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Review

Modeling human muscle disease in zebrafish☆

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Abstract

Zebrafish reproduce in large quantities, grow rapidly, and are transparent early in development. For these reasons, zebrafish have been used extensively to model vertebrate development and disease. Like mammals, zebrafish express dystrophin and many of its associated proteins early in development and these proteins have been shown to be vital for zebrafish muscle stability. In dystrophin-null zebrafish, muscle degeneration becomes apparent as early as 3 days post-fertilization (dpf) making the zebrafish an excellent organism for large-scale screens to identify other genes involved in the disease process or drugs capable of correcting the disease phenotype. Being transparent, developing zebrafish are also an ideal experimental model for monitoring the fate of labeled transplanted cells. Although zebrafish dystrophy models are not meant to replace existing mammalian models of disease, experiments requiring large numbers of animals may be best performed in zebrafish. Results garnered from using this model could lead to a better understanding of the pathogenesis of the muscular dystrophies and the development of future therapies.

Keywords: Zebrafish; Muscular dystrophy; Dystrophin; Animal models; Therapy

1. Introduction

Muscular dystrophy is a progressive muscle degenerative disease in which the muscle forms normally, but degenerates faster than it can be repaired. Mutations in the dystrophin gene have been shown to cause Duchenne Muscular Dystrophy and the less severe Becker Muscular Dystrophy, the most common forms of the disease [1]. Interestingly, dystrophin was one of the first human disease genes discovered through positional cloning [2–4]. The human dystrophin gene encodes a large protein that localizes to the intracellular portion of the muscle sarcolemmal membrane [5–7]. Further analysis showed that dystrophin is part of a large membrane-bound complex called the dystrophin associated protein complex (DAPC) [8–10]. Mutations in many of the DAPC proteins have since been shown to cause different forms of muscular dystrophy, suggesting that this complex is important for maintaining muscle integrity [11–15]. Linkage analysis has been used in human patient samples to show that mutations in non-DAPC genes can also cause muscular dystrophy (reviewed in [16]).

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disorder presenting in approximately 1 in 3500 live male births. DMD is caused by mutations in the dystrophin gene that typically result in the near complete loss of the protein at the sarcolemmal membrane. Loss of dystrophin expression destabilizes the entire DAPC, likely weakening the sarcolemma to allow local perforation and calcium influxes, and potentially disrupting DAPC-associated signaling pathways (reviewed in [17]). DMD patients first show symptoms between 1 and 5 years of age, beginning with weakening of the proximal muscles and quickly extending to distal muscles, particularly the legs. Eventually, the disease affects almost all voluntary muscles and patients may also present with cardiac involvement to varying degrees.

The limb-girdle muscular dystrophies show a similar pattern of affected muscle, but are inherited in either

☆ Due to space limitations, many references were not included. We sincerely apologize to the authors whose work was not cited.

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autosomal dominant (LGMD 1A–G) or autosomal recessive (LGMD 2A–K) inheritance modes. In addition, LGMDs can vary in age of onset, rate of progression, and presence of accompanying mental retardation. Several other muscular dystrophies have been identified, including congenital MDs with frequent central nervous system involvement and far less progressive weakening, as well as distal myopathies which show more limited muscle group participation.

Mutations in over 25 genes have been shown to be the underlying basis of many of the muscular dystrophies (reviewed in [16]). In addition, there remain at least 5 forms of muscular dystrophy for which the causative mutant gene has yet to be identified suggesting that additional dystrophy genes remain unknown. The known genes encode proteins which position to four main cellular compartments in muscle cells, including the nucleus, the Golgi apparatus, the sarcomere, and the sarcolemmal membrane. The fact that mutations in seemingly unrelated proteins positioned in various cellular compartments can all cause clinically similar forms of disease suggests that additional adaptor molecules remain to be identified. Given the likely importance of these adaptor molecules, it is also possible that mutations in these genes could be lethal in mammals. For this and other reasons, many laboratories have resorted to using animal models like zebrafish in which all developmental stages can be easily assayed (reviewed in [18]).

2. Animal models for muscular dystrophy

2.1. Current animal models

There are many well established animal models for muscular dystrophy (reviewed in [19]). Animal models are frequently used to model human disease because their environment and reproduction can be controlled, their generation times are typically short, and they can be easily used to evaluate the effectiveness of potential therapies. Between different animal species, these advantages can vary widely such that specific animals are often selected to address specific questions.

While there are many vertebrate models of muscular dystrophy, the most commonly used is the *mdx* mouse [20]. This mouse carries a nonsense mutation in exon 23 of the dystrophin gene and is predominantly dystrophin-null [20]. However, exon 23 is prone to skipping and therefore the number of revertant muscle fibers in this model can be a concern. Revertant fibers can successfully circumvent the mutation and express a modified form of dystrophin. While the *mdx* mutant mouse was isolated naturally, other mouse models were isolated as part of a genetic screen [21,22]. The mdx^{5cv} mouse has a point mutation in exon 10 affecting splicing and shows fewer revertant fibers than the *mdx* mouse [22]. The resulting phenotype in the mdx^{5cv} mouse is more severe than the *mdx* mouse, potentially making the mdx^{5cv} mutant a better model for evaluating the effectiveness of different therapies.

While the mouse is the most commonly used dystrophy model, dog models of muscular dystrophy are thought to have a phenotype more similar to that of humans [23]. For example, the muscle in the *mdx* dystrophin-null mouse undergoes less degeneration and regeneration than the muscle of dystrophin-null humans or dogs. While there are obvious limitations for maintaining large animal models, dogs can serve as excellent models for evaluating specific therapies (reviewed in [24,25]).

Currently, many potential therapies are evaluated by introducing fluorescently tagged proteins or cells into a diseased animal and then following their fate using fluorescence to determine if the introduced proteins or cells localize to muscle and participate in the repair process. While systems are available for monitoring transplanted cells in living mouse models, these systems can be relatively expensive and/or difficult to get resolution high enough to identify single cells in the host animal. As such, expression of the introduced protein is often evaluated by euthanizing the animal several months after the therapeutic intervention and sectioning the muscle to determine whether any of the muscle fibers are marked as arising from the donor cells. Since the assay is terminal, the effectiveness of the therapy is evaluated at a single time-point which can make it difficult to deduce exactly how the transplanted cells produced the resultant phenotype.

2.2. Advantages and disadvantages using zebrafish models of disease

Some of the obvious advantages for using zebrafish as a disease model include its small size (about 1.5 in. full grown), rapid *ex-utero* development, optical clarity of the embryos and early larvae, high reproductive capacity (a single female may produce up to 300 eggs once every 5–7 days), and short generation time (3 months). In addition, there is strong similarity between the zebrafish and human genomes with significant degrees of synteny between conserved genes [26] (see also http://www.sanger.ac.uk/Projects/D_rerio/ and http://genome.ucsc.edu/cgi-bin/hgGateway). In addition, orthologous genes in both organisms have been shown to regulate similar developmental processes such as T cell development (reviewed in [27]). All of these traits make the zebrafish an excellent model for examining vertebrate development, and for performing genetic screens requiring large numbers of animals (reviewed in [28]). In addition, zebrafish are ideally suited for performing real-time analysis to assay the fate of transplanted cells. Cells labeled with GFP can be transplanted into transparent early zebrafish embryos and the fate of the transplanted cells can be monitored real-time without harm to the animal (see Section 5.4).

While zebrafish can be an ideal model for vertebrate development (perspective in [29]), there are some intrinsic disadvantages to the system. Because zebrafish are evolutionarily more distant from humans than mammalian dystrophy models, findings from fish experiments will likely have to be replicated in mammals before being directly correlated to human therapy. However, the fact that zebrafish express many of the same dystrophy associated proteins as humans and that mutations in these proteins can cause phenotypes similar to those seen in human patients suggests that findings from fish experiments are likely to be transferable to mammals (see below).

While it is relatively easy to generate transgenic fish, it has been problematic to generate zebrafish with specific mutations, although procedures for performing homologous recombination in zebrafish ES cells have recently been reported [30]. In addition, the zebrafish genome underwent an additional duplication event after the fish and mammalian lineages diverged [31,32] such that, in some instances, the zebrafish can be polyploid for specific genes. Since many zebrafish mutants have been genetically isolated over the last 20 years using traditional diploid screens, it is thought that many of the duplicated genes have either been inactivated or have divided function between the duplicated genes. Other disadvantages include a limited number of cross-reacting antibodies and the modest costs associated with starting a zebrafish facility. Despite these limitations, the advantages for establishing zebrafish models of human disease can far outweigh the costs such that the simultaneous use of both fish and mouse models would be faster and more economical than relying solely on mammalian models.

3. Zebrafish muscle development

3.1. Early muscle development

Over the years, zebrafish have been used as an outstanding developmental model. With regards to muscle, zebrafish have been utilized to distinguish which progenitor cells are destined to differentiate into different muscle fiber types since fast and slow muscle fibers position to different parts of the developing fish. Fish slow muscle is generally found just underneath the skin whereas the fast muscle is located more internally [33,34]. In mammals, slow and fast muscle fibers are intermingled. In both fish and mammals, fast muscle fibers function anaerobically and are used for short powerful bursts whereas slow muscle fibers function aerobically and are used for sustained activity.

During zebrafish development, slow muscle originates from adaxial cells which migrate from the presomitic mesoderm through the developing myotome (reviewed in [35–37]). Adaxial cells have been shown to express early myogenic markers like *MyoD* [38] and *Myf5* [39], and their migration through the myotome has been shown to be dependant on the expression of M- and N-cadherin [40]. Adaxial cells were named due to their initial location near the axial mesodermal cells that later derive the notochord. The position of the adaxial cells suggested that hedgehog factors, which are notochord derived signaling factors, could direct the fate of adaxial cells (reviewed in [41]). The analysis of the zebrafish mutant *sonic-you* showed that mutations in the sonic hedgehog signaling pathway could adversely effect muscle development [42]. In slow muscle mutants, the smoothened protein was found to be critical for hedgehog signaling and the development of slow muscle [43,44]. Interestingly, the differentiation of certain fast muscle fibers in zebrafish has also been shown to be indirectly regulated by the same hedgehog signaling pathways. Henry et al. recently showed that migrating cells transiting the myotome to generate the superficial slow muscle induce a wave of fast fiber differentiation in the cells through which they migrate [45]. Fast muscle differentiation has also been shown to be driven by Fgf8 expression [46].

3.2. Muscle development in adult zebrafish

While many developmental pathways are well established in mammals, less is known about adult muscle development and maintenance. It has been established that satellite cells are the main muscle precursor cell in adults. Muscle satellite cells are committed mononuclear muscle precursors that position to the inside of the basal lamina in adult muscle tissue [47]. When signaled, they can divide to generate cells capable of contributing to myofiber repair (reviewed in [48]). Experiments in mice and chicks have shown that adult muscle satellite cells are originally derived from the dermomyotome of the developing embryo [49,50]. Specifically, skeletal muscle satellite cells in the limb [50] and trunk [51] have been shown to develop from Pax3 expressing cells. Pax3 is a paralogue of Pax7, an early marker of muscle satellite cells [52,53]. In adult muscle, it is possible that satellite cells could also originate from other less committed cells within muscle [54,55] or blood [56,57]. There are significant therapeutic ramifications associated with the positive identification of these cells as they could theoretically be modified and transplanted into patients for cell therapy to treat muscular dystrophy.

While it is unknown exactly how muscle regenerates in adult zebrafish, satellite cells have been identified in other fish species, including Atlantic salmon [58,59]. Although salmon are clearly different than zebrafish, the identification of c-met (a satellite cell marker) positive mononuclear cells within muscle suggests that muscle development in other fish is likely to be conserved [58]. Unlike mammals, zebrafish retain their somitic structure throughout development and each muscle fiber stretches from one myosepta to the next. This difference suggests that satellite cells in zebrafish could position anywhere in the somite and still enable fusion-competent cells access to existing myofibers. As in mammals, zebrafish blastulae or adult muscle mononuclear cell cultures can differentiate into myofibers in culture indicating the presence of muscle precursor cells ([60], Kunkel, unpublished data). Like mammals, fully developed zebrafish muscle fibers are multinucleated, although muscle striation can occur in certain muscle fibers before fusion in developing zebrafish [61,62].

4. Muscular dystrophy in zebrafish

4.1. Isolation of zebrafish muscle mutants

One of the early zebrafish mutational screens was published in 1990 and described the isolation of the muscle mutant *fub-1* (fibrils unbundled) [63]. Gamma ray irradiation was used as the mutagen and, in a screen of 225 fish, two different *fub-1* alleles were identified. This mutant showed disorganized muscle fibers early in development [64], although the causative mutation has yet to be identified.

In 1996, as part of a large genetic screening effort in Tuebingen, Germany, almost 3,000 zebrafish mutant families

were screened and grouped into various categories [65]. One of the categories included 166 motility mutants which were further subgrouped into 14 phenotypically distinct groups [66,67]. From this categorization, 4 unique alleles and 5 unresolved alleles were found to exhibit normal muscle at 2 dpf, but decreased muscle organization by 5 dpf [67]. Muscle disorganization was assayed using birefringence (Fig. 1A), a technique used in the initial characterization of muscle [68]. Birefringence is a phenomenon in which the highly ordered somitic muscle has the unique property of being able to rotate polarized light. This can be easily monitored by placing muscle (or a whole fish) between two polarizing filters and aligning the filters until only the rotated light is visible. Using this assay, a decrease in the amount of rotated light could be indicative of the loss of the sarcomeric structure within muscle whereas dark patches in the muscle could be indicative of muscle tearing or muscle fiber disorganization.

4.2. Using zebrafish to understand the pathogenesis of muscular dystrophy

Since the muscle in one particular Tuebingen subgroup of fish appeared to develop normally and then degenerate, these mutants were labeled as potentially dystrophic [67]. It was not until 2003 that the first of these dystrophic mutants was characterized [69]. By mapping the mutation and using a candidate gene approach, Bassett et al. found that the *sapje* mutant contained a nonsense mutation in exon 4 of the zebrafish dystrophin gene [69]. They demonstrated that the muscle in the *sapje* fish was degenerating due to the failure of the somitic muscle attachments at the embryonic myotendinous junctions (reviewed in [70]).

Earlier work showed by immunohistochemistry that zebrafish express dystrophin at the sarcolemmal membrane in adult zebrafish [71,72]. Bolanos-Jimenez et al. used *in situ* hybridization to localize dystrophin expression in developing embryos to the transverse myosepta [73] and mapped the dystrophin gene to linkage group 1 [74]. Using short interfering RNA mediated gene targeting, Dodd et al. inhibited dystrophin expression and showed that the developing embryos had disorganized sarcomeres and body defects [75]. When morpholinos were used to inhibit dystrophin translation in developing embryos, the zebrafish were inactive at 5 dpf and a fraction of them were bent [71]. Western blot and immunohistochemistry analysis of the dystrophin morphants confirmed that dystrophin was down-regulated, as were other members of the dystrophin associated protein complex [71]. This is similar to what happens in mammals with mutations in dystrophin [76] and suggests that the biochemistry of the zebrafish dystrophin associated protein complex is similar to that of humans. In addition, when δ-sarcoglycan (a member of the muscle DAPC) was down-regulated in early embryos, the sarcoglycan subcomplex was destabilized, but dystrophin expression was normal [77]. Again, this is exactly as occurs in patients with Limb-Girdle Muscle Dystrophy type 2F due to mutations in δ-sarcoglycan [14] suggesting that the overall biochemical nature of the complex is conserved.

When the dystrophin morphant was compared to the *sapje* mutant, both fish were found to be inactive with somitic lesions [69], most had a delay in swim bladder inflation (Fig. 1B), and

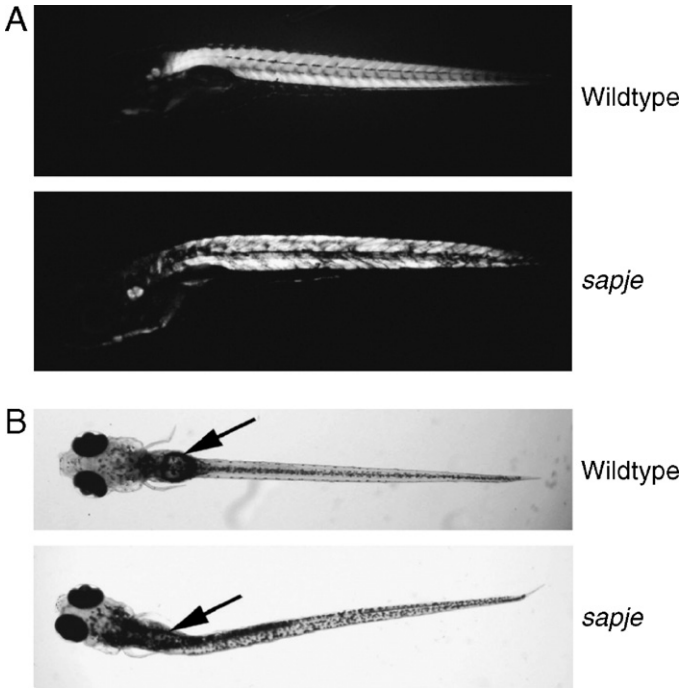

Fig. 1. *Sapje* mutants show decreased birefringence, inactivity and bending at 7 dpf. (A) As reported previously [84], the zebrafish dystrophin-null mutant (*sapje*) shows patchy skeletal muscle birefringence. Birefringence is assayed by placing the zebrafish between two polarizing filters rotated 90 degrees relative to each other and measuring the light rotated by the muscle. (B) 7 dpf *sapje* mutants are relatively inactive, have an un-inflated their swim bladder, and adopt an energy efficient bent conformation on the bottom of the Petri dish. The location of the swim bladder is indicated with an arrow.

many were temporarily bent (Fig. 1B). In contrast to the *sapje* mutant, the dystrophin morphants showed a less severe birefringence phenotype [69]; however, this may be because the effectiveness of the morpholino was near its limit at 5 dpf when the defects in birefringence were most apparent in the s*apje* mutant.

Dystroglycan was one of the first members of the dystrophin associated protein complex that was investigated in zebrafish [78]. When dystroglycan was down-regulated using morpholinos to inhibit translation of the protein, the morphant embryos were developmentally delayed, had a hooked tail, and showed compromised muscle integrity [78]. While dystroglycan-null mice die during development due to defects in the Reichert's membrane [79], zebrafish develop outside the mother and therefore dystroglycan-null fish developed and provided novel insight into dystroglycan's role in vertebrate muscle development. Morpholino experiments have also been used to down-regulate δ-sarcoglycan, laminin α2, and caveolin-3 [77,80–82], all genes in which mutations in the mammalian orthologue have been linked to muscular dystrophy. In all instances, down-regulation of these proteins early in development results in fish with decreased muscle organization [77,80–82].

Interestingly, zebrafish that lack proteins associated with muscular dystrophy show a phenotype relatively earlier in development than humans. For example, the *sapje* mutant shows muscle degeneration as early as 3 dpf [67] whereas Duchenne patients normally do not normally show symptoms of disease until they are 3–4 years old. This might be explained since utrophin, a dystrophin homologue than can substitute for dystrophin early in mammalian development [83], is not expressed in early zebrafish muscle fiber ends like dystrophin [84]. As such, it is likely that zebrafish lacking functional dystrophin will show a severe phenotype more similar to mice lacking both dystrophin and utrophin [85].

As mentioned previously, a number of dystrophic fish were isolated as part of the first Tuebingen screen [67]. To date, only one of them has been characterized and this fish was shown to have a mutation in dystrophin [84]. The characterization of this family of mutants and mutants isolated from other genetic screens will be very useful to uncover additional genes involved in the pathogenesis of muscular dystrophy. In addition to identifying additional dystrophy associated genes, dystrophic zebrafish mutants can also be used to evaluate future therapies.

5. Using zebrafish to evaluate therapies for muscular dystrophy

5.1. Background

While significant inroads are being made to treat muscular dystrophy, there are currently no curative treatment options for those afflicted with the disease. At this time, patients diagnosed with muscular dystrophy are treated with various catabolic steroids such as prednisone and deflazacort [86]. Although the mechanism(s) by which these drugs work is not completely clear, studies have shown that these glucocorticoids might mitigate symptoms associated with muscle degeneration, stimulate muscle repair, and/or inhibit muscle degeneration [87–89]. Other treatments currently being tested or considered, include gentamycin treatment (which encourages read-through of nonsense mutations) [90], myostatin down-regulation (encourage muscle development by down-regulating myostatin) [91,92], and drug therapy using catabolic steroids (like prednisone) or anabolic steroids (like oxandrolone) [93–95].

As with many genetic disorders, there are two main ways to potentially treat the muscular dystrophies. First, drug therapy can be used to either mitigate the symptoms of disease (indirect) or supply the missing chemical that the cell needs (direct). Second, protein therapy can be used to supply the missing gene or protein using either various DNA vectors (gene therapy) or by transplanting whole cells (cell therapy). All therapies have the potential to correct the symptoms of disease although each has its inherent disadvantages. For example, it can be difficult to identify potential drugs from libraries of millions of different chemicals. Currently, drugs are often identified based on positive effects found for one specific cell population, but these same drugs can often have deleterious effects on other "non-target" cells. Gene therapy can be associated with both an adverse immune response to the foreign delivery vectors and also the inherent problems associated with random genome integration events (reviewed in [96]). Cell therapy can be used to circumvent both the immune and integration problems associated with gene therapy, yet tests in dystrophic mice have found it difficult to deliver therapeutic levels of protein to the diseased cells ([97,98], reviewed in [99]).

5.2. Drug screens in zebrafish

As drug discovery using mammals can be very expensive, mammalian disease models are normally only used to test limited numbers of compounds, typically those that are first screened using cell culture. While it is more efficient to test larger numbers of compounds in cell culture, this is an artificial environment in which the cells may respond very differently than in a living organism. These problems can be circumvented in zebrafish as they are small and reproduce in large enough numbers such that they can be efficiently used to assay for therapeutic changes in the context of a living disease model. To illustrate, the muscle degeneration phenotype in the dystrophin-null *sapje* mutant zebrafish is transmitted in a recessive manner such that 25% of the offspring become dystrophic after 3 dpf. As such, in a clutch of 300 offspring, 75 would show phenotype. By crossing many fish, it is possible to quickly produce large numbers of mutant offspring that can then be arrayed on plates and exposed to different chemicals in their water. Chemicals which diffuse into the dystrophic mutant and mitigate the symptoms of disease would be considered for potential treatments.

To date, a number of chemical/drug screens have been published using zebrafish embryos (reviewed in [100]). These screens have demonstrated the ability to isolate small molecules capable of altering wildtype embryonic zebrafish development of several organ systems, including the central nervous system, cardiovascular system, eye, and ear. The most recent two

screens, however, use zebrafish mutants modeling human diseases to identify chemical suppressors of disease phenotypes. Peterson et al. employed the hey2 gene mutant, *gridlock* (*grl*), which presents with aorta malformations similar to human aortic coarctation [101]. Exposure of mutant embryos to dissolved chemical compounds in 96-well plates identified a class of chemicals that upregulates the angiogenic gene VEGF, suppressing the *grl* phenotype. Stern et al. recently showed the suppression of a cell-cycle mutant, *crash&burn* (*crb*), which carries a bymb mutation [102]. Roughly 16,000 chemical compounds were screened in 16 weeks using *crb* mutants, allowing for the identification of a novel cell-cycle regulating drug. These results demonstrate the feasibility of chemical screens to ameliorate disease phenotypes using zebrafish models.

In mammals, mutations that affect splicing, translation, or transcription have all been shown to cause different forms of muscular dystrophy. Therefore, different drugs may have different effects based on the origin of the causative mutation. Establishing representative zebrafish models that have muscular dystrophy due to mutations in different genes is therefore just as important as establishing zebrafish models with different mutations in the same gene. This is similar to having multiple mouse models with different mutations in dystrophin (see discussion regarding *mdx* versus mdx^{5CV} mouse models). Established zebrafish disease models would then be ideally suited for use in chemical screens to select drug candidates capable of correcting the phenotype.

5.3. Gene therapy approaches for treating muscular dystrophy

Gene therapy holds significant promise for treating people with genetic disorders like muscular dystrophy. The goal would be to introduce a corrective gene into muscle to either address the cause or treat the symptoms of disease. While gene therapy holds great promise, there are a number of technical issues that are still being addressed. For instance, current limitations include the insertion size limit of the delivery vector, the immune response associated with the delivery vector and/or the newly expressed wild-type gene, random integration events for certain vectors that could disrupt the expression of normal genes, and the vast numbers of skeletal, smooth, and cardiac muscle cells that would have to be "corrected".

Gene therapy can either be direct (addressing the cause of disease) or indirect (addressing the symptoms of disease). While zebrafish have not yet specifically been used to investigate gene therapy approaches to treat muscular dystrophy, there are a number of potential strategies that could be helpful using zebrafish as disease models. For instance, dystrophin is a large gene and extensive work has been done in mice to identify a smaller form of the gene that could fit into viral delivery vectors [103,104]. This work could be continued in zebrafish mutants or morphants to either (1) further delimit the functional size of large genes like dystrophin or (2) to perform domain analysis to study protein function. Similarly, zebrafish can be used to discover whether DNA anomalies identified in humans are disease causative or simply random polymorphisms. In all cases, these experiments can be performed by injecting specific morphants or mutants with mRNA constructs (human or zebrafish) in an attempt to rescue the early fish dystrophic phenotype. This approach has been used recently to confirm that a mutation in TRIM32 was causative for Bardet–Biedl syndrome [105]. In this instance, human TRIM32 mRNA with a Bardet–Biedl syndrome causing mutation failed to complement TRIM32 morphants which showed a disruption in the Kupffer's vesicle and a delay of intracellular transport. Importantly, a control human mRNA co-injected in a separate experiment could rescue the phenotype [105].

In a recent review, Engvall and Wewer described the utility of modifying the expression of booster genes for treating muscular dystrophy [106]. These are genes that are not mutated in patients with muscular dystrophy but whose expression can address the symptoms of disease. For example, integrin $\alpha7\beta1$ and the DAPC are the two major cell adhesion complexes found in muscle [107]. In patients with Duchenne Muscular Dystrophy, the DAPC is lost and it has been hypothesized that upregulating the integrin $\alpha7\beta1$ complex could help stabilize the muscle cell membrane. In testing this hypothesis, Burkin et al. showed that over-expression of integrin $\alpha7$ in *mdx/utr* deficient mice helps prolong the lifespan and ameliorates their dystrophy symptoms [107,108]. Due to the ease of making transgenic zebrafish or of transiently expressing the protein using mRNA injection, zebrafish are an ideal platform for testing hypotheses for treating muscular dystrophy via altered expression of booster genes.

While over-expressing integrin $\alpha7$, GalNAc transferase, NOS, Adam12, and calpastatin have been shown to mitigate dystrophy symptoms in mice (reviewed in [106]), down-regulation of negative regulators of muscle differentiation like myostatin can have the same effect (reviewed in [109]). Myostatin is a secreted factor with sequence similarity to the transforming growth factor-beta subfamily of proteins. Mutations in myostatin in mice and cattle cause these animals to have increased muscle mass [110,111], although this can also be associated with negative health effects such as increased mortality [112]. Down-regulation of myostatin in the *mdx* mouse can help mitigate the symptoms of muscular dystrophy [91,92] suggesting that decreased expression of myostatin might be a viable therapy to treat people with muscle disease, especially those in which muscle degeneration is a secondary effect. Zebrafish also express myostatin and may have two different forms of the gene [113,114]. Using zebrafish models, it should be possible to down-regulate one or both forms of the gene in an attempt to rescue phenotype in *sapje* or one of the other dystrophic mutants. By performing these experiments in an animal model, it is possible to assay effects (positive or negative) of gene expression in all tissues.

5.4. Evaluating cell therapy in zebrafish models

Muscle is a normally regenerating tissue with satellite cells providing an immediate precursor cell population. Upon activation, satellite cells are able to divide and expand both *in vitro* and *in vivo*, giving rise to later stage precursors and new

muscle myofibers (reviewed in [115]). Following the cloning of the dystrophin gene, a cell-based approach was proposed for delivery of normal dystrophin to diseased muscle [116,117]. Culture-expanded myogenic cells derived from normal muscle were shown to fuse into *mdx* mouse muscle and produce normal dystrophin [116,118]. These initially promising mouse studies led to human trials in Duchenne Muscular Dystrophy patients (reviewed in [119]). Unlike the mouse studies though, the human experiments showed little, if any, donor-derived dystrophin expression. This led in the late 1990s to a rethinking of how the cells should be isolated, expanded and delivered to correct the symptoms of disease.

It has become clear that there exist cells within muscle likely to be more primitive than satellite cells [98,120–122]. These putative muscle stem cells have been purified from mouse skeletal muscle using different methods and techniques, including pre-plating [117,122,123], cell sorting [55,98,120,124,125] and fractionation based on specific antigens [126]. Although progenitor cells can be enriched using the above purifications, selected cell populations are still heterogeneous suggesting that additional purifications could help further enrich for cells with muscle engraftment capability.

Zebrafish are well suited for testing cell transplantation potentials at any developmental stage. Early in development, the animals are optically clear, allowing for tracking the fate of transplanted fluorescently labeled cells [127]. Later in development, as the fish become more pigmented, they become less transparent and develop a fully functional immune system. In this case, pigment mutants which retain their optical clarity throughout development can be used to facilitate *in vivo* tracking of fluorescently labeled transplanted cells at all development stages. In addition, the immune system can be suppressed through temporary exposure to irradiation. If necessary, Traver et al. has also shown that the immune system can be reconstituted in lethally irradiated zebrafish by transplanting hematopoietic cells [128], almost exactly as in mice.

Procedures for cell transplantation at all stages of zebrafish development are well-established (reviewed in [129]). Since zebrafish can be grown and irradiated in large numbers, it is possible to prepare large numbers of animals for analysis very quickly. In addition, since zebrafish are smaller than mice, it is possible to transplant smaller quantities of cells for analysis. While any labeled cell can be used, transgenic zebrafish expressing GFP under different promoters have been created [130]. Zebrafish muscle cells can be fractionated and injected into hosts to assay their ability to engraft into muscle. Using this assay, cells can be sequentially fractionated using many sorting methods such as rhodamine 123 staining, alcohol dehydrogenase fractionation, forward and side scatter FACS analysis and preplating before assaying for muscle engraftment potential. Purified muscle progenitor cells can then be characterized by available microarray probe sets to identify cell-specific markers that can then be used to isolate similar cell populations from mammals.

To assay muscle engraftment potential in zebrafish, cells can be efficiently transplanted directly into dechorinated blastulae (500 cell stage), injected directly into the circulation of a 48 h post-fertilization (hpf) embryo, or injected into the muscle of an adult zebrafish (reviewed in [129]). In each case, 100 transplantations can be easily performed in an afternoon. After transplantation, the fate of the injected GFP+ cells can be assayed by anesthetizing the hosts and monitoring cell fate under a fluorescent dissecting scope. After analysis, the fish can be revived and reanalyzed at a later date. In our experience, muscle engraftment can be assayed as early as 3–4 days after transplantation, although 2 weeks is more optimal (Kunkel laboratory, unpublished data). These advantages make the zebrafish an ideal model to screen cell populations for muscle engraftment potential.

6. Conclusion

Muscular dystrophies are a group of genetic diseases that vary significantly in age of onset, severity of the disease, rate of progression, modes of inheritance, and pattern of affected muscle. More than 25 human genes have been identified that when mutated, lead to various dystrophies; however, the underlying pathways that result in shared dystrophic muscle pathology are not completely understood. Studies suggest that the small vertebrate zebrafish *Danio rerio* have muscle structure and development similar to that of mammals, and that the majority of muscle-related genes tested are present in zebrafish. Genetic screens in zebrafish have already provided a model for the most common human dystrophy, DMD. We propose that additional dystrophic mutants will provide models for other dystrophies, or may uncover additional dystrophy-associated genes that will further our understanding of the pathology of muscular dystrophy. In addition, the use of zebrafish will allow us to analyze several different therapeutic methods on a time scale not possible in mammals. The ability to easily transplant labeled cells into zebrafish and analyze the results will enable rapid screening of many cell populations to help identify cells with the greatest engraftment potential for cell-based therapy. While the activity of analogous cell populations will have to be verified in mice, the screening capabilities of zebrafish make the fish an attractive model. The ability to quickly generate large numbers of tiny dystrophic zebrafish also makes this animal ideal for screening chemical libraries to identify new pharmacological agents for the treatment of dystrophy. While the fish will never completely replace other animal models of muscular dystrophy, the many advantages of the zebrafish model make it an effective complement to established mammalian systems helping to provide further insight into specific questions related to muscular dystrophy.

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