective treatment for DMD was *in vitro* [52]. After that, a number of additional but fundamental *in vitro* studies using AONs on murine [53] and human [54-56] dystrophic cells clearly highlighted their potential. Indeed, AONs have been extensively and successfully tested *in vivo* in DMD mouse models [57-64] and in dystrophic dogs [65-67], showing amelioration of the pathological phenotype.

Among all the existing chemistries, two classes of AONs are under clinical experimentation: 2'O-methilphosphorothioate oligoribonucleotide (2'OMe) e phosphorodiamidate morpholino oligomers (PMOs). Pioneer clinical trials were conducted testing intra-muscular administrations of 2'OMe (PRO051/GSK 2402968, Drisapersen) [68] and PMO (AVI-4658/Eteplirsen) [69] respectively. In both cases, local injections were well tolerated and exon skipping was detected together with dystrophin restoration. The success of exon-skipping intramuscular clinical trials lead to two following systemic phase I/II studies for DMD whose results demonstrated a safety profile combined with a remarkable production of dystrophin protein [70, 71]. Recently, a double-blind placebo-controlled clinical trial to test Eteplirsen (the trade name for the oligonucleotide specific for exon 51 skipping) showed that systemic injections of the PMO induced dystrophin production (24 weeks post treatment) together with some functional improvement (63.7 meters advantage in the 6 minutes walking distance test) [72]. However, very recently GlaxoSmithKline and Prosensa announced that a phase III clinical trial on DMD patients with Drisapersen (2'OMe oligonucleotide for exon 51 skipping) did not meet the primary endpoint of a statistically significant improvement in the 6-minute walking distance test compared to placebo. (http://www.gsk.com/media/press-releases/2013/gsk-and-prosensaannounce-primary-endpoint-not-met-in-phase-iii-.html). Results from other placebo-controlled studies for AONs are expected and other clinical trials are currently ongoing or planned [73, 74].

On the other hand, one disadvantage of exon skipping is the need for repeated AONs administrations. To overcome this limitation the feasibility of

using designed chimeric small nuclear RNAs (snRNAs) to guarantee continuous in loco production of AONs has been investigated *in vitro* [75, 76] and *in vivo* [77-80]. Even though exon skipping is one of the most promising approaches to treat muscular dystrophies, it is a mutation specific strategy. Though theoretically suitable for 70%-80% of DMD mutations [81-83], a significant number of mutations, such as large deletions, cannot benefit from this strategy.

Apart from DMD, there are other muscular dystrophies where the genetic defect can be repaired using exon skipping: Myotonic dystrophy 1 (MD1), Limb-girdle muscular dystrophy 2B (LGMD 2B), Fukuyama congenital muscular dystrophy (FCMD) and merosin-deficient congenital muscular dystrophy (CMD1A).

One of the most prominent features of MD1 is myotonia (i.e. slow relaxation of muscles after contraction) which is specifically caused by the inclusion of exon 7a into ClC-1 mRNA transcripts, leading to a premature stop codon and a non-functional chloride channel. AONs targeting exon 7a have been used *in vitro* to produce an almost normal protein. Intramuscular injections of AONs into disease animal models restored ClC-1 and reduced myotonia [84]. Moreover, a recent *in vitro* study demonstrated the feasibility of a UsnRNA mediated exon-skipping approach for this type of muscular dystrophy [85].

LGMD 2B is a form of muscular dystrophy caused by mutations spread all over the 55 exons of the dysferlin gene, whose protein is involved in repairing skeletal myofibers membrane. AONs inducing exon 32 skipping in

patient-derived cell cultures have now been identified, as well as AONs targeting other dysferlin exons in control cell cultures [86-88].

In the case of FCMD, the disrupted interaction with extracellular matrix proteins, which is at the basis of the pathology, are caused by SVA retrotransposon insertion in the 3'UTR of the fukutin gene. This insertion led the loss of 38 amino acids from fukutin C-terminal and the addition of 129 amino acids coming from SVA gene. 2'OMe AONs targeting the splice acceptor, the predicted exonic splicing enhancer and the intronic splicing enhancer restored fukutin expression and functionality *in vitro* and *in vivo* [89].

Therapeutic potential of PMOs was also successfully tested in a mouse model for CMD1A, a merosin-deficient congenital muscular dystrophy in which there is an active muscle regeneration process. Moreover, the authors demonstrated that regenerating fibers are more permissive for PMO entry [90].

1.3.2 RIBOSOMAL READ-THROUGH OF NONSENSE MUTATIONS

A nonsense mutation occurs when a single nucleotide substitution in mRNA coding region converts an amino acid-codifying triplet into a premature termination codon (PTC). This lead to: I) mRNA instability by nonsense-mediated mRNA decay (NMD); II) premature translational termination; III) production of a truncated and mostly not functional protein product [91]. The NMD pathway depends on different factors [92], whose inactivation stabilizes nonsense-containing transcripts and promotes nonsense codon read-through [93]. As a consequence, the modulation of either NMD rate or premature translational termination could lead to the production of a functional protein

from nonsense-containing mRNAs. Indeed, read-through for nonsense mutation is a transcript repair strategy based on the use of small molecules able to allow the ribosomal subunits to substitute the PTC with a different amino acid resulting into the production of the full-length protein [94]. Being a mutation specific strategy, it has been estimated that small molecule-mediated read-through could be theoretically applied to 13-15% of DMD/BMD mutations [95, 96].

Two “read-through” molecules have undergone pre-clinical and clinical investigation: gentamicin and Ataluren (PTC124; PTC Therapeutics) [94]. Starting from early 1960s, pioneer studies conducted on bacteria [97], yeasts [98, 99] and subsequently on mammalian cells [100, 101] clearly highlighted the potential of aminoglycoside antibiotics in interfering with the translation termination process and suppressing PTCs. Indeed, more than ten years ago it was reported that aminoglycosides, and in particular gentamicin, allowed the production of the full-length dystrophin protein *in vivo* [102]. Based on these promising pre-clinical results, clinical trials started in DMD and BMD patients. The first follow-up clinical study in DMD/BMD patients with nonsense mutations reported a failure in producing full-length dystrophin [103]. In order to better investigate the potential of gentamicin and aminoglycoside antibiotics, subsequent *in vitro* and *in vivo s*tudies analyzing the efficiency level of nonsense mutation read-through were performed on DMD/CMD individual nonsense mutations [104, 105]. In both studies, the results emphasized that only a minority of PTCs found in patients show a significant level of read-through, implying that only a subset of DMD and CMD patients with nonsense mutation would potentially benefit from aminoglycoside

treatment. This also suggested a possible explanation for the failure of the first clinical trial with gentamicin [103]. Few years later, other clinical trials showed that read-through occurred and full-length dystrophin was produced, even if not at a high extent [106, 107]. Beside an improvement in creatine kinase levels, no other clinical benefits have been achieved. Discrepancy of results among these studies and the previous one could potentially relate to differences in drug treatments or permissiveness of different PTCs.

In addition to these studies, Allamand et al showed that the dipeptide antibiotic negamycin but not gentamicin allowed mRNA read-through of PTC responsible for a subset of CMD patients. However, neither gentamicin nor negamycin enabled re-expression of the corresponding full-length protein [108]. Overall, these results underline that several issues regarding nonsense mutation read-through, such as PTC nucleotide context, have still to be elucidated.

Furthermore, the severe side effects (i.e. ototoxicity and nephrotoxicity) associated with gentamicin treatment [109, 110] and the need for a long-time treatment, strongly limited its clinical application. In this direction, an extensive high-throughput screening for other PTC ribosomal read-through molecules lead to the discovery of Ataluren, which was shown to be safe and promising in a DMD pre-clinical model [111] and in a phase I clinical trial [112]. Subsequent data collected from phase IIa and phase IIb clinical trials in DMD/BMD patients, showed once again safety but no functional improvements (www.clinicaltrials.gov NCT00592553). Currently subgroup analysis is underway. At the moment, DMD patients with nonsense mutations are being recruited for a phase III clinical trial (www.clinicaltrials.gov

NCT01826487). Ataluren was also effective in reverting *in vitro* the pathological phenotype of LGMD2B/Miyoshi patient-derived myotubes [113]. Nevertheless recent reports highlighted the need for further studies on PTC124-mediated read-through efficacy and specificity [94, 114-116].

Up top now, two other read-through compounds named RTC13 and RTC14 respectively have been identified and shown to restore full-length dystrophin *in vitro* [117]. Pre-clinical studies in mdx mice confirmed the therapeutic potential of RTC13 but not RTC14 [118].

1.3.3 RARE-CUTTING ENDONUCLEASES FOR GENOME EDITING

Homing endonucleases are enzymes able to generate double-strand breaks (DSBs) in a specific DNA sequence playing a crucial role in genome repair processes. Endonucleases have been promptly and extensively engineered for gene therapy purposes thanks to their peculiar ability of precise DNA-cutting. As a matter of fact, endonuclease-induced DSBs can be repaired by non-homologous end-joining (NHEJ) error-prone repair [119], which frequently results in micro-insertions/deletions (INDELs) at the sites of the breaks. These INDELs can restore the correct gene reading frame.

Rare-cutting endonucleases differ for chemistries and structures [120, 121], it is indeed reasonable to hypothesize that in relation to their different specificity, efficiency, and spectrum of targeting sequences and outcomes they can exhibit a different application potential. Based on these features, endonucleases can be divided into three major groups: meganucleases (MNs) [122, 123], zinc fingers nucleases (ZFNs) [124-126] and transcription activator-like nucleases (TALENs) [127, 128].

MNs and ZFNs are currently the most well known and widely used for gene targeting. Lately, the first genome-wide study-mediated generation of a MN platform demonstrated that chromatin accessibility controls the efficiency of rare-cutting endonucleases. These results suggest that the chromosomal context (e.g. chromosomal positional effect) and epigenetic mechanisms act as key players in endonuclease-mediated genome editing [129].

The feasibility of meganuclease-mediated gene correction of dystrophin mutations has been demonstrated by re-establishing dystrophin expression both in myoblasts *in vitro* and in muscle fibers *in vivo* [130]. In addition, Rosseau and colleagues detected, characterized and quantified INDELs produced *in vitro* by six MNs and one pair of ZFNs targeting the dystrophin gene. The results demonstrated that both MNs and ZFNs could target the dystrophin gene restoring the correct reading frame. Moreover they observed that the frequency in INDEL generation was strongly increased after serial and consecutive exposures to the same endonuclease [131]. Very recent work published by Dickson and colleagues showed MN-induced locus-specific genome cleavage and homologous recombination knock-in of deleted exons in DMD cells [132].

As for MNs and ZFNs, also TALEN-mediated gene editing in DMD myoblasts restored dystrophin expression in dystrophic cells [133].

1.4 GENE REPLACEMENT

As a general scheme, gene replacement is based on delivering an additional functional copy of a named gene into skeletal muscle with the aim of restoring muscle function. There are two main gene-delivering tools: viral or non-viral

vectors. Safe and efficient gene delivery to skeletal muscle is challenging mainly because it is the most abundant tissue of the body and it is composed of non-dividing fibers surrounded by layers of connective tissue. Additionally, dystrophin is the largest gene in the human genome (2.4 mb); as a result, it is particularly difficult to even clone its cDNA (14 kb) [134] into conventional integrating viral vectors.

The paragraphs below discuss the various vectors currently used, with an emphasis on the ones that do not require to be shuttled to muscle by previous transfer into stem cells (see 1.4.2 *Cell Therapy: Allogeneic and Ex-Vivo-Corrected Progenitor/Stem Cells* section for further details on *ex vivo* gene transfer in stem cells for autologous strategies).

1.4.1 VECTOR-MEDIATED GENE THERAPY

1.4.1.1 VIRAL VECTORS

In order to engineer replication-defective viruses to efficiently deliver transgenes to muscle, many groups have exploited their natural ability to insert genetic sequences into cells. Several vectors, such as those based upon adeno, lenti, and adeno-associated viruses (AAVs) have been studied for gene replacement strategies. However, with the exception of AAVs, the majority of them do not efficiently transduce adult, dystrophic muscle [135].

1.4.1.1.1 ADENO-ASSOCIATED VIRAL VECTORS

AAVs have a limited cloning capacity (approximately 4.5 kb) and this is a major hurdle for dystrophin cDNA. To this end, the discovery that in humans shorter dystrophins lead to a milder dystrophic phenotype [136] fuelled the

idea that mini- (mDYS) or micro-dystrophins (μDYS) containing only some spectrin-like domains could be still functional. This idea was extensively demonstrated in mice [137-143]. This development, together with AAV high efficiency of transduction of skeletal and cardiac muscle, places AAVs among the best viral vectors for the gene therapy of muscular dystrophies [144-146]. As a matter of fact, AAVs and shorter dystrophins have given good results also in different large pre-clinical animals, even if further functional data are needed [147-151]. In rodents AAV vectors carrying short dystrophins infrequently cause cellular immune responses against the capsid proteins or the transgene products, at variance with large animal models where different immunological outcomes have been detected [147, 150, 152-154].

Immunological problems have also hampered the outcome of two recent clinical trials based on intramuscular injection of AAV vectors. In the first case, this was due to the presence of capsid-specific T-cells [155], although recently some of these hurdles have been overcome by novel optimized AAVs, which have already been tested in DMD patients [156]. In another clinical trial, mini-dystrophin-specific T lymphocytes have been found in some of the treated patients. In this last study two out of six DMD patients presented a few fibers, which were positive for mDYS 42 days after injection, but not after three months. Despite viral genomes were found in all the treated muscles, the presence of mini-dystrophin-specific T lymphocytes was probably responsible for the lack of long-term expression of the dystrophin transgene [157]. Very recently, a study published by Mendell and colleagues confirmed the presence in a large number of DMD patients of preexisting circulating anti-dystrophin T-cells. Moreover, they showed that the presence of

anti-dystrophin T-cell immunity increases with age and decreases in DMD patients treated with corticosteroids [158]. These results raise the hypothesis of a possible self-antigenicity of dystrophin, suggesting the need for further studies and for monitoring the immune response against therapeutic genes also in autologous therapies.

Another recent approach consists of a series of dual-vector systems that allowed an efficient delivery of a larger mDYS. The dystrophin cDNA is split into two different parts and packaged separately into two AAV vectors to be then reconstituted after co-infection. Although its delivery efficiency is still lower when compared with a single AAV vector, pre-clinical studies with dystrophic animal models have been encouraging [159-162].

AAV-based gene therapy has been adopted also for some forms of Limb girdle muscular dystrophies (LGMDs). Mutations in the calpain 3 gene are responsible for LGMD2A. The systemic delivery of an AAV carrying the calpain 3 cDNA under a skeletal muscle-specific promoter lead to the recovery of the dystrophic phenotype and prevented the toxicity of ubiquitous calpain 3 expression (i.e. in the heart) [163]. In the case of LGMD2B, an autosomal recessive muscular dystrophy caused by mutation in the dysferlin gene, the large size of dysferlin (> 150 kb) represents a major issue. To bypass this restriction, mini-dysferlin [164] and novel AAVs [165, 166] have been utilized. Autosomal recessive LGMD type 2D (LGMD2D) is caused by mutations in the gene encoding alpha-sarcoglycan, a member of the DGC [42]. In this case, a clinical trial showed persistent expression of alpha-sarcoglycan in two out of three LGMD2D patients which received AAV-mediated gene transfer to the extensor digitorum brevis muscle using a

muscle-specific promoter [167]. LGMD2C is a limb girdle muscular dystrophy with autosomal recessive inheritance, which is triggered by mutations in the gamma-sarcoglycan gene. After showing the efficacy of gene therapy based on the use of AVV vector expressing the human γ-sarcoglycan cDNA in a mouse model for LGMD2C, a phase I clinical trial has began. Following intra-muscular injections of an AVV serotype 1 vector (AVV1) expressing the human gamma-sarcoglycan cDNA under a muscle promoter, expression of protein and no sever adverse events have been observed. Nevertheless, one patient developed a cytotoxic response to the AVV1 capsid [168]. Genetic mutations in the FKRP gene and responsible for reduced α-dystroglycan glycosylation can result phenotypically either into mild LGMD2I or severe Walker-Warburg congenital muscular dystrophy. Recently, the intra-peritoneal delivery of serotype 9 AAV (AAV9) carrying FKRP cDNA in a mouse model of the disease restored α-dystroglycan glycosylation and resulted in functional amelioration of the dystrophic phenotype [169]. AAVs gene therapy protocols have been tested also in other dystroglycanopathies, which are caused by post-translational modifications of α-dystroglycan and belong to CMD family [170, 171]. In particular, it has been recently shown that mutations in LARGE [172] and fukutin [39] genes are responsible for a α-dystroglycan hypoglycosylation. In vivo systemic injections of AAV-9 carrying respectively LARGE [171] and fukutin [170] gene have been restored protein expression, functional α-dystroglycan glycosylation and improved the dystrophic phenotypes of murine animal models for CMDs.

In the case of facioscapulo-humeral muscular dystrophies (FSHD), a mouse model over-expressing FRG1, one of the candidate causative genes for FSHD, was generated [173] and lately two independent groups showed amelioration of the dystrophic phenotype upon FRG1 mRNA knockdown using an AAV-mediated delivery of RNA interference [174, 175]. Another experimental approach for treating FSHD is based upon the inhibition of the myopathic DUX4 gene, which is overexpressed in FSHD. In this direction a study demonstrated that AAV vector-mediated DUX4-RNA interference reverted the DUX4-related myopathy in a preclinical mouse model [176].

Overall, although the results obtained with AAV vectors are very encouraging for the development of AAV-mediated gene therapy for muscular dystrophies, their effective clinical application will greatly benefit from further long-term studies on immunity and improvements in delivery methods.

1.4.1.1.2 ADENOVIRUSES AND HERPES SIMPLEX VIRUS TYPE 1-BASED VECTORS

Adenoviral vectors and herpes simplex virus type 1-based vectors can carry large cassettes and have been used to transfer the entire dystrophin cDNA in cells and muscle. However, their use is hampered by their immunogenicity, the stability of transgene expression *in vivo* and the physical impediment of the myofibers basal lamina [177-182]. More recently, capsid-modified helper-dependent adenoviral vectors have attracted some interest because of their enhanced safely profile and large cloning capacity. Indeed, dystrophic mice showed stable expression of a marker gene together with improved motor

performance and life span upon delivery of the full-length dystrophin cDNA [183, 184].

1.4.1.1.3 LENTIVIRAL VECTORS

At variance with AAVs, lentiviral vectors have a relatively large size capacity (up to 7.5 kb transgenes) and although their direct injection into muscle targets also progenitor cells, the overall efficiency is limited [185]. Indeed, lentiviral vectors are currently being used to genetically modify myogenic stem cells *ex vivo*, which can then be transplanted into pre-clinical animal models of muscular dystrophy [186-192] or eventually into patients. Although the use of lentiviral vectors offers great potential, a possible risk of tumorigenicity due to insertional mutagenesis needs to be - and is currently being - carefully assessed [193].

1.4.1.2. NON VIRAL VECTOR-MEDIATED STRATEGIES

1.4.1.2.1 PLASMIDS

The application of plasmids coding for both truncated and full-length dystrophin is certainly attractive since they are produced easier and faster than viral vectors. Indeed, previous studies based upon direct intramuscular injection of plasmids highlighted the feasibility of this approach as well as the need to increase gene-transfer efficiency and to perform long-term analyses [139, 194]. Therefore, trans-venous high pressure increases efficiency in delivering plasmids carrying the dystrophin gene into rodents and primates [195, 196]. In addition, safety of this technique has been recently tested using

saline in dystrophic patients [197]. However, this method is less efficient than systemic injection of AAV vectors and can be used only for certain muscles.

An alternative system to improve plasmid transfer into muscle is electroporation [198, 199] and transgene expression by local plasmid electroporation into muscle has been shown [187, 200]. However, in some methods this can result in tissue damage and it is hard to envisage in a large muscle [201]. Anyway, in some of these studies a variable immune response has been found highlighting the need of further elucidating this point [202-207].

1.4.1.2.2 TRANSPOSONS

Transposons are mobile DNA repetitive sequences that have been found in the genome of almost all the species, mammals included. During evolution process, they have played a key role in genome modeling by “jumping” across it [208-210]. Recent evidence suggests that transposons are still actively mobilizing DNA sequences across genomes [211].

Transposons are divided into two groups based upon their different structure and mechanism of action: class I RNA-based retrotransposons and Class II DNA-transposons. Thanks to their intrinsic ability to insert genetic material, their remarkable cargo capacity (more than 10kb), the relative low immunogenicity and the fact that up to now no active class II human transposases have been identified, class II DNA transposons have been proposed for gene therapy strategies [212-218].

Among the various transposons, the Sleeping Beauty (SB) system, which combines the class II DNA salmon transposon with its related transposase, resulted particularly suitable for gene therapy thanks the fact

that transposase cannot recognize human but only class II fish transposons (reviewed in [219]). This avoids inter-species transposase activity that can be potentially deleterious for genomic stability. In addition, SB needs a thymine and adenine (TA) sequence for integration into the host genome, which being an incredibly common site it guarantees a widespread and random distribution of integration sites all over the genome [220]. Moreover, the engineering of novel hyperactive SB transposase increases transposition efficiency and transgene expression [221, 222].

Based on these premises, SB system was used to successfully deliver μDYS cDNA into dystrophic myogenic murine cells without affecting their myogenic potential *in vitro*. Once transplanted into mdx nude mice, SB transposon-genetically corrected cells were able to give rise to μDYS-positive skeletal myofibers [223].

Very recently, myogenic progenitors have been derived from induced pluripotent stem cells (iPSCs, see *1.4.2.3 Embryonic and Induced Pluripotent Stem Cells in Making Skeletal Muscle* section), which have been obtained from a dystrophic mouse lacking both dystrophin and utrophin, a dystrophin related protein. These cells have been genetically corrected with SB system carrying the micro-utrophin gene and once transplanted back into dystrophic mice they were able to ameliorate the pathological phenotype [224].

In conclusion, SB system represents a good candidate for ex vivo gene therapy of muscular dystrophies. However, although a recent study calculated that the chances of a transposon-related adverse event due to insertional mutagenesis or re-mobilization are very low [225], careful

evaluation of this risk in the actual candidate cells will be necessary before clinical translation.

1.4.1.2.3 HUMAN ARTIFICIAL CHROMOSOMES (HACs)

Ideally, the perfect tool for gene delivery is represented by I) a non-integrating vector with II) very high cargo capacity and III) able to be stably maintained into the host nucleus.

Human artificial chromosomes (HACs) fulfill all these criteria and present many advantages over conventional gene delivery platforms. HACs are artificial mini-chromosomes generated by using top-down (engineered pre-existing chromosome) or bottom-up (*de novo* artificial chromosome) approaches. The presence of telomeres and a centromere guarantees autonomous replication and segregation, coupling the need for a stable maintenance with no risk of insertional mutagenesis.

HACs have a large cargo capacity allowing the cloning of entire genetic loci together with their regulatory elements and native promoters, mimicking a physiological gene expression [226, 227]. In the case of DMD, where the mutated gene is the largest of the human genome with a complex regulation, the use of HACs represents a unique opportunity.

In this direction, a HAC suitable for gene therapy was generated few years ago by top down approach starting from human chromosome 21 [228]. Afterwards Hoshiya et al. used it as a template for the cloning of the 2.4 MB dystrophin genomic locus (DYS-HAC), including its promoters and regulatory elements [229]. Moreover, it has been demonstrated that the DYS-HAC could

be stably transferred into target cells, paving the way for a future DYS-HAC mediated gene therapy for DMD [229].

Subsequently, DYS-HAC has been transferred to dystrophic induced pluripotent [230] and mesoangioblast stem/progenitor cells [231] (see 1.4.2 *Cell therapy: Allogeneic and Ex-Vivo Corrected Progenitor/Stem Cells* section). This last paper provided the first evidence of safe and efficacious pre-clinical ex vivo gene therapy strategy based upon the use of a HAC [231]. More recently, it was also shown the successful derivation of mesoangioblast-like cells from DMD induced pluripotent stem cells previously genetically-corrected with the DYS-HAC [192].

In conclusion, HACs represent an ideal gene-delivery tool for DMD and maybe other forms of muscular dystrophy, such as LGMD2B, which is caused by mutation in another very large gene, dysferlin. However, also in this case the roadmap to clinical translation will probably need their validation in large animal models and increasing transfer efficiency in human cells.

1.4.2 CELL THERAPY: ALLOGENEIC AND EX-VIVO CORRECTED PROGENITOR/STEM CELLS

The existence in skeletal muscle of cells responsible for growth and regeneration was reported many years ago [232-236]. After two decades, it was hypothesized that these cells, called satellite cells, could be used in cell therapy protocols for muscular dystrophies replacing the ones containing the genetic defects and then allowing the generation of “healthy” skeletal muscle fibers. Later studies supported the notion that satellite cells are the “main” resident muscle stem cells (reviewed in [237]). From then onwards, a variety

of stem/progenitors cells with myogenic potency from skeletal muscle itself or from other tissues have been isolated, characterized and some of them transplanted (summarized in Table 1).

1.4.2.1 STEM/PROGENITOR CELLS FROM SKELETAL MUSCLE

1.4.2.1.1 SATELLITE CELLS AND MYOBLASTS

Satellite cells (SCs) are the main skeletal muscle stem cells involved in muscle growth and regeneration. They have been described more than fifty years ago as mononucleated cells positioned between myofiber's plasma membrane and basal lamina [236].

SCs originate from progenitors located in embryonic somites [238, 239] and are mitotically quiescent in adult healthy skeletal muscle [240]. In their quiescent state SCs express: Pax7 [241, 242], a transcription factor essential for their specification and survival; Pax3, mainly expressed in quiescent SCs and only in specific muscles [243]; Myf5 (myogenic regulatory factor 5), whose expression in the large majority of quiescent SCs [244, 245] is still controversial [246]. Others markers have been identified for SCs [247], however human SC markers do not always match with their murine counterpart [248].

SCs become activated and proliferate as a result of acute or chronic muscle injury. In this status they are usually referred to as myoblasts [249]. Activated SCs will usually give rise to a small number of $Pax7^{+}MyoD^{-}$ cells, which will return to quiescence to maintain a reservoir of progenitors, and to a majority of $Pax7^{-}MyoD^{+}$ cells which will fuse together or with pre-existing skeletal myofibers [250].

Thanks to their intrinsic myogenic potency, SC-derived myoblasts were soon considered as the ideal candidate cell population for the cell therapy of muscular dystrophies. In this direction, experiments performed on *mdx* mice showed that intra-muscular transplantations of myoblasts lead to their fusion with host fibers and dystrophin production [251]. A number of clinical studies in the early 90' showed safety, some dystrophin production but no efficacy (reviewed in [247, 252]); this was probably related to problems not yet completely solved, such as poor survival and migration of donor cells as well as host immune rejection [253-255]. Nevertheless, the need for solving the above issues generated a number of basic, pre-clinical and clinical studies to improve isolation, delivery, survival, engraftment, proliferation and differentiation [256-270].

Intriguingly, the above limitations of myoblasts may not constitute a major hurdle for those forms of muscular dystrophy in which only a limited and easily accessible group of muscles are affected, such as oculopharyngeal muscular dystrophy (OPMD). Myoblasts isolated from muscles not affected by OPMD (i.e. vastus lateralis) did not show defects compared with OPMD muscles from the very same patient. This original discovery paved the way for a possible injection of autologous myoblasts (from non OPMD muscles) as a new strategy of cell therapy for this type of muscular dystrophy [271]. Very recently, the results of a phase I/IIa clinical trial based on autologous transplantation of myoblasts have been published. The authors showed short and long-term safety and tolerability in all OPMD patients. Moreover, they reported an improvement in swallowing and in the quality of life of the patients [272].

In recent years a large number of studies on satellite cell biology and their role in muscle disease have been published (reviewed [273]). In particular, it was showed that when SCs are locally or systematically ablated, skeletal muscle is not able to regenerate after an acute injury [274-277]. These results support the claim that SCs are the only essential muscle stem cell for acute skeletal muscle regeneration. On the other hand, lineage-tracing experiments also showed that pericytes contribute to muscle growth and regeneration (Dellavalle et al., 2011). Therefore, a unifying model for both these observations still needs to be developed.

Another interesting set of observations show that in several forms of muscular dystrophies both satellite and/or other muscle stem cell compartments are exhausted or defective [192, 278-282].

1.4.2.1.2 PERICYTES AND MESOANGIOBLASTS

Mesoangioblasts (MABs) are vessel-associated progenitors of mesoderm origin that were initially isolated from murine embryonic dorsal aorta [283, 284]. MABs were shown to be able to differentiate into skeletal muscle and cross the vessel wall, an ability that lead them to be delivered through the arterial circulation. Intra-arterial transplantation of wild type or genetically corrected murine MABs improved the pathological phenotype of the α-sarcoglycan null mouse, a pre-clinical mouse model for LGMD2D [285]. Moreover, transplantation of allogeneic and autologous adult canine MABs gene-corrected with a micro-dystrophin lentivirus was effective in ameliorating the dystrophic pathology in a large animal model for DMD [186].

Lately, we combined the use of mesoangioblasts with a new gene

delivery tool, a HAC carrying the entire dystrophin locus (DYS-HAC). In details, we gene-corrected murine dystrophic MABs with DYS-HAC. Once transplanted back into immunodeficient dystrophic *scid/mdx* mice, DYS-HAC corrected cells were able to improve the dystrophic phenotype [231].

MABs have been isolated also from human skeletal muscle biopsies were they have been considered to be a subset of pericytes [286]. Indeed they express CD146 and Alkaline Phosphatase (ALP), the latter being specifically expressed by pericytes in skeletal muscle. Transplantation of human pericyte-derived MABs in *scid/mdx* mice resulted in dystrophin-positive muscle fibers and subsequent amelioration of function and morphology of the pathological phenotype [286]. In addition, lineage-tracing study in mice demonstrated that pericytes are able to fuse with developing skeletal fibers and enter the satellite cell pool during normal murine postnatal development and regeneration [287]. These results supported the evidence of a myogenic progenitor resident in adult human skeletal muscle but distinct from SCs.

For this reason a phase I/II clinical trial centered on intra-arterial allogeneic transplantation of healthy-donor-HLA-matched-MABs into DMD patients is currently on going (EudraCT no. 2011-000176-33). As propaedeutic for this study, motor capacity and force of contraction of 5 enrolled DMD patients and other 23 DMD patients have been tested for one year and half before the start of the clinical trial [288]. Indeed, even if the major goal of this clinical trial is to test safety for mesoangioblasts-based cell therapy protocols, this accurate analysis will provide the possibility to evaluate improvements in DMD patient's motor capacity after cell administration.

Lately, in scid/blAJ mice, a mouse model of dysferlinopathy

mesoangioblast-based cell therapy result into a functional recovery [289].

1.4.2.1.3 CD133+ MUSCLE CELLS

After publishing that human CD133+ circulating cells could differentiate into skeletal muscle (see 1.4.2.2.3 *Hematopoietic and CD113+ Stem Cells* section), Torrente and colleagues isolated CD133+ cells with myogenic properties from the microvasculature of skeletal muscle [290]. These cells express myogenic markers such as Pax7, Myf5, MyoD, m-cadherin, MRF4 and myogenin. Interestingly a small percentage of muscle-derived CD133+ cells also expressed CD45, a hematopoietic marker. In the case of CD133+ cells isolated form DMD patients, the gene defect was corrected using a lentivirus expressing U7snRNA in order to skip exon 51 [290]. These results set the conditions for a double–blind phase I clinical trial in DMD patients based upon intra-muscular autologous CD133+ cell transplantation [291]. Results confirmed the safety of this cell therapy protocol.

1.4.2.1.4 PW1+ INTERSTITIAL CELLS

Cells situated in the interstitium of murine skeletal muscle and characterized by the expression of the transcription factor PW1 (PICs) have been recognized as myogenic progenitors capable to participate in muscle regeneration [292]. Since PICs express Pax7, a marker for SCs, Sassoon and colleagues were particularly interested in explaining the relation existing among PICs and SCs. Indeed by means of lineage-tracing studies they demonstrated that PICs I) are able to generate satellite cells, which are PW1+ too; II) can generate other PICs; III) do not derive from satellite cells [292].

1.4.2.1.5 MUSCLE-DERIVED STEM CELLS

Muscle derived stem cells (MDSCs) have been isolated from muscle biopsies using different approaches and appear to be similar to muscle SCs [293, 294]. MDSCs express myogenic markers such as desmin, MyoD, and myogenin whereas they are weakly positive for CD34 and negative for c-Kit and CD45 [294]. Although these findings suggest that MDSCs are different from SCs, a population of SCs expressing m-cadherin, a marker for SCs, has been observed to express CD34 as well in isolated myofibers [240].

However, it remains unclear whether MDSCs isolated with different techniques represent the same population or the same population at a different stage of maturation within the myogenic lineage [295]. Moreover, in vivo experiments based on MDSCs transplantation resulted into variable outcomes [294, 296-298]. Recently, the systemic delivery in GRMD dogs of poorly adherent MDSCs named MuStem cells resulted to be effective [299].

1.4.2.1.6 MYOGENIC ENDOTHELIAL CELLS

The observation that human cord blood endothelial progenitors cells (EPCs) [300] could differentiate not only into endothelial cells but also into muscle cells [301], led to the identification in the human skeletal muscle of a myogenic cell population associated with vessels and expressing both myogenic ($CD56^+$) and endothelial markers ($CD34^+$, $CD144^+$) [302]. These cells were found able to fuse and to form donor-derived-skeletal myofibers in an immunodeficient mouse model for DMD [302].

1.4.2.2 UNORTHODOX MYOGENIC PROGENITORS FROM OTHER TISSUES

1.4.2.2.1 AMNIOTIC FLUID STEM CELLS

The amniotic fluid is mainly composed of water (99%-98%), chemicals (e.g. lipids, proteins) and cells. Amniotic fluid cells, whose origin is still unclear, derive both from embryonic and extra-embryonic tissues and express the markers of all the three germ layers [303, 304].

Despite the fact that the vast majority of amniotic fluid cells are terminally differentiated or with no more proliferation potential [305, 306], in the 1990s pioneer studies demonstrated the presence in the amniotic fluid of both hematopoietic [307] and non-hematopoietic [308] progenitors. Indeed, few years later Prusa and colleagues formally showed the existence of amniotic fluid stem cells (AFSCs)[309].

AFSCs present high proliferation ability and are able to differentiate into several tissues (reviewed in [310], among these skeletal muscle. Indeed, it was published that a fraction of AFSCs positive for CD117 surface marker (c-kit) could acquire a myogenic phenotype in vitro [311, 312] but they were not able to generate skeletal muscle fibers in vivo [312].

Recently, independent groups showed that CD117+ AFSCs could differentiate *in vitro* into myogenic cells and actively contribute to muscle regeneration when transplanted into mouse models of acute [313] or chronic muscle injury [314]. Intriguingly, De Coppi and colleagues showed for the first time a functional improvement of a pre-clinical model of muscular dystrophy after CD117+ AFS cell transplantation via tail vein injection [314]. Moreover, they demonstrated that AFSCs can occupy the stem cell niche and that in

vitro cultured AFSCs are less efficient in regenerating dystrophic skeletal muscle compared to freshly isolated ones [314].

1.4.2.2.2 MESENCHYMAL STEM CELLS

In the last years, it has been shown that mesenchymal stem cells (MSCs) isolated from different sources have some myogenic capability *in vitro* and *in vivo* [315-317]. On the other hand, it was also demonstrated that although Pax3 activation lead to the *in vitro* differentiation of both murine and human MSCs into myogenic cells, these cells were able to engraft with a good efficiency the skeletal muscle of dystrophic *mdx* mice but not to produce a functional muscle [318]. Other recent studies showed that MSCs were not able to restore the pathological phenotype of dystrophic mouse models [319, 320].

Nevertheless, even if MSCs did not contribute muscle regeneration *in vivo*, it was proposed that they could produce extracellular matrix molecules [320] or they could help in contrasting inflammation [321].

Recently, the widespread use of MSCs in clinical trials for different types of diseases has been questioned. This still represents a delicate issue due to uncertainties on MSC identity, function, isolation and transplantation methods [322, 323].

1.4.2.2.3 HEMATOPOIETIC AND CD133+ STEM CELLS

In the late 1990s it was reported that donor-derived skeletal myofibers could be detected after bone marrow transplantation in mice with injured skeletal muscle [324]. This data demonstrated that a fraction of murine bone marrow

progenitors could be recruited in muscle regeneration. Unfortunately, the frequency of the event was very low, resulting in no amelioration of the pathological phenotype [297, 325].

Following studies identified the hematopoietic $CD45^+$ fraction of the bone marrow as the population with myogenic potential [326] and a subsequent report clarified that the progenies of a mouse hematopoietic progenitor can both reconstitute the hematopoietic system and contribute to muscle regeneration [327].

Interestingly, a retrospective long-term analysis of a DMD patient that received bone marrow transplantation confirmed what emerged in the pre-clinical studies: long persistence of a few donor nuclei into skeletal myofibers without functional amelioration [328].

Recently, it has been showed that bone marrow transplantation slightly ameliorate the pathological phenotype of A/J $Dysf^{prmd}$ mouse [329], a model of dysferlinopathy characterized by loss of dysferlin expression in skeletal myofibers and monocytes. Nevertheless, dysferlin expression was seen in monocytes but not in the skeletal muscle of A/J $Dysf^{prmd}$ bone marrow transplanted mice. Gallardo and co-workers suggest that dysferlin absence in peripheral blood could be partially responsible of disferlinopathy progression and therefore bone marrow-derived monocytes expressing dysferlin could help in ameliorating the dystrophic phenotype. Moreover, the authors proposed that bone marrow transplantation could have a preventive effect on the progression of the disease by restoring HGF (hepatocyte growth factor) expression, a factor involved in muscle stem/progenitor cell activation/mobilization [330-332].

Almost ten years ago Torrente and colleagues identified a subpopulation of circulating cells expressing the hematopoietic stem cell marker CD133 as well as early myogenic markers [333]. These cells showed to be effective in ameliorating the pathology of *scid/mdx* mice. Lately $CD133^{+}$-mediated cell therapy protocol has been also tested into scid/blAJ mice, an immunodeficient mouse model of dysferlinopathy. In this direction, Torrente and colleagues gene-corrected patient-derived CD133+ stem cells with a lentivirus carrying the full-length dysferlin gene. Following transplantation, dysferlin expression was detected as well as recovery of the membrane integrity in single fibers preparation from transplanted scid/blAJ mice. On the other side, the dystrophic murine phenotype was not restored *in vivo* [334]

1.4.2.3 EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS IN MAKING SKELETAL MUSCLE

Embryonic stem (ES) cells are pluripotent cells derived from the early embryo that are capable of differentiating into all tissues of the three germ layers [335, 336]. Myoblast- or satellite-like cells have been derived from mouse and human ES cells with the aim of regenerating injured muscles [337-339]. Similarly, Darabi et al. [340, 341] showed the generation and transplantation of PDGFRa+Flk myogenic cells from murine ES cells. However, the generation of ES cells involves ethical concerns related to the destruction of human blastocysts.

In this scenario, the reprogramming of adult somatic cells to an ES cell-like state (induced pluripotent stem, iPS cells) revolutionized the field of cell therapy and regenerative medicine [342]. Indeed, the possibility of

deriving patient-specific iPS cells for autologous cell therapies is one of the most promising strategies for future personalized medicine [343].

Recent work has described the generation of myogenic progenitors from iPS cells able that are able to play an active role during muscle regeneration in pre-clinical models for both DMD and LGMD2D [192, 224, 344-346]. Among these studies, Perlingueiro and colleagues [224, 344] have demonstrated protein restoration, improved contractility and contribution to the satellite cell pool upon transplantation of healthy donor/dystrophic human ES/iPS-derived myogenic progenitors into different mdx mouse models.

In parallel, our group reported evidence that human iPS cells generated from patients with LGMD2D give rise to myogenic stem/progenitor cells that can be genetically corrected ex vivo, restoring a-sarcoglycan expression upon xenotransplantation into a novel immunodeficient a-sarcoglycan-null mouse model [192]. Notably, we also showed functional phenotype amelioration and re-establishment of progenitors upon intra-specific transplantation, together with an extension of this strategy to DMD using HACs [192].

Deriving patient-specific iPS cells and expanding their differentiated progeny provides a unique tool for gene and cell therapies, even if further safety studies and improvements in protocols will be necessary to avoid any potential risk that could hamper the translation of these promising strategies into future clinical trials (e.g. transgene expression, genome stability and immune response)

1.5 CONCLUDING REMARKS

The search for a cure for muscular dystrophy has brought innovative approaches (Figure 1) to start tackling these incurable diseases, together with considerable progress in the understanding of muscle cell biology, physiology and regeneration dynamics. However, we are still away from a definitive cure, particularly in the case of complex autologous ex vivo stem cell-mediated gene therapies. Promising technologies, such as iPS cells, are already providing novel fuel for this journey and future efforts of the research community will certainly address the various hurdles of this challenging quest.

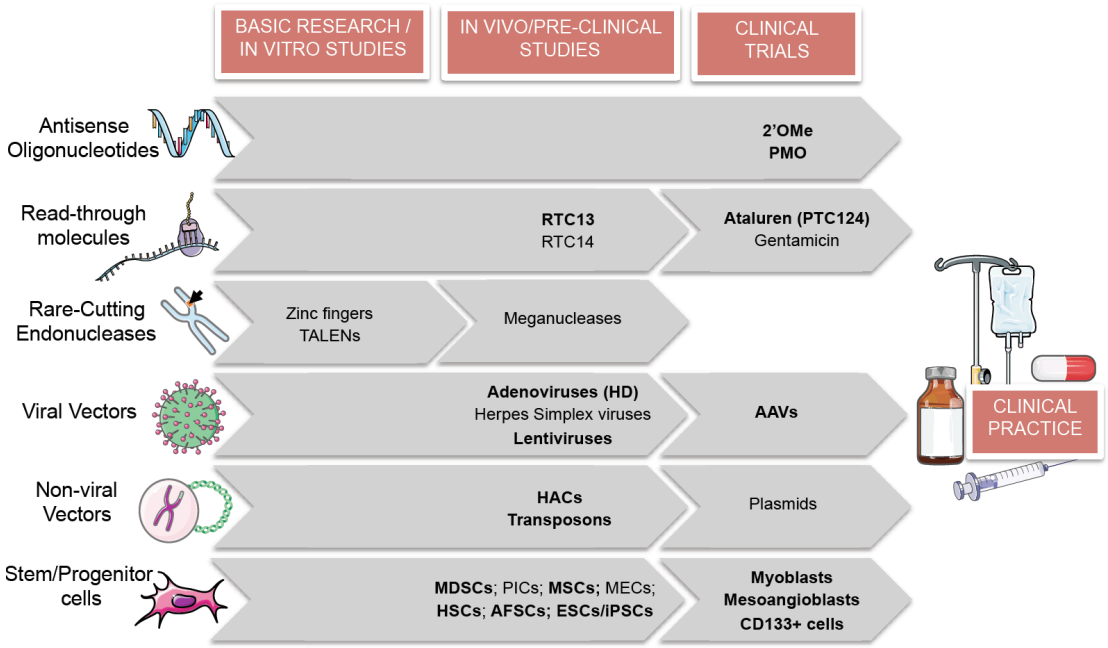

Figure 1. Gene and cell therapies strategies for the treatment of muscular dystrophies. The figure illustrates the current strategies of gene repair (antisense oligonucleotides, read-through molecules and rare cutting

endonucleases) and gene replacement (viral and non vectors, stem/progenitors cells) and their progression from basic research (left) through pre-clinical studies (middle) and then, in some cases, into clinical trials (right). In the in vivo/pre-clinical trials column (middle column), strategies tested for efficacy have been highlighted in bold. In the clinical trials column (right column), only recent clinical trials have been highlighted. 2'OMe: methylphosphorothioate oligoribonucleotide; PMO: phosphorodiamidate morpholino oligomer; TALENs: transcription activator-like nucleases; HD: helper dependent; AAV, adeno-associated virus; HACs: human artificial chromosomes; MDSCs: muscle-derived stem cells; PICs: Pw1+ interstitial cells; MSCs: mesenchymal stem cells; MECs: myogenic endothelial cells; HSCs: hematopoietic stem cells; AFSCs: amniotic fluid stem cells; ESCs: embryonic stem cells; iPSCs: induced pluripotent stem cells. Adapted (updated) from Benedetti et al. 2013, FEBS Journal. Permission granted from the publisher (Wiley-Blackwell Production).

Cell type	Derivation	Delivery mode	Animal model (disease)	Clinical Trials
Satellite Cells/Myoblasts (SCs)	Skeletal muscle	Local	mdx mice (DMD) [251]	DMD: Phase II Completed [269] OPMD: Phase I/IIa Completed [272]
Pericytes and Mesoangioblasts (MABs)	Skeletal muscle/vessels	Local/Systemic	mdx/scid (DMD) [231, 286]; sgca-null mice (LGMD2D) [285]; scid/blAJ (LGMD2B) [289]; GRMD dogs (DMD) [186]	DMD: Phase I/II Ongoing
CD133+ Cells	Blood and/or Skeletal muscle	Local/Systemic	mdx/scid mice (DMD)[290,333]; scid/blAJ (LGMD2B) [334]	DMD: Phase I Completed [291]
PW1+ Interstitial Cells (PICs)	Skeletal muscle	Local	Injured nude mice [292]	None
Muscle-derived Stem Cells (MDSCs)	Skeletal muscle	Local	mdx mice (DMD)[294,296,297]; mdx nude mice (DMD)[298]; GRMD dogs (DMD)[299];	None
Myogenic Endothelial Cells (MECs)	Skeletal muscle vasculature	Local	Injured scid mice [301,302]	None
Amniotic Fluid Stem Cells (AFSCs)	Amniotic Fluid	Systemic	Injured scid mice [312]; injured NOD/scid mice [313]; HSA-Cre, Smn $^{F7/F7}$ mice (spinal muscle atrophy mouse model with signs of muscular dystrophy) [314]	None
Mesenchymal Stem Cells (MSCs)	Bone marrow vessels and other sources	Local/Systemic	Injured rats and mdx nude mice (DMD) [315]; injured NOD/scid and mdx/scid mice (DMD) [316]; injured nude mice [317]; mdx mice (DMD) [317, 318, 319]; injured Rag2$^{-/-}$Yc$^{-/-}$/C5- mice [320]	None
Hematopoietic Stem Cells (HSCs)	Bone marrow and blood	Local	Injured scid/beige mice [324]; mdx ^{4cv} (DMD) [325]; mdx mice (DMD) [297]; injured mice [327]; A/J Dysf^{prmd} mouse (LGMD2B) [329]	Retrospective analysis of a DMD patient after bone marrow transplantion [328]

Embryonic Stem Cell- (ESC-) derived Progenitors	Embryo	Local/ systemic	Injured scid/beige mice [337]; injured mdx mice (DMD) [338,340,341]; injured nude mice [339]; injured $Rag2^{-/-}Yc^{-/-}$ mice [340]; injured NSG mice and NSG-mdx^{4Cv} (DMD) [344]; $Rag2^{-/-}$/mdx (DMD) and injured $Rag2^{-/-}$/mice [345]	None
Induced Pluripotent Stem Cell- (iPSC-) derived Progenitors	Dermis and other tissues	Local/ systemic	sgca-null/scid/beige mice (LGMD2D) [192]; sgca-null (LGMD2D) [346]; mdx/utrophin$^{-/-}$ mice (DMD) [224]; injured NSG mice and NSG-mdx^{4Cv} (DMD) [344]; $Rag2^{-/-}$/mdx (DMD) and injured $Rag2^{-/-}$/mice [345]	None

Table 1. **Stem and Progenitor cells with myogenic properties.** The table described the origin, the delivery mode, the pre-clinical studies and the recent/ongoing clinical trials of different type of stem/progenitor cells identified and used so far for the cell- and gene-therapy of muscular dystrophies. In the case of Satellite cells/Myoblasts, only recent clinical trials have been described whereas past clinical trials have been extensively reviewed in 247, 252 and 273. Adapted (updated) from Benedetti et al., 2013. Permission granted from the publisher (Wiley-Blackwell Production).

1.6 ACKNOWLEDGEMENTS

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