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Recent advances using zebrafish animal models for muscle disease drug discovery

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Abstract

Introduction—Animal models have enabled great progress in the discovery and understanding of pharmacological approaches for treating muscle diseases like Duchenne muscular dystrophy.

Areas covered—With this article, the author provides the reader with a description of the zebrafish animal model, which has been employed to identify and study pharmacological approaches to muscle disease. In particular, the author focuses on how both large-scale chemical screens and targeted drug treatment studies have established zebrafish as an important model for muscle disease drug discovery.

Expert opinion—There are a number of opportunities arising for the use of zebrafish models for further developing pharmacological approaches to muscle diseases, including studying drug combination therapies and utilizing genome editing to engineer zebrafish muscle disease models. It is the author's particular belief that the availability of a wide range of zebrafish transgenic strains for labeling immune cell types, combined with live imaging and drug treatment of muscle disease models, should allow for new elegant studies demonstrating how pharmacological approaches might influence inflammation and the immune response in muscle disease.

Keywords

birefringence; drug screen; Duchenne muscular dystrophy; muscle function; muscle structure; myopathy; zebrafish

1. Introduction

Myopathies are muscle diseases in which dysfunction of muscle fibers results in muscle weakness. The diversity of inherited myopathies, which include the muscular dystrophies, is

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reflected in the diversity of genetic mutations that are implicated in these diseases [1]. No cures are currently available for inherited myopathies. Gaining increased understanding of the cellular and molecular mechanisms underlying the muscle defects seen in inherited myopathies will help provide insight into potential therapies [2,3]. Here, I review how the zebrafish animal model has been employed to both identify, and study the mechanisms of, pharmacological approaches to muscle disease therapies.

Many animal models, including mice, dogs, pigs, fruit flies, worms and zebrafish, have contributed to our understanding of the genetic basis of, and molecular and cellular mechanisms behind, inherited muscle diseases [4-9]. In particular, one muscle disease that is well characterized in animal models is Duchenne muscular dystrophy (DMD). DMD is the most common and severe form of muscular dystrophy, affecting about 1 in 3500 males, and is caused by mutations in the X-linked *DMD* gene, which encodes dystrophin [10]. Dystrophin contributes to the multi-protein dystrophin–glycoprotein complex (DGC), also known as the dystrophin-associated protein complex, which links the extracellular matrix with the cytoskeleton and is critical for muscle cell membrane stability and cell signaling [2,11]. Many types of muscular dystrophy are caused by mutations in genes that encode components of the DGC [2,11]. Muscular dystrophies are characterized by progressive muscle degeneration and progressive loss of muscle function. Mechanisms that contribute to muscle degeneration in the muscular dystrophies include muscle membrane instability, disrupted calcium homeostasis and oxidative stress [2,11]. The congenital myopathies (sometimes also referred to as ‘inherited myopathies’), such as nemaline myopathy, are characterized by muscle weakness but do not usually show progressive muscle degeneration. Congenital myopathies typically show characteristic structural defects in the contractile apparatus that lead to reduced myofiber contractile function [5]. Disruption of excitation–contraction coupling appears to be a common pathological mechanism in the congenital myopathies [3,5].

Animal models have also contributed to the development of therapeutic approaches for muscle diseases. For DMD in particular, gene therapy and stem cell-mediated therapeutic strategies hold tremendous promise, but these approaches still face many obstacles [12,13]. Therefore, many different pharmacological therapies are currently being pursued [14-17]. One advantage of pharmacological therapy is that systemically delivered drugs could reach all muscle groups, including the heart, which undergoes cardiomyopathy in DMD patients [13-15]. Animal models from fruit flies [18] to dogs [19] have helped advance our knowledge of potential pharmacological therapies for DMD. The zebrafish animal model, in particular, offers many advantages for drug discovery and for understanding drug mechanisms of action not only for DMD therapies but for therapies for other muscle diseases as well.

2. Zebrafish as a model for muscle development and disease

2.1 Zebrafish model organism advantages

The zebrafish, *Danio rerio*, offers several advantages as a model organism for human disease and drug discovery [20]. Zebrafish can be produced readily in large numbers such that hundreds of embryos can be obtained in a single day. The embryos are transparent and

develop rapidly outside of the mother, allowing the earliest stages of development to be examined. Also, zebrafish are readily manipulated by both genetic and chemical approaches (Figure 1). I will discuss genetic manipulations below (Sections 2.3 and 2.4). For chemical approaches, zebrafish embryos raised in a petri dish can readily absorb drugs that are simply added to the embryo bath (Figure 2) [21]. Embryos can be raised in 96- or 384-well plates, allowing for high-throughput chemical screening (Figure 1) [21]. Significantly, drugs identified through screening in the zebrafish have led to clinical trials [22,23], underscoring the relevance of pharmacological studies in zebrafish to human disease. In addition to these general advantages, there are several additional characteristics that have made zebrafish an outstanding model for human muscle disease, as I describe below.

2.2 Zebrafish skeletal muscle features and motor behaviors

One advantage of studying zebrafish muscle is that development of the main body musculature is rapid, such that by 24 h post-fertilization, all 30 segmental blocks of muscle, or myotomes, are present and easily visible along the trunk and tail of the embryo. The transparency of the zebrafish embryo has facilitated cellular observations of early muscle cell lineages, cellular migrations and muscle fiber morphogenesis [24-26]. Many of the molecular events of skeletal muscle development and differentiation are conserved between zebrafish and mammals [25,26]. Structurally, zebrafish skeletal muscle is very similar to human muscle, and zebrafish share the same muscle disease genes with humans, including genes encoding components of the sarcomere, the DGC and excitation–contraction coupling [6,27-32]; (see Section 2.4 Zebrafish muscle disease models).

The mechanisms of skeletal muscle repair and regeneration are similar in zebrafish and mammals. Defective muscle membrane repair can lead to myopathy, and both zebrafish and mammals share requirements for dysferlin and associated proteins such as annexins for sarcolemmal repair [33,34]. Pax7-expressing skeletal muscle stem cells, or satellite cells, are required for mammalian skeletal muscle regeneration [35]. Zebrafish have Pax7-expressing cells that migrate into areas of muscle damage [36]. Whether these Pax7-expressing cells function as true ‘satellite cells’ and contribute to new muscle fibers during zebrafish muscle regeneration has not yet been definitively shown [37]. However, zebrafish muscle does appear to regenerate from proliferative stem cell-like cells and not from de-differentiated fibers [37]. These studies help support the idea that investigating the mechanisms of zebrafish muscle response to injury and disease will inform our understanding of human muscle.

Early motor behaviors during zebrafish embryonic and larval stages have been well characterized and can be easily observed and measured [38-41]. The first movements begin at about 17 h post-fertilization as spontaneous contractions. At about 24 h, embryos will coil in response to touch. At about 26 h, embryos exhibit swimming movements in response to touch, and by 96 h the larva is freely swimming. These stereotyped movements have formed the basis for genetic screens for zebrafish mutant strains with muscle defects, as I describe next, and for characterization of zebrafish muscle disease models (see Section 5.2 Assessing muscle function).

2.3 Zebrafish genetic screens for muscle defects

The rapid development of zebrafish and the ability to readily obtain large numbers of animals have facilitated forward genetic screens that have significantly contributed to the use of zebrafish as an animal model for human muscle disease. Some of the earliest genetic screens in zebrafish used embryonic motility to identify loci required for muscle function [39,42]. The stereotypic touch-evoked escape response in zebrafish larvae has formed the basis of several genetic screens in an effort to identify genes involved in motor control of behavior [39,40,43,44]. Additional zebrafish forward genetic screens have utilized muscle birefringence, a measure of skeletal muscle structural integrity (see Section 5.1 Assessing muscle structure; Figure 1), to identify genes relevant to skeletal muscle disease [40,45]. Many of the genes responsible for the phenotypes in the mutant strains recovered from these screens have been identified, and most are orthologs of human muscular dystrophy and myopathy genes [28,30,45-48].

2.4 Zebrafish muscle disease models

These genetic screens and the resulting mutant zebrafish strains have thus provided a wealth of zebrafish models of human muscle diseases. Additional approaches to generating zebrafish models of human muscle diseases have been the use of antisense morpholinos (Figure 1) or, in cases where the human disease results from a dominant mutation or misexpression, transgenic zebrafish or transient overexpression in zebrafish embryos. Recent reviews provide tables with comprehensive lists of known zebrafish models of human muscle diseases [6,32,49]. Genome editing technology now opens up new approaches to generating zebrafish muscle disease models, as I will discuss further below (Section 8 Expert opinion; Figure 1). The identification of zebrafish mutant strains for human muscle disease genes has revealed that zebrafish represent excellent models of human myopathies [32,50]. For example, zebrafish *dmd* mutants show severe muscle pathology and motor defects by 4 days and die by about 30 days post-fertilization, while mouse DMD (*mdx*) mutants are viable and have only mild muscle defects [28,50,51].

The identification of zebrafish models of human muscle diseases has provided opportunities to use these models for directly testing genetic and pharmacological approaches for ameliorating the skeletal muscle defects associated with each disease. For example, studies of zebrafish acetylcholinesterase (*ache*) mutants and acetylcholine (ACh) receptor (*nic1* and *sop*) mutants were among the earliest to show that zebrafish models can be employed for investigating genetic and pharmacological suppression of myopathies [43,52,53]. ACh is the major neurotransmitter in the nervous system. ACh binds post-synaptic ACh receptors (AChR) on muscle cells and is degraded by acetylcholinesterase (AChE). Zebrafish *ache* mutant embryos show progressive motor and muscle structural defects [43]. The *ache* mutant muscle phenotype can be suppressed by loss of the *nic1* AChR or by treating *ache* embryos with the drug eserine, suggesting that eserine acts not only as a known AChE inhibitor but also an antagonist for the AChR [43,52]. Additional studies showed that the *sop* AChR mutation could suppress the myopathy observed in zebrafish *dmd* mutants [53]. Taken together, these studies showed that zebrafish can serve as a screening system for drugs that can suppress myopathies and also showed that modifiers of the zebrafish *dmd*

mutant phenotype could be discovered and readily examined in easily accessible young animals.

3. Pharmacological therapies for DMD studied in zebrafish

Recent pharmacological approaches, including large-scale drug screens as well as targeted compound tests, that have demonstrated the rescue of the zebrafish *dmd* mutant are reviewed below. These examples illustrate how zebrafish embryos can serve as a vertebrate high-throughput screening platform for new drug therapies for DMD. These examples also illustrate how zebrafish can be used to examine the mechanism of action of specific drugs and bridge the gap between cell culture and mammalian models. Table 1 provides a list of these drug studies in zebrafish *dmd* models, and Figure 2 illustrates a drug treatment strategy.

3.1 Large-scale drug screens

To establish zebrafish as a significant vertebrate model for identifying pharmacological approaches for DMD, the Kunkel laboratory has performed large-scale drug screens for small molecules capable of suppressing the zebrafish *dystrophin*-null (*dmd*, also referred to as *sapje*) mutant phenotype [54,55]. In zebrafish, the *dmd* gene is not sex chromosome linked, as in humans and mice, and shows autosomal recessive inheritance [31]. With the zebrafish *dmd* mutant strain, about 25% of the larvae from a cross of heterozygote carriers show the *dmd* muscle lesion phenotype [28,29,54]. These muscle lesions can be observed through a simple, high-throughput birefringence assay in 4-day-old larvae (Figure 2; [54]; see also Section 5.1 Assessing muscle structure). In their initial large-scale screen, Kawahara *et al.* tested 1120 small molecules from the Prestwick library of bioreactive compounds approved for human use [54]. First, 140 chemical pools were tested and selected if < 7.5% of the progeny exhibited muscle lesions, compared with 25% in the untreated control embryos. In the second step, the selected pools were separated and 48 chemicals were tested individually. Seven chemicals from this two-step screen rescue the *dmd* mutant muscle lesion phenotype [54]. The chemical that promoted the highest long-term survival in *dmd* fish was aminophylline, a non-selective phosphodiesterase (PDE) inhibitor [54]. They further tested a series of other PDE inhibitors and found that sildenafil, a PDE5 inhibitor, improved muscle lesions in zebrafish *dmd* larvae [54]. Because PDE5 inhibitors, including sildenafil, have been shown to ameliorate the mouse *mdx* model [56-58], this study showed that zebrafish *dmd* drug screening is highly relevant to identifying potential mammalian DMD therapies.

More recently, Kawahara *et al.* have expanded their drug screening on zebrafish *dmd* models with an additional 1520 chemicals, identifying 8 more compounds, including sildenafil [55]. Out of the 15 total drugs identified from both screens, 6 compounds target heme oxygenase signaling [55]. These compounds improve the zebrafish *dmd* muscle phenotype through upregulation of Heme oxygenase 1 (Hmox1; [55]). The authors further show that sildenafil treatments in *mdx* mice, as in the *dmd* fish, increase Hmox1 expression [55]. These studies thus reveal Hmox1 as a novel potential target for DMD therapy. These large-scale screens from the Kunkel laboratory highlight the potential of zebrafish models for identifying new

therapeutic compounds and targets as well as for understanding the molecular mechanisms behind muscle disease.

3.2 Targeted drug studies

Two recent studies have directly tested whether compounds that ameliorate the mouse *mdx* model can also ameliorate the zebrafish *dmd* model. In one study, Winder *et al.* tested the proteasomal inhibitor MG132, because it had been previously shown that inhibition of the proteasome by MG132 was able to restore components of the DGC in both *mdx* mice and DMD patient extracts and also showed signs of ameliorating disease symptoms in these models [59-61]. When zebrafish *dmd* mutant larvae are exposed to MG132, reduced muscle damage, as assessed by birefringence (see below Section 5.1 Assessing muscle structure), is observed [59]. In a second study, my laboratory tested the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), because it and other HDAC inhibitors can ameliorate the *mdx* model [17,62,63]. We showed that TSA robustly rescues muscle fiber damage in the zebrafish *dmd* mutant [62]. These studies further underscore that zebrafish is an appropriate model for testing pharmacological therapies for DMD.

The ability of TSA to ameliorate muscular dystrophy in the mouse *mdx* model may work through more than one mechanism. Initial studies of TSA-treated *mdx* mice suggested that TSA acted through promoting upregulation of follistatin expression in satellite cells [64]. A recent study, however, showed that fibro-adipogenic progenitor cells mediate the ability of TSA to ameliorate muscular dystrophy in young *mdx* mice [63]. The recent zebrafish TSA studies now provide an additional model system for further mechanistic analysis of how HDAC inhibitors function to ameliorate dystrophic muscle.

One limitation of the zebrafish *dmd* mutant model for chemical screening is that phenotypic rescue can be assessed on only the 25% of larvae in the clutch that are mutant, while 75% of the larvae are phenotypically normal [54]. Furthermore, to accurately assess rescue, the mutant individuals must be genotyped. Therefore, my laboratory developed a robust zebrafish *dmd* antisense morpholino (*dmd*-MO) knock-down model that closely resembles the zebrafish *dmd* mutant phenotype and achieves almost 100% penetrance [62]. We showed that this *dmd*-MO model is useful for identifying small molecules that rescue the *dmd* phenotype by showing that TSA can rescue muscle fiber damage similarly in both *dmd*-MO and *dmd* mutant larvae (Figure 2; [62]). Thus, *dmd*-MO larvae could be employed as a rapid and robust model for initial chemical screening. Candidate drugs that rescue *dmd*-MO larvae can subsequently be retested on *dmd* mutants. I discuss further advantages and disadvantages of using morpholinos below (Section 8 Expert opinion).

4. Additional zebrafish muscle disease models and pharmacological studies

Here, I would like to highlight examples where pharmacological studies in other zebrafish muscle disease models have helped provide new insights into mechanisms of drug action, therapeutic targets and disease pathogenesis. Table 1 provides a list of these and other examples.

4.1 Collagen VI myopathies and cyclosporine A treatment

Deficiencies in collagen VI, an essential component of the extracellular matrix in skeletal muscle, lead to Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) [65]. Collagen VI myopathies share a common pathogenesis of mitochondrial dysfunction. Mitochondrial defects and associated apoptosis seen in the mouse *Col6a1*^{-/-} mouse model and in muscle cell cultures from UCMD patients can be rescued by treatment with the proton pump modifier cyclosporine A (CsA; [66,67]). In order to better understand these myopathies and the mechanism of CsA action, Telfer *et al.* generated zebrafish models of the collagen VI myopathies [68]. Antisense morpholinos designed to target either exon 9 or exon 13 of the zebrafish *col6a1* gene caused motor defects and skeletal muscle structural defects, including abnormal mitochondria, consistent with either a mild BM-like (exon 13) or severe UCMD-like (exon 9) phenotype [68]. Treatment with CsA improves the mitochondrial and apoptosis defects and improves the motor deficits in the UCMD-like zebrafish [68]. However, CsA does not improve muscle fiber integrity observed using birefringence, suggesting that CsA acts ‘downstream’ of muscle membrane integrity. Reducing apoptosis through p53 suppression does not improve the motor phenotype of UCMD fish, suggesting that CsA is not working solely through reducing mitochondrial-mediated apoptosis [68]. These studies raise the question of whether the ability of CsA to improve motor function in UCMD fish is related to other effects of improving mitochondrial function or is due to additional roles of CsA. Because the mouse *Col6a1*^{-/-} model shows no clear functional defects [69], the zebrafish UCMD-like model can serve for further studies of muscle structural and functional pharmacological rescue.

4.2 RYR-1-related myopathy and oxidative stress

Mutations in the skeletal muscle ryanodine receptor (RYR1), a calcium release channel needed for excitation–contraction coupling, are associated with several congenital myopathies (RYR1-related myopathies; [70]). To identify novel disease mechanisms in RYR1-related myopathies, Dowling *et al.* used the zebrafish *relatively relaxed* (*ryr*) mutant, a zebrafish model of RYR1-related myopathies [30,71]. *ryr* zebrafish carry a mutation in *ryr1b* and show decreased RyR1 levels, impaired excitation–contraction coupling in fast twitch muscles, and a slow-swimming phenotype. RNA expression analysis of zebrafish *ryr* mutant larvae reveals significant changes in several pathways associated with cellular stress, in particular increased expression of markers of oxidative stress [71]. Treatment of *ryr* zebrafish with the antioxidant *N*-acetylcysteine rescues the swimming defects and improves the muscle histological appearance [71]. This study further showed that myotubes derived from patients with RYR1-related myopathies also display increased oxidative stress and that *N*-acetylcysteine could reduce oxidative stress and improve survival of cultured patient myotubes [71]. Thus, the zebrafish RYR1-related myopathy model identified oxidative stress as an important pathophysiological mechanism in RYR1-related myopathies. Treatment with *N*-acetylcysteine now represents a potential therapy for RYR1-related myopathies.

4.3 Vitamin rescue of dystroglycanopathy and muscle fiber adhesion

Genetic mutations that disrupt adhesion of muscle fibers to their extracellular environment can lead to several myopathies [72,73]. The receptors used by muscle fibers to adhere to the basement membrane in the extracellular matrix are the DGC (see Section 1 Introduction) and integrin $\alpha 7\beta 1$ heterodimers. Weakened links between these receptors and the basement membrane can make muscle fibers more susceptible to damage and degeneration. Because their previous studies show that the ubiquitous coenzyme nicotinamide adenine dinucleotide (NAD⁺) helps promote basement membrane organization in zebrafish muscle, Goody *et al.* tested whether NAD⁺ treatments could improve muscle fiber attachment in zebrafish muscular dystrophy models [73,74]. Indeed, they find that in zebrafish lacking either dystroglycan or integrin $\alpha 7$, basement membrane organization, muscle fiber attachment and motility are all significantly improved by treating embryos with NAD⁺ [73]. An NAD⁺ precursor, niacin (vitamin B3), is similarly able to rescue zebrafish embryos lacking dystroglycan or integrin $\alpha 7$ [73]. The niacin source chosen was Emergen-C, an over-the-counter nutritional supplement powder that easily dissolves in the zebrafish embryo medium. Interestingly, the Emergen-C concentration that rescues the dystroglycan-null zebrafish is calculated to be roughly equivalent to what the concentration of niacin would be in an adult human's bloodstream after consuming an Emergen-C packet [73]. Furthermore, Goody *et al.* used the NAD⁺ treatments to help demonstrate the requirement for a novel skeletal muscle adhesion receptor, integrin $\alpha 6$ [73]. Altogether, this study combines many advantages of zebrafish (cell biology, imaging, genetics and pharmacological treatments), to support the ideas that modulation of muscle fiber–extracellular matrix adhesion may be a therapeutic avenue for many myopathies and that vitamin supplementation warrants further investigation.

4.4 Dynamin-2-related centronuclear myopathy and neuromuscular transmission

Centronuclear myopathies (CNM) are characterized by muscle weakness and abnormal centralized nuclei and are most commonly caused by dominant mutations in dynamin-2 (DNM2; [75]). The mechanisms by which DNM2 mutations lead to myopathy are not well understood. Gibbs *et al.* generated a zebrafish model for DNM2–CNM by overexpressing one of the most common and severe DNM2 mutations (DNM2-S619L; [76,77];). They find that overexpression of DNM2-S619L in zebrafish embryos impairs motor behavior, causes perinuclear muscle structural abnormalities and decreases AChR clustering in muscle fibers [76], and also causes defects in T-tubule and sarcoplasmic reticulum formation and in excitation–contraction coupling [78]. Clinical studies of patients with DNM2–CNM mutations also suggest defects in neuromuscular transmission [76]. Treating the DNM2–CNM zebrafish larvae with an AChE inhibitor greatly improved their motor behavior [76]. These results suggest a new mechanism of neuromuscular junction defects in DNM2–CNM pathology.

5. Analyses of zebrafish muscle disease and assessment of pharmacological rescue

The examples presented above of both large-scale drug screening and targeted drug studies have not only identified classes of drugs that ameliorate zebrafish *dmd* and other

myopathies, but also identified optimal approaches for analyzing and quantifying zebrafish muscle disease and its rescue. The zebrafish system is an exceptional animal model for the range of extremely simple to sophisticated approaches for assessing muscle structure and function. Here, I discuss studies of zebrafish muscle structure and function that have enhanced zebrafish as a model for pharmacological approaches to muscle disease.

5.1 Assessing muscle structure

A critical aspect of using zebrafish as a model for pharmacological approaches to muscle disease is the ability to quantitatively assess muscle structural damage and rescue by drug treatment. The Kunkel group has successfully used a simple birefringence assay through a stereomicroscope for large-scale drug screening with the zebrafish *dmd* model [54,55]. The highly organized sarcomere pattern of zebrafish skeletal muscle appears bright under polarized light, but zebrafish with muscle disease show darkened disruptions in this birefringence due to muscle fiber disorganization, detachment or degeneration (Figures 1 and 2; [6,32,39,40]). Imaging birefringence in zebrafish larvae can be rapid, is noninvasive, and can be performed on live or on fixed specimens [62,79]. However, birefringence is very dependent on orientation of the larva between two polarizing filters, and improper alignment of larvae may prevent correct scoring of the muscle phenotype. Also, overall birefringence may not allow for distinguishing subtle effects of a drug treatment on muscle structure. Three studies have addressed these issues by describing highly quantitative approaches to birefringence. Selected areas of muscle birefringence images taken from a stereomicroscope can be analyzed for mean pixel intensity using freely available software [79]. The Currie group described a polarizing light microscope system that rapidly generates an unbiased birefringence map of a fish larva that is independent of the specimen orientation [80]. Subsequent measuring of the pixel brightness allows quantification of the birefringence. Also, zebrafish *dmd* muscle damage can be quantified by subjecting gray-scale birefringence intensities over the length of the larva to Fourier analysis [59].

In addition to birefringence, skeletal muscle can be readily visualized in whole zebrafish larvae using dyes, stains or transgenic skeletal muscle fluorescent reporters. Evans Blue Dye, a vital stain that only enters cells with damaged membranes, can reveal sarcolemmal damage in dystrophic zebrafish [28,48]. Immunocytochemistry, histology and electron microscopy can provide insight into muscle fiber and sarcomere structure defects in zebrafish myopathy models [27,48,53,68,73]. Skeletal muscle actin labeling with the transgene *acta1a:gfp* or with phalloidin, or muscle myosin labeling with immunocytochemistry, can reveal muscle lesions in dystrophic zebrafish (e.g., Figure 2; [28,48,54,62,73]). The Henry group used phalloidin staining to quantitate dystrophic muscle lesions by scoring the percent of myotomes (muscle segments) affected per larva [73]. The Henry group has also used two-dimensional wavelet transform modulus maxima, a measure of muscle fiber organization, in combination with phalloidin staining and immunocytochemistry, to quantify changes in muscle structure in zebrafish muscular dystrophy models [73,81]. Elegant high-resolution imaging of the damage response in myofibers in live zebrafish larvae has been used to identify a novel sequential sarcolemmal repair process [34]. Thus, the zebrafish system has a range of simple to sophisticated approaches for assessing muscle structure.

For large-scale chemical screening, it is ideal to optimize the cost and speed of the screening approach while still allowing for reliable phenotypic scoring. Assessment of the muscle birefringence pattern, in which larvae are simply scored as affected versus unaffected using a stereomicroscope, provides a measurement of *dmd* phenotypic rescue, following treatment with the drug TSA, that is comparable to the more quantitative, but time-consuming, approach of counting myotome lesions per larva, visualized with muscle actin labeling using the confocal microscope [62]. This study supports the large-scale birefringence screening approach used by the Kunkel group [54,55] as an easy and reliable assessment of drug-treatment rescue of dystrophic zebrafish larvae.

5.2 Assessing muscle function

Another critical aspect of using zebrafish as a model for pharmacological approaches to muscle disease is the ability to quantitatively assess rescue of muscle function following drug treatment. Zebrafish are quite amenable to assessing muscle function, and, as with muscle structure, the assays range from the simple to the more complex. Perhaps the simplest and most important assessment of muscle function is animal survival, which Kawahara *et al.* used to show improvement of zebrafish *dmd* mutants following treatment with selected chemicals from their large-scale drug screens [54,55]. Spontaneous embryo coiling and larval touch-evoked escape response are two straightforward motor assays that have been used to characterize zebrafish myopathy models [47], and several studies have used these assays to demonstrate motor function rescue of zebrafish myopathies after drug treatments [6,68,73]. Dowling *et al.* assessed motor function in *N*-acetylcysteine-treated *ryr*-mutant zebrafish larvae using video monitoring to measure swim velocity and distance travelled (Figure 1; [71]). To further assess motor function, they measured multiple parameters of skeletal muscle contractile properties in whole larvae by mounting larvae to a force transducer [71]. Electrophysiological recordings from muscle in whole larvae can assess muscle function in mutant and drug-treated animals [30,47]. Another option for muscle functional analysis in zebrafish is the study of cultured isolated myofibers, in which analyses such as electrophysiological measurements can be performed [82,83]. To assess locomotor abilities of swimming adult zebrafish under the demanding conditions of a water current, Blazina *et al.* developed a Spinning Task in a beaker using a magnetic stirrer and were able to quantitatively assess motor impairments induced by drug treatments [84]. This variety of approaches thus allows for the exquisite analysis of many aspects of muscle function and for the identification of key pathological properties of different myopathies.

6. Using zebrafish to study pharmacological approaches to muscle disease gene modification

Exon-skipping agents and stop-codon read-through are potential therapeutic approaches for muscle diseases that have been tested in zebrafish models. Table 1 includes these approaches as examples of drug studies in zebrafish *dmd* models.

6.1 Exon skipping

One promising therapeutic approach for DMD is exon skipping, in which antisense oligonucleotides are administered to sterically block splicing of the *DMD* pre-mRNA

transcript, leading to exclusion of targeted exons and restoration of truncated, but partially functional, dystrophin protein [85]. It is estimated that about 83% of all *DMD* mutations could potentially be corrected by the skipping of specific exons [86]. Although mouse and dog animal models for DMD exon skipping have already provided valuable guides for clinical trials [12,15,87], one question that has been tested in the zebrafish model is the level of dystrophin needed for functional rescue [88]. The Currie laboratory used a forward genetic screen to identify new zebrafish *dmd* alleles that are amenable to testing exon skipping strategies [88]. They injected *dmd* splice-site targeted antisense morpholino oligos into these *dmd* mutant embryos and then assayed *dmd* phenotype and dystrophin expression. Their study showed that robust morpholino activity, in which exon-skipped *dmd* transcript is induced to levels of at least 30 – 40% of total *dmd* transcript, is needed to significantly improve the zebrafish *dmd* muscle phenotype [88]. Their study was also able to identify a conserved cryptic splice site in the *dmd* gene [88]. Thus, this analysis in zebrafish could help inform antisense oligo design and strategies for human DMD exon-skipping therapies. Modulation of pre-mRNA splicing has been investigated as a therapeutic option for several muscle diseases [85]. Because of the ease of testing antisense oligos in zebrafish, and because of the ability to engineer zebrafish carrying specific disease gene alleles found in humans (see below 8. Expert opinion), zebrafish should serve as an excellent preclinical animal model for testing exon-skipping strategies.

6.2 Stop-codon read-through

About 10 – 15% of DMD patients have a mutation that generates a premature stop codon [89]. Drugs that enable ribosomal read-through of stop codons have thus been investigated as DMD therapies [14]. PTC Therapeutics identified Ataluren (PTC124) as a drug that promotes read-through of premature stop codons in the *DMD* gene and improves muscle function in the mouse *mdx* model [90], although the read-through effectiveness of Ataluren has been questioned [91,92]. A Phase IIb clinical trial has suggested some success in patients' physical functioning [93]. Li *et al.* have used zebrafish to further investigate the ability of Ataluren to ameliorate DMD [94]. They find a significant improvement of muscle structure and function in *dmd* larvae after two days of Ataluren treatment, accompanied by increased expression of Dystrophin protein [94]. Importantly, Ataluren shows dosedependent effects in zebrafish, in which increasing doses can begin to show reduced effects or even negative effects on muscle force production [94]. These findings may provide insight into the results from the Ataluren Phase IIb clinical trials, where patients' walking improvement was observed at lower doses but not at higher doses [93].

Thus, even for drug therapies whose mechanism of action is relatively well understood, zebrafish studies can provide further insight into their design and use. Although the importance of human-specific issues such as target-sequence specificity and cellular delivery need to be considered, zebrafish is a valuable model for rapidly testing the efficacy and reproducibility of these gene-product modification approaches.

7. Conclusion

The zebrafish system has a long history of providing insight into skeletal muscle development and function. The last couple years have generated an exciting flurry of activity using the zebrafish model for the discovery and analysis of pharmacological approaches to human muscle diseases. In particular, the large-scale drug screens using the zebrafish DMD model [54,55] have not only provided new insight into pharmacological therapies for DMD but also, for the future, provided a model with which to approach large-scale drug screens with zebrafish models for additional myopathies. The ability to take a variety of simple as well as sophisticated approaches to assess muscle structure and function in zebrafish has also recently allowed for novel insights into the pathological mechanisms of many muscle diseases. This insight gained in zebrafish can provide clues to novel, rational pharmacological approaches for muscle disease therapies, which, likewise, can then be directly tested in zebrafish.

8. Expert opinion

A major advantage of the zebrafish system for future pharmacological approaches for muscle diseases is in the study of combinatorial drug treatments. Because corticosteroids are now widely used for treating DMD patients, any new potential pharmacological therapies for DMD should be tested for efficacy with or interactions with corticosteroids [15]. The ability to easily obtain many animals and rapidly assess the effects of drug treatments makes zebrafish an ideal model for optimizing the timing and dosage of drugs alone and in combinations.

Because of the significance of addressing cardiac muscle function in DMD patients [13], it will be important to determine whether *dmd* zebrafish can serve as a model for cardiac muscle defects in addition to the skeletal muscle defects. There are not yet any reports of dystrophin expression in the zebrafish heart, nor have there been any reports of heart defects in *dmd* mutant zebrafish [95]. This lack of data may be due to zebrafish dystrophin being expressed or required in the heart at later stages than are typically examined.

As discussed earlier, most zebrafish models of human muscle diseases have come from forward genetic screens and through the use of morpholinos. Genome editing technology, in particular the CRISPR/Cas system [96], now opens up the possibility of engineering zebrafish models for human muscle diseases that have not been identified through forward genetics, such as *mtm1* for myotubular myopathy [95,97]. Another use of genome editing approaches could be to engineer zebrafish strains with muscle disease gene alleles that mimic the specific mutations found in humans [96]. With genome editing, zebrafish strains can be created that carry a spectrum of mutations within an individual gene, to reflect the diversity of human mutations that have been identified within certain muscle disease genes [1,2]. Such engineered zebrafish could be useful for testing muscle disease gene modification therapies such as exon skipping (see Section 6 Using zebrafish to study pharmacological approaches to muscle disease gene modification) and also would be valuable for in-depth cellular, structural and muscle functional analyses to better understand disease mechanisms. Engineered zebrafish strains can also be used in drug screens to more

directly screen for drugs that ameliorate the phenotypes caused by specific muscle disease gene mutations.

Even with the increasing development of genome editing technology for reverse genetics, antisense morpholinos can continue to provide important tools for zebrafish models of muscle disease. Morpholino knock-downs in zebrafish embryos can provide a rapid means to test new candidate genes for human muscle diseases or new modifier loci for existing muscle disease genes. Two of the main potential disadvantages of morpholinos, incomplete knock-down and off-target effects, can be alleviated through careful validation controls or by comparing the morpholino phenotype to that of a mutant allele, identified through forward genetics, TILLING [98], or genome editing. As described above (Section 3.2 Targeted drug studies), zebrafish muscle disease models generated using morpholinos can provide advantages over a mutant allele for drug screening because about 100% of morpholino-injected embryos should show a muscle disease phenotype, as opposed to only 25% for a genetic recessive mutant cross. Because the efficacy of morpholinos, typically injected at the one-cell stage, lessens as larvae get older, it is better to perform long-term (> 4 – 5 days) drug studies using mutant strains instead of morpholinos.

One outstanding opportunity for use of the zebrafish model is in the study of inflammation and the immune response in muscle disease. The inflammatory response plays an important role in normal muscle repair, but chronic inflammation contributes to the progressive pathology of muscle diseases such as DMD [99]. Understanding how, and when, different cell types of the immune system respond to, and influence, muscle degeneration and regeneration would provide insight for improved pharmacological treatment of muscle diseases with chronic inflammation. Zebrafish are a powerful system with which to address these issues. Zebrafish *dmd* larvae show signs of acute inflammatory response in their dystrophic muscle [50], and it would be informative to address whether and how drug treatments that ameliorate the zebrafish *dmd* phenotype affect this inflammatory response. The availability of many zebrafish transgenic strains for labeling immune cell types [100], combined with live imaging and drug treatment of muscle disease models, should allow elegant studies of how pharmacological approaches might influence inflammation and the immune response in muscle disease.

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Article highlights

- The zebrafish animal model offers many advantages for drug discovery and for understanding drug mechanisms of action for muscle disease therapies.
- Zebrafish has served as a vertebrate high-throughput screening platform for new drug therapies for Duchenne muscular dystrophy.
- Pharmacological studies in zebrafish muscle disease models have helped provide new insights into mechanisms of drug action, therapeutic targets and disease pathogenesis.
- Zebrafish is an exceptional animal model for the range of extremely simple to sophisticated approaches for assessing muscle structure and function.
- The use of new approaches, such as genome editing technologies, will further enhance the use of zebrafish for muscle disease drug discovery.

This box summarizes key points contained in the article.

Figure 1. Zebrafish offer many advantages as an animal model for muscle diseases

There are several ways to generate zebrafish muscle disease models, including using forward genetic screens and birefringence, injecting antisense morpholinos into zebrafish one-cell embryos to cause targeted gene knock-down, and injecting CRISPR/Cas9 mRNAs to induce targeted gene editing. Zebrafish muscle disease models, such as *dmd* mutants, can show disruptions in the muscle birefringence pattern (center figure). Zebrafish muscle disease models can be used in high-throughput drug screens to identify compounds that ameliorate the muscle disease phenotype, for *in vivo* muscle fiber imaging for muscle structure analyses, and for muscle function analyses, such as time-lapse tracking of larval movements in petri dishes.

DMD: Duchenne muscular dystrophy.

Figure 2. Example of a strategy for testing pharmacological rescue of a zebrafish muscle disease model

In this example, embryos are collected from *dmd*^{+/-} parents and raised in a water bath in petri dishes. Drugs, and vehicle control such as dimethyl sulfoxide (DMSO) can be added to the water bath at any time, typically at 1 day post-fertilization [54,55,59,62]. Following incubation, larvae can be scored for muscle lesions using birefringence (shown in low magnification views; [39,54]) or muscle stains such as phalloidin (larval trunk myotomes shown in higher magnification views; [62,73]). At 4 days post-fertilization, about 25% of larvae from DMSO-control treated *dmd*^{+/-} crosses show disruptions in the muscle fiber pattern, whereas lower frequencies of larvae with muscle defects appear upon treatment with drugs that improve the *dmd* phenotype (Table 1). Arrows point to larvae with disrupted birefringence pattern. Phalloidin images were previously published in [62] and appear with permission of PLOS Currents.

DMD: Duchenne muscular dystrophy.

Table 1

Zebrafish muscle disease models with drug therapies studied in zebrafish.

Zebrafish gene	Protein product	Human disease model	Type of zebrafish model treated	Drug therapy	Ref.
<i>ache</i>	Acetylcholinesterase	Unknown	Loss of function mutant	Eserine	[52]
<i>col6a1</i>	Collagen VI	Ullrich congenital muscular dystrophy, Bethlem myopathy	Morpholino	Cyclosporine A	[68]
<i>dag1</i>	Dystroglycan 1	Limb-girdle muscular dystrophy	Morpholino	Nicotinamide Adenine Dinucleotide (NAD+), Emergen-C	[73]
<i>dmd</i>	Dystrophin	Duchenne muscular dystrophy, Becker muscular dystrophy	Two loss of function mutants, Morpholino	15 chemicals from screens	[54,54]
			Loss of function mutant	MG132	[59]
			Loss of function mutant, Morpholino	Trichostatin A	[62]
			Loss of function mutant	Splice-altering morpholinos	[88]
<i>dnm2</i>	Dynamin 2	Centronuclear myopathy, dominant	Loss of function mutant	Ataluren	[94]
			Overexpression of human <i>DNM2</i> mutant RNA	Edrophonium	[76]
			Morpholino	NAD+, Emergen-C	[73]
			Overexpression of mutant <i>lmna</i>	Farnesyltransferase inhibitor L-744832	[101]
<i>lma</i>	Lamin A	Multiple types of muscular dystrophy	Overexpression of mutant <i>lmna</i>	Farnesyltransferase inhibitor L-744832	[101]
<i>ryr1b</i>	Ryanodine receptor 1 (skeletal)	RYR1-related congenital myopathies	Loss of function mutant	N-acetylcysteine	[71]