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Prospect of gene therapy for cardiomyopathy in hereditary muscular dystrophy

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Abstract

Introduction—Cardiac involvement is a common feature in muscular dystrophies. It presents as heart failure and/or arrhythmia. Traditionally, dystrophic cardiomyopathy is treated with symptom-relieving medications. Identification of disease-causing genes and investigation on pathogenic mechanisms have opened new opportunities to treat dystrophic cardiomyopathy with gene therapy. Replacing/repairing the mutated gene and/or targeting the pathogenic process/mechanisms using alternative genes may attenuate heart disease in muscular dystrophies.

Areas covered—Duchenne muscular dystrophy is the most common muscular dystrophy. Duchenne cardiomyopathy has been the primary focus of ongoing dystrophic cardiomyopathy gene therapy studies. Here, we use Duchenne cardiomyopathy gene therapy to showcase recent developments and to outline the path forward. We also discuss gene therapy status for cardiomyopathy associated with limb-girdle and congenital muscular dystrophies, and myotonic dystrophy.

Expert opinion—Gene therapy for dystrophic cardiomyopathy has taken a slow but steady path forward. Preclinical studies over the last decades have addressed many fundamental questions. Adeno-associated virus-mediated gene therapy has significantly improved the outcomes in rodent models of Duchenne and limb girdle muscular dystrophies. Validation of these encouraging results in large animal models will pave the way to future human trials.

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Keywords

AAV; adeno-associated virus; gene therapy; cardiomyopathy; Duchenne muscular dystrophy; DMD; dystrophin; capsid engineering; vector; capsid; muscular dystrophy; gene therapy; heart failure; limb-girdle muscular dystrophy; congenital muscular dystrophy; myotonic dystrophy

1. Clinical presentation, pathogenic mechanism and therapeutic challenge of dystrophic cardiomyopathy

Dystrophic cardiomyopathy refers to cardiac manifestations of muscular dystrophies. Muscular dystrophies are a clinically, genetically, and biochemically heterogeneous group of disorders. They are characterized by progressive muscle wasting, force loss and dystrophic muscle pathology[1, 2]. Muscular dystrophies can be classified in many different ways such as the age of onset (congenital/neonatal, adolescent, or adult), disease progression (rapid or slow), the muscle groups involved (such as limb girdle, facioscapulohumeral and oculopharyngeal etc.), the mode of inheritance (such as X-linked/autosomal, recessive/dominant). Some muscular dystrophies are named after people who discovered the disease (such as Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and Emery-Dreifuss muscular dystrophy etc). Despite the unique clinical features of each type of muscular dystrophy, cardiac involvement has been a common finding in most muscular dystrophies and often represents a major cause of morbidity and mortality[3–7]. Interestingly, the cardiac phenotype varies in different types of muscular dystrophies and even in different patients or disease stages of the same type muscular dystrophy. Some present with dilated/hypertrophic/restrictive cardiomyopathy with eventually heart failure while others exhibit conduction defects leading to arrhythmia and sudden cardiac death. In the case of DMD and BMD, MRI studies have revealed a unique pattern of subepicardial fibrosis predominantly in the left ventricular lateral wall[8–10].

The pathogenic mechanisms of dystrophic cardiomyopathy are not completely understood[11, 12]. However, it may at least involve destabilization of the cardiomyocyte membrane, or sarcolemma. Unlike other cells in the body, muscle cells undergo continuous calcium-regulated contraction/relaxation cycles. A consequence of this unique physiology is the repeated cycles of shrinking and expansion of the cell. This dynamic deformation process places enormous stress on the sarcolemma. Such stress is especially problematic for cardiomyocytes because of the repetitive pumping activity of the heart. To relieve contraction-induced stress, muscle cells have evolved specialized trans-membrane protein complexes such as the dystrophin-associated glycoprotein complex (DGC) and the integrin complex. These protein complexes constitute physical connections between the cytoskeleton and the extracellular matrix. Mutations in the genes encoding the components of these complexes result in various forms of muscular dystrophies. Failure to maintain sarcolemmal integrity leads to membrane leakage, myocyte degeneration, necrosis and eventual replacement by fibrofatty tissue. Clearly, strengthening the destabilized sarcolemma holds the key for treating dystrophic cardiomyopathy. Unfortunately, this cannot be achieved with conventional medical/surgical treatments[13]. Gene therapy, however, provides a great opportunity to address this therapeutic challenge.

2. Strategies to deliver a therapeutic gene to a dystrophic heart

Disease-causing genes for many muscular dystrophies have been discovered. The identification of the genetic underpinning makes it possible to treat dystrophic cardiomyopathy with gene therapy. The first step of gene therapy is delivery of a therapeutic gene to the heart. A number of viral and nonviral vectors have been tested[14]. So far the most effective and least immunogenic vector is adeno-associated virus (AAV). AAV is a 20-nm single stranded DNA virus[15]. Recombinant AAV vector contains no wild type viral genes. The vector genome can be readily packaged into naturally existing or synthetic capsids to meet specific therapeutic needs. The nano-size AAV particle creates a packaging dilemma. The maximal carrying capacity of a single AAV particle is 5-kb[16]. This is too small for many genes required for muscular dystrophy gene therapy (such as the dystrophin gene and the dysferlin gene). To overcome this limitation, we and others have invented a series of dual and tri-AAV vectors[17]. The basic idea is to fragment a large therapeutic gene and package each segment into an AAV particle. The full-length gene is reconstituted by cellular recombination machinery after co-infection. These multi-vector strategies have made it possible to deliver the 6 to 8-kb mini-dystrophin gene and even the 12-kb full-length dystrophin coding sequence to dystrophin-deficient mdx mice, the most commonly used animal models for DMD[18–22].

Over the years, a number of different strategies have been developed to achieve effective AAV gene transfer in dystrophic hearts. Early studies were mainly based on AAV-2 using invasive and complicated methods such as direct myocardial injection[23], intracavity injection[24], transcoronary perfusion[25], and ex vivo coronary perfusion[26]. The identification and development of novel AAV capsids has opened the door to transduce dystrophic hearts with peripheral vein injections[27–32]. This simple method not only greatly reduces the risks associated invasive heart gene transfer but also allows simultaneous treatment of both cardiac and skeletal muscle disease in muscular dystrophy.

The tissue tropism of the AAV vector is largely determined by the viral capsids. Experimenting with natural and engineered AAV capsids has proven to be a fruitful approach in identifying cardiotropic AAV vectors. For example, a comparison of AAV-1 to 9 revealed AAV-9 as the most potent vector for the mouse heart[33]. Indeed, AAV-9 results in robust widespread myocardial transduction in mdx mice irrespective of the age and the route of delivery (intravenous or intra-arterial)[34–36]. Directed evolution and cardiotropic peptide insertion have also yielded novel AAV variants with enhanced cardiac transduction in rodent models of limb girdle muscular dystrophy (LGMD) 2F, an extremely rare type of muscular dystrophy caused by δ-sarcoglycan deficiency[32, 37, 38].

3. Disease gene-specific gene therapy

3.1. Dystrophin-based Duchenne cardiomyopathy gene therapy

The dystrophin gene was the first muscular dystrophy-associated gene cloned[39]. Its mutation leads to DMD. The 2.4-mb full-length dystrophin gene contains 79 exons and it transcribes into a ~ 12-kb cDNA. The full-length dystrophin protein has four major functional domains including the N-terminal, rod, cysteine-rich and C-terminal domain. The N-terminal domain

binds to cytosolic γ-actin. The rod domain consists of 24 spectrin-like repeats. Within the rod domain, there are several important subdomains including one for γ-actin-binding, one for neuronal nitric oxide synthase (nNOS)-binding and one for microtubule-binding[40–43]. The cysteine-rich domain links dystrophin to the extracellular matrix through dystroglycan, a transmembrane glycoprotein. The C-terminal domain binds to syntrophin and dystrobrevin.

The enormous size of the dystrophin gene presents a delivery challenge because it is beyond the packaging capacity of most viral vectors. Interestingly, some naturally-existing, internally-deleted dystrophins (e.g. Δ17–48) are quite functional[44]. These mini-dystrophin genes are about 6 to 8-kb in length and their expression in humans and animals has greatly mitigated skeletal muscle disease[19–21, 44–47]. The therapeutic implication of mini-dystrophin in the heart has only been investigated in transgenic mice[48]. We expressed mini-dystrophin specifically in the heart of mdx mice. This cardiac-restricted expression completely corrected cardiac histopathology, improved exercise performance and enhanced myocardial contractility[48]. Whether mini-dystrophin gene therapy can achieve similar effectiveness remains to be seen. In this regard, dual AAV vectors have been developed to express the mini-dystrophin gene[18–21, 49–51]. Further, systemic injection of dual AAV vectors has been shown to transduce the myocardium at high efficiency in mdx mice[52, 53].

A single vector therapy would be more advantageous. To package dystrophin into AAV, highly abbreviated micro-dystrophin genes have been developed. The microgene is about 3.5 to 4-kb in length and contains ~30% of the dystrophin coding sequence. In contrast to mini-dystrophin, micro-dystrophin does not carry the C-terminal domain. Additionally, it has a shorter rod domain with only 4 to 5 spectrin-like repeats. AAV-mediated micro-dystrophin gene therapy has been extensively studied in various mouse models and more recently in the canine model[47, 54–59]. Direct or systemic AAV microgene therapy significantly ameliorated skeletal muscle disease in dystrophic mice and dogs. The first study to evaluate therapeutic effect of micro-dystrophin in the heart was performed by Yue et al[24]. In this study, an AAV-5 microgene vector was directly injected into the cardiac cavity of neonatal mdx mice. Micro-dystrophin restored the DGC complex in the heart and enhanced the membrane stability of cardiomyocytes[24]. In subsequent studies, newly developed AAV capsids (such as AAV-6 and AAV-9) were utilized to delivery micro-dystrophin to the heart through peripheral vein injection[34–36, 54, 60–62]. Of particular interest are studies by Bostick et al in which an AAV-9 microgene vector was delivered to the heart of aged female mdx mice. This study is noteworthy because aged female mdx mice develop a cardiac phenotype nearly identical to that observed in dilated cardiomyopathy of human patients[34, 35, 63, 64]. Despite the advanced heart disease in very old mice, surprisingly, cardiomyocytes were efficiently transduced[34, 35]. The average lifespan of mdx mice is ~ 22 months[65, 66]. In pre-terminal mdx mice (16 to 20-m-old), microgene therapy reduced myocardial fibrosis, improved the electrocardiographic profile and hemodynamic function[34]. In terminal age mdx mice (> 21-m-old), neither fibrosis nor hemodynamic function was improved[35]. However, some ECG parameters were partially corrected and dobutamine stress-induced acute cardiac death was reduced[35].

Expression of a full-length or near-full-length dystrophin protein may lead to a better recovery. This is feasible with tri-AAV vectors but the efficiency is too low to be of practical use[22]. Editing the mutated RNA transcript or genome offers alternative approaches to reach this goal. Exon skipping is a potent method to achieve RNA-level editing. Briefly, antisense oligonucleotides (AONs) are delivered to modulate RNA splicing so that the mutated (and some times adjacent) exons are removed. The resulting mRNA, though abbreviated, is in-frame and can yield a near-full-length protein[67]. Several chemically distinctive classes of AONs have been developed including 2-O-methylated phosphorothioated (2-OMePS), phosphorodiamidate morpholino oligomers (PMOs), peptide/polymer/nanoparticle–conjugated PMOs, and most recently tricycle-DNA (tcDNA). 2-OMePS and PMOs are currently in clinical trials[68–73]. However, these AONs cannot reach the heart[74–76]. Peptide/polymer/nanoparticle–conjugated PMOs can induce exon-skipping in the heart of mdx mice and improve heart function[77–87]. However, there are issues related to potential toxicity and immunogenicity[88]. The newly developed tcDNA represents the most advanced AON formulation[89]. Because of its unique pharmacological property, systemic delivery of tcDNA-AONs resulted in phenomenal uptake in many tissues including the heart and brain. Treatment in mdx mice and more severe utrophin/dystrophin double knockout (u-dko) mice improved cardiac, respiratory and behavioral function[89]. Importantly, no overt toxicity was detected with tcDNA[89]. An alternative strategy to deliver AONs is to use the AAV vector. AAV-9 mediated systemic AON delivery resulted in high efficient dystrophin expression in the heart of u-dko mice[90]. More recently, two independent groups achieved long-term dystrophin restoration in the heart of the canine DMD model with AAV-6 mediated local exon-skipping[91, 92].

Compared to RNA editing with exon-skipping, targeted editing of the mutated dystrophin gene has just entered an exciting time due to recent development of highly versatile genome engineering tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and most importantly, the clustered regularly interspaced palindromic repeat (CRISPR)-associated endonuclease 9 (Cas9)[93]. A series of elegant studies from the Gersbach laboratory has provided compelling proof-of-concept evidence in correcting cells from DMD patients using these new technologies[94–96]. It is highly anticipated that genome editing will soon be used to treat skeletal muscle disease and cardiomyopathy in animal models of DMD[97].

3.2. Disease gene-based therapy for cardiomyopathy in other muscular dystrophies

3.2.1. Targeting disease gene to treat LGMD cardiomyopathy—LGMD refers to a group of muscle disorders with a wide range of clinical and genetic heterogeneity[98, 99]. Based on the inheritance pattern, they are classified as autosomal dominant type 1 (LGMD1) and autosomal recessive type 2 (LGMD2). Each type of LGMD is further classified according to the time the disease gene was discovered. For LGMD1, the goal of the gene therapy is to decrease the expression of the mutated gene. This can be achieved with RNA interference (RNAi) to silence the mutated gene[100, 101]. So far, only one study has tested gene therapy for dominant LGMD. LGMD1A is caused by myotilin gene mutation. Liu et al targeted mutant myotilin with an AAV-6 microRNA vector[102]. Treatment significantly reduced expression of the mutated myotilin protein and ameliorated skeletal muscle

myopathy. Although LGMD1A patients do not exhibit cardiac abnormalities[4], the RNAi approach described by Liu et al may treat cardiac manifestations in other dominant myopathies such as lamin A/C gene mutation-induced LGMD1B and Emery-Dreifuss muscular dystrophy.

There has been significant progress in LGMD2 gene therapy. AAV-mediated gene therapy has been tested in animal models of at least seven different subtypes of LGMD2 (2A to 2F, and 2I). LGMD2A and 2B are caused by mutations in the calpain-3 gene and the dysferlin gene, respectively. According to Hermans, LGMD2A and 2B do not show cardiac manifestations[4]. However, cardiomyopathy has been seen in dysferlin-deficient mice and there are also a few reports of cardiac involvement in some LGMD2B patients[103–106]. Three different approaches have been explored to express a function dysferlin gene. These include delivering a minimized dysferlin gene with a single AAV vector, delivering a full-length dysferlin cDNA with dual AAV vectors and exon-skipping or pre-mRNA trans-splicing to repair defective dysferlin RNA transcript[107–113]. Defective membrane repair has been considered as the major pathogenic mechanism for LGMD2B. The in vitro membrane repair assay has been used as a surrogate endpoint to evaluate the therapeutic efficacy. Surprisingly, a recent study by Lostal et al found that a correction of membrane repair by the in vitro assay did not correlate with the correction of muscle pathology. The authors overexpressed myoferlin, a homolog of dysferlin, in dysferlin-null mice by the transgenic approach and they also expressed the mini-dysferlin gene in 4-week-old dysferlin-null mice. Neither transgenic overexpression of myoferlin nor AAV-mediated expression of mini-dysferlin improved muscle histology although both corrected membrane repair deficit in vitro[114].

LGMD2C to 2F are often referred to as sarcoglycanopathies because they are caused by mutations in the sarcoglycan genes. The most common sarcoglycanopathy is α-sarcoglycan-deficient LGMD2D. Cardiac involvement is rare in LGMD2D[6]. LGMD2C, which is caused by mutations in the γ-sarcoglycan gene, usually exhibits mild cardiomyopathy. Deficiency of β-sarcoglycan and δ-sarcoglycan results in LGMD2E and LGMD2F, respectively. These two diseases are associated with dilated cardiomyopathy[3, 6]. The molecular weights of sarcoglycans are 35 to 50 kD. The small size makes sarcoglycan genes perfect candidates for AAV delivery. Sarcoglycanopathies were among the first few inherited diseases proposed for AAV gene therapy[115]. Recently, AAV gene therapy for LGMD2C and 2D has entered into clinical trials[116–118]. Very few studies have explored AAV β-sarcoglycan gene transfer for treating LGMD2E[119, 120]. However, therapeutic delivery of the δ-sarcoglycan gene by AAV has been tested extensively in the mouse and hamster models of LGMD2F. Systemic or direct myocardial delivery of the δ-sarcoglycan gene not only reduced histological lesions in the heart (such as myocardial necrosis, inflammation, calcification and fibrosis) but also improved heart function and extended lifespan[38, 121–125]. Collectively, these preclinical studies suggest that AAV δ-sarcoglycan gene transfer is an effective treatment for dilated cardiomyopathy in LGMD2F.

LGMD2I is caused by mutations in the fukutin-related protein (FKRP) gene. FKRP is located in the Golgi apparatus and it is essential for post-translational glycosylation of α-dystroglycan, the protein that directly interacts with the extracellular matrix in the DGC complex. More than half of LGMD2I patients have cardiac abnormalities and a quarter of

them develop heart failure[126]. Gene therapy for LGMD2I has been hindered by the lack of a good animal model. Nonsense mutations and whole gene deletions are embryonically lethal[127]. To overcome this hurdle, Lu and colleagues recently generated a FKRP L276I knock-in mouse[128]. This nonsense mutation model mimics the clinical phenotype of LGMD2I. To determine whether systemic delivery of the FKRP gene with AAV can protect the heart, Qiao et al performed intraperitoneal injection in newborn FKRP L276I knock-in mice using an AAV-8 vector. Dobutamine-stressed echocardiography in 7-m-old treated mice showed significantly higher ejection fraction and fractional shortening than those of untreated mice[128].

3.2.2. Targeting disease gene to treat heart disease in other muscular dystrophies—Dystroglycanopathies are a group of congenital muscular dystrophies (MDC). They are caused by mutations in the genes involved in the glycosylation pathway of α-dystroglycan[129, 130]. Fukuyama muscular dystrophy, a dystroglycanopathy caused by retrotransposon insertion in the 3′-untranslated region of the fukutin gene, is associated with severe cardiomyopathy and congestive heart failure[131, 132]. Blockade of pathogenic exon-trapping by a cocktail of AONs restored fukutin expression and α-dystroglycan glycosylation in the mouse model and human cells[132]. Whether this therapy can rescue heart function remains to be determined by future studies.

FRKP gene mutation not only causes LGMD2I but also causes congenital muscular dystrophy type 1C (MDC1C). Similar to LGMD2I, cardiac involvement is also a frequent finding in MDC1C patients[133]. A mouse model for MDC1C has been generated with FKRP P448L knock-in[134]. AAV-9 mediated FKRP expression normalized α-dystroglycan glycosylation in the heart of MDC1C mice. Unfortunately, cardiac function was not assessed due to mild heart disease at the age of euthanization (5 months)[134].

Myotonic dystrophy (DM), the second most common muscular dystrophy, is an autosomal dominant disease. It is caused by pathogenic RNA gain-of-function toxicity due to CTG (for DM1) or CCTG (for DM2) expansion. Cardiac conduction deficits (conduction block and arrhythmia) contribute significantly to the morbidity and mortality[135]. About 20 different mouse models have been developed to reveal various aspects of the disease[136]. Among these, tamoxifen-inducible EpA960 mice and tetracycline-inducible GFP-DMPK-$(CTG)_5$ mice are considered as good models to test cardiac interventions for DM[137, 138]. The field of DM gene therapy has been particularly active in recent years. RNAi, ribozyme, AONs and more recently site-specific RNA endonuclease have all been explored for DM gene therapy[139–144]. However, most of these studies have not examined therapeutic efficacy in the heart. The in vivo proof of principle for reversing cardiac conduction defects has only been shown in GFP-DMPK-$(CTG)_5$ mice. In this model, administration of doxycycline induced myotonia and cardiac conduction abnormalities. Discontinuation of doxycycline dramatically reduced myotonic symptoms and conduction block in the heart[137].

4. Expanding the armory of dystrophic cardiomyopathy gene therapy by targeting pathogenic mechanisms

4.1. Dystrophin-independent Duchenne cardiomyopathy gene therapy

4.1.1. Stabilization of cardiomyocyte membrane with endogenous cellular genes—Given membrane weakening is a primary pathogenic mechanism, strategies that enhance sarcolemmal stability should theoretically ameliorate Duchenne cardiomyopathy. Utrophin is a dystrophin homolog[145]. Despite some differences[43, 146, 147], utrophin shares significant structural and functional similarity to dystrophin and assembles the utrophin-associated glycoprotein complex (UGC). As is the case for dystrophin, micro-utrophin has been generated for AAV delivery[40, 148]. More recently, AAV-mediated expression of jazz, an artificial zinc finger transcription factor, was found to activate the utrophin promoter and enhance utrophin expression[149]. So far these utrophin-based strategies have only been shown to protect skeletal muscle. Their therapeutic efficacy in the heart remains to be tested experimentally. Several components of the DGC and UGC, including sarcoglycans, sarcospan and nNOS, were recently shown to reduce the skeletal muscle phenotype in mdx mice[66, 150, 151]. Of these, only nNOS has been shown to treat Duchenne cardiomyopathy[152]. Specifically, Lai et al delivered a PDZ domain truncated version of the nNOS gene to the heart of 14-m-old mdx mice and examined the cardiac phenotype when mice reached 21 months of age. Supra-physiological ΔPDZ-nNOS expression significantly reduced myocardial fibrosis, inflammation and apoptosis. Importantly, treatment partially ameliorated ECG abnormalities and improved hemodynamic performance[152].

Besides the DGC and UGC, the integrin complex (especially α7β1) is another membrane-crossing complex that stabilizes the sarcolemma[153]. Expression of the α7-integrin gene by AAV was recently shown to reduce limb muscle disease in mdx mice and extend the life span of u-dko mice[154, 155]. The cardiac benefit of AAV-mediated α7-integrin expression remains to be demonstrated.

4.1.2. Treating Duchenne cardiomyopathy with calcium regulating genes—Cytosolic calcium overload is a pivotal pathogenic event leading to muscle damage and force reduction in DMD[156]. Restoring calcium homeostasis holds great promise for treating Duchenne cardiomyopathy. The sarco/endoplasmic reticulum calcium ATPase (SERCA) is a calcium pump that removes calcium from the cytosol and transports it into the lumen of the sarcoplasmic reticulum (SR). SERCA accounts for ≥ 70% of calcium removal from the cytosol in muscle cells. SERCA2a is expressed in the heart and slow twitch skeletal muscle. We found SERCA2a expression is reduced in the heart of mdx mice by immunostaining[157]. When the AAV-9 SERCA2a vector was delivered to the heart of 12-m-old mdx mice, it increased myocardial SERCA2a expression and significantly improved cardiac electrophysiology[157]. Encouragingly, similar protection was observed when the AAV-9 SERCA2a vector was administrated to terminal aged (22-m-old) mdx mice[158].

4.1.3. Additional dystrophin-independent gene therapy strategies—Besides strengthening the sarcolemma and restoring calcium homeostasis, investigators have explored many other creative gene therapy strategies that are not dependent on dystrophin.

These include AAV-mediated inhibition of the myostatin pathway, AAV-mediated overexpression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), the cytotoxic T cell GalNAc transferase (Galgt2) and miR486, and AAV-mediated blocking of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway[159–165]. However, most of these studies only demonstrated disease amelioration in skeletal muscle. Whether cardiac muscle can be protected is yet to be seen. Among these strategies, the myostatin inhibition approach is especially intriguing because this approach aims at increasing muscle mass. This raises two concerns: (a) muscle hypertrophy may increase stress on the sarcolemma and hence worsen muscle disease, and (b) myostatin inhibition may lead to hypertrophic cardiomyopathy. Indeed, different results have been achieved depending on the models used. In animal models for DMD (mice and dogs), myostatin inhibition has consistently improved skeletal muscle pathology and function[163, 164, 166–168]. In a phase I trial, AAV-mediated regional expression of the myostatin antagonist follistatin improved walking distance in 5 out of 6 BMD patients[169]. However, the results of myostatin inhibition appears less promising in preclinical studies of some other muscular dystrophies such as LGMD2B, LGMD2C, LGMD2F and congenital muscular dystrophy type 1A[170–173]. Cohn et al examined whether myostatin deficiency can cause myocardial hypertrophy in normal C57BL/6 mice and mdx mice[174]. Surprisingly, myostatin elimination did not affect heart weight and heart weight/body weight ratio in either strain[174]. A major protective mechanism of myostatin inhibition is to reverse muscle fibrosis by inducing fibroblast apoptosis[175]. For reasons yet unknown, this mechanism appears to be deficient in the heart[174]. Collectively, there is a lack of clear evidence suggesting that myostatin blockade benefits a dystrophic heart. Myostatin inhibition-based gene therapy strategies have to be carefully weighted against potential undesirable side effects[170–173, 176].

4.2. Disease gene-independent gene therapy for cardiomyopathy in other muscular dystrophies

4.2.1. Disease gene independent gene therapy for dilated cardiomyopathy in LGMD2E and 2F—MicroRNAs (miRs) are regulatory non-coding RNAs. Recent studies suggest that miRs play crucial roles in myocardial remodeling[177]. Sampaolesi and colleagues found that miR669 is down regulated in the heart of β-sarcoglycan null LGMD2E mice[178]. In a subsequent study, they evaluated preventive miR gene therapy in β-sarcoglycan knockout mice[179]. After intra-ventricular delivery of an AAV-2 miR669a vector to neonates, they quantified survival, cardiac fibrosis and function at the age of 18 months. AAV injected mice showed significantly better survival, less myocardial fibrosis and better heart function[179].

Several disease gene-independent approaches have been tested to treat dilated cardiomyopathy in rodent models of LGMD2F[25]. Mitsugumin 53 (MG53) is a 53 kD membrane repair protein and also a ubiquitin E3 ligase[180]. Mice lacking MG53 show increased susceptibility to sarcolemmal injury and develop a slow but progressive myopathy[181]. He et al introduced MG53 to neonatal and young adult LGMD2F hamster model with AAV-8. Supra-physiological MG53 expression in the heart and limb muscle partially reduced the serum creatine kinase level, stabilized the sarcolemma, and slowed

muscle degeneration and fibrosis. It also improved treadmill performance and heart function[182]. Since sarcolemmal disruption is a common pathogenic process, it is suggested that MG53 therapy may server as a broadband therapeutics for a wide range of muscular dystrophies[180]. Unfortunately, there are some important safety concerns for long-term use. In one study, authors noticed elevation of hepatic enzymes due to leaky MG53 expression in the liver[182]. Most alarmingly, two recent studies found that the E3 ligase function of MG53 targets the muscle insulin receptor and insulin-receptor substrate 1 for degradation[183, 184]. Transgenic over-expression of MG53 in striated muscle and heart resulted in metabolic syndrome and diabetic cardiomyopathy, respectively[183, 185].

Defects in sarcoplasmic reticulum calcium cycling plays a pivotal role in the pathogenesis of inherited and acquired cardiomyopathy[186]. As eluded before, SERCA2a is the primary calcium pump in the heart. AAV-mediated SERCA2a over-expression ameliorates some cardiac manifestations in the mdx model of Duchenne cardiomyopathy[157, 158]. The activity of SERCA2a is regulated by phospholamban. Unphosphorylated phospholamban inhibits SERCA2a activity but phosphorylated phospholamban does not. A single amino acid change (Ser16 Glu) locks phospholamban in a conformation that resembles the phosphorylated form. Hoshijima et al delivered this pseudo-phosphorylated phospholamban to the heart of δ-sarcoglycan deficient hamsters using AAV-2[25]. Chronic expression of pseudo-phosphorylated phospholamban markedly improved heart function in this LGMD2F dilated cardiomyopathy model[25].

Apoptosis has been implicated in the progression of heart failure. In particular, activation of apoptosis signal-regulating kinase 1 (ASK1) induces cardiomyocytes apoptosis. Hikoso et al tested whether delivery of the dominant mutant form of ASK1 can reduce cardiomyopathy in the LGMD2F hamster model[187]. They delivered dominant mutant ASK1 by AAV-2 via transcoronary perfusion to 10-week-old affected hamsters. Evaluation at the age of 24 weeks revealed remarkable improvements of systolic and diastolic function as well as a reduction of chamber dilation and myocardial fibrosis.

4.2.2. Disease gene independent gene therapy for cardiomyopathy in other muscular dystrophies—Merosin (laminin α2) is an extracellular matrix protein. Deficiency in merosin leads to congenital muscular dystrophy MDC1A. Although MDC1A patients usually do not have clinically significant cardiomyopathy[4], cardiac involvement has been documented in atypical patients and laminin α2-null dy/dy mice[188–191]. Agrin is also an extracellular matrix protein but it has no structural similarity to laminin α2. Interestingly, AAV-1 mediated systemic expression of a miniature version of agrin greatly reduced myocardial fibrosis in dy/dy mice[192].

LGMD2I and MDC1C are caused by mutations in the FKRP gene and both diseases display prominent cardiac manifestations. FKRP knock-in mice L276I and P448L have been developed to model LGMD2I and MDC1C, respectively[128, 134]. The pathway of α-dystroglycan glycosylation involves a series of glycosyltransferases. Like-acetylglucosaminyltransferase (LARGE) acts downstream of FKRP. Activation of a downstream enzyme presumably should correct the disease phenotype caused by upstream enzyme deficiency. Vannoy indeed found that AAV-mediated LARGE over-expression not

only reduced myopathy in LARGE-deficient congenital muscular dystrophy mice but also improved α-dystroglycan glycosylation in the heart and skeletal muscle of FKRB P448L knock-in mice[193].

5. Expert opinion

The cloning of the dystrophin gene in 1986 started a flood of discoveries on genes whose mutations cause various forms of muscular dystrophies[39]. All of a sudden, it appears we may cure many muscular dystrophies and their associated cardiomyopathy by either fixing the mutated gene or introducing a functional copy of the normal gene. While conceptually straightforward, the journey thus far has turned out to be long and winding. Research in dystrophic cardiomyopathy and its gene therapy has made significant progress in the last decade[194–196]. Several fundamental issues have been addressed. These include the establishment of a large collection of animal models to test experimental gene therapy in various forms of dystrophic cardiomyopathy, the development of noninvasive AAV delivery methods to efficiently transduce the heart, and the expansion of therapeutic schemes from simply delivering a functional cDNA to dystrophic muscle to the modulation of the RNA/DNA structure and expression using a variety of coding and noncoding sequences, even oligonucleotides. Some critical parameters for dystrophic cardiomyopathy gene therapy have also been clarified. For example, studies in the mdx model of Duchenne cardiomyopathy have provided compelling evidence that we may achieve a near wild-type protection by treating half of the cardiomyocytes instead of every single cell[63, 197]. On the other hand, debates on whether treating skeletal muscle disease will alleviate or aggravate cardiomyopathy have settled down on the conclusion that both should be treated either together or separately[76, 198, 199].

There is no doubt that Duchenne cardiomyopathy gene therapy has led the way for the entire field. First, a number of models have been generated for Duchenne cardiomyopathy gene therapy studies such as aged mdx mice, Cmah/mdx mice, u-dko mice, myoD/dystrophin double knockout mice and telomerase RNA/dystrophin double-null mdx/mTR mice[63, 64, 200–204]. Importantly most of these rodent models are commercially available[205]. In terms of large animal models, besides the commonly used golden retriever muscular dystrophy dogs (GRMDs), additional dog models have been identified and colonies established[206–209]. Second, we have successfully treated the cardiac phenotype in symptomatic u-dko mice and aged mdx mice using micro-dystrophin and exon-skipping[34, 89]. We even achieved widespread myocardial AAV gene transfer and some ECG improvements in terminal stage mdx mice[35]. For scaling up, efficient myocardial transduction has been achieved in newborn dogs and adult affected dogs with systemic and percutaneous transendocardial AAV delivery[57, 91, 92, 210, 211]. Third, many previously under-appreciated disease targets (such as nNOS and SERCA2a) and revolutionary technologies (such as tcDNA, ZENs, TALENs and CRISPR/Cas9) are now on the horizon for Duchenne cardiomyopathy gene therapy. Despite this substantial progress, we still do not have answers to a lot of important questions. For example, it is not clear whether supra-physiological dystrophin expression in the heart is toxic, whether there exists heart-specific domain(s) in the dystrophin gene that should be included in micro-dystrophin, and whether cardiotropic features of some existing AAV serotypes can cross the species boundary and result in

efficient heart transduction in humans. For this last point, some recent developments in the generation of the xenograft model using dystrophic human muscle and forced evolution of human tissue tropic AAV capsids may provide some hints[32, 212, 213]. It should be noted that emerging new technologies such as genome editing with CRISPR/Cas9 not only brings in new hopes, they are also accompanied with new questions such as potential toxicity from off-target editing.

There is a long to-do list for the field of dystrophic cardiomyopathy gene therapy. Some of these may include (1) continued development and characterization of large animal models for dystrophic cardiomyopathy. In light of recent success in creating rat, pig and monkey models using the CRISPR/Cas9 technology, model generation may no longer represent a formidable barrier as it was before[214]; (2) thorough evaluation of the most promising gene therapy strategies in large animal models[215]. Lack of solid large animal data has been an important factor limiting the translation of rodent study results to human patients. In this regard, there is an urgent need to thoroughly evaluate therapeutic efficacy in large mammals. For example, treating heart disease with tcDNA exon skipping and AAV micro-dystrophin gene therapy in dystrophin-deficient dogs[216]; (3) establishment of cardiac specific biomarkers that can be used to monitor disease progression and responses to gene therapy in animal models of dystrophic cardiomyopathy; (4) investigations of gene therapy for cardiac manifestations in muscular dystrophies other than DMD and LGMD2F. For many of these muscular dystrophies, gene therapy strategies have been developed for treating skeletal muscle myopathy. We need to test if similar approaches can attenuate cardiac disease.

In summary, gene therapy for dystrophic cardiomyopathy has taken a slow but steady path towards preclinical and eventually clinical studies. These efforts will undoubtedly be complicated by issues related to vector manufacturing, host immune responses, and the lack of enough patients for large-scale clinical trials due to the relatively low incidence of the disease. Nevertheless, we already have a solid foundation. The future of dystrophic cardiomyopathy gene therapy is very bright.

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Highlights box

- Cardiomyopathy is a common complication in inherited muscular dystrophies.
- Gene therapy holds great promise to reduce heart-related morbidity and mortality in muscular dystrophies.
- Adeno-associated virus (AAV) is the most effective cardiac gene delivery vector.
- Micro-dystrophin and sarcoglycan gene therapies have significantly improved the cardiac outcome in animal models of Duchenne muscular dystrophy and limb girdle muscular dystrophy, respectively.
- Targeting pathogenic mechanisms with disease gene independent gene therapy opens exciting new opportunities.
- Preclinical test in large animal models will pave the way to human trials.