The zebrafish as a model for muscular dystrophy and congenital myopathy

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The muscular dystrophies and congenital myopathies are inherited diseases of the skeletal muscle, which lead to a loss of muscle function and are often fatal. While many of the loci involved are already known, these conditions remain incurable, and genetic models are being developed in an effort to understand the pathological mechanisms involved. Recently, several papers have shown that the zebrafish, which is now widely used in developmental genetic studies, will provide a useful addition to our toolkit in this regard. Here we describe these studies, including a zebrafish model of what is potentially the novel pathological mechanism of muscle attachment failure in Duchenne and other muscular dystrophies.

INTRODUCTION

Muscle tissue can be subject to a tremendous variety of diseases, both inherited, such as the muscular dystrophies, in which cycles of de- and re-generation destroy skeletal muscles, and acquired, such as the cardiomyopathies in which abnormal growth of cardiac muscle compromises heart function. In order to develop the necessary preventative strategies and treatments for conditions like these, it is likely to be of great benefit to understand their pathology at the level of the genes and cellular processes affected. Molecular genetic studies, focussing on developmental processes are currently providing a torrent of such information, and during the last decade the zebrafish, Danio rerio, has come to rank alongside the nematode Caenorhabditis elegans, the fruit fly, the frog Xenopus laevis, and the mouse, as a model system of choice in the burgeoning field of developmental genetics. It shares with invertebrate models the advantage of quickly producing high numbers of externally developing, diploid embryos, making large-scale random mutagenesis screens a feasible, almost routine, undertaking. It also conforms to the same fundamental body plan as humans, containing an equivalent to nearly every human organ, cell and molecular pathway, allowing the examination of gene function in a context likely to produce data applicable to human development and disease. This formidable combination of ‘forward’ genetics, which trawls a genome for new gene functions, wherever they may reside, and ‘reverse’ genetics, which entails functional studies of known genes including disease loci, is now being employed in the study of genetic diseases of skeletal muscle. Here we concentrate on a recent series of papers have begun to employ the zebrafish in the study of muscular dystrophies of the types caused by disruption of the dystrophin-associated protein complex (DAPC).

MUSCLE DEVELOPMENT IN THE ZEBRAFISH

Zebrafish embryos are particularly suited to the study of muscle development for several reasons, most importantly they develop externally, are transparent, somitic muscle comprises a large proportion of the body and is accessible, and they begin to move very soon after gastrulation. Both embryological and genetic studies have taken advantage these qualities to examine early stages of muscle development in the zebrafish with great success, such as the specification of slow-twitch muscle fibres (1). However, the later stages of development have been relatively little studied beyond the initial identification of mutations that affect muscle fibre differentiation, function and integrity.

Somitic muscle in fish forms initially from segmented paraxial mesoderm, with the posterior compartment of each somite expressing myogenic basic–helix–loop–helix transcription factors from the end of gastrulation. Muscle fibres differentiate to span an entire somite in the anterior–posterior axis. The first somitic muscle cells to differentiate are slow-twitch fibres that are specified medially by midline-derived hedgehog family signalling proteins, differentiate by 16 h

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post-fertilisation (hpf) and subsequently migrate to the lateral periphery of the somites (reviewed in 2). The bulk of the somite develops as fast-twitch muscle fibres once this migration has occurred. The somite adopts its distinctive chevron shape early on, by 24 hpf, with the dorsal and ventral halves being separated by a sheet of matrix material called the horizontal myoseptum and each pair of adjacent somites being separated by a sheet of matrix called the vertical myoseptum (see Fig. 1 for a schematic representation). These myosepta, along with the notochord (a primitive stiffening rod common to all chordates) serve as the attachment sites for somitic muscle fibres. These structures are essentially laminar tendons that transmit force to the notochord, and later to the vertebral column. These muscle attachment sites have now come under the spotlight with the finding that their mechanical failure is the pathological mechanism in a zebrafish mutation that provides the first zebrafish model of an inherited disease of skeletal muscle (3).

THE DAPC IN HUMAN DISEASE AND MOUSE MODELS

The DAPC is a multi-protein assembly that provides a transmembrane link between the cytoskeleton and the extracellular matrix. This complex also acts as an anchor for certain signalling components such as nitric oxide synthase (4–6). The complex consists of several sub-complexes of a few proteins, with the main structural axis being provided by a series of three proteins that link intracellular F-actin below the sarcolemma to laminin outside the cell. The laminin receptor within the DAPC is dystroglycan, which is formed by the cleavage of a precursor protein into extracellular α within the DAPC is dystroglycan, which is formed by the cleavage of a precursor protein into extracellular α and transmembrane β subunits. Dystrophin is a large rod-like protein related to the spectrin family, which binds to β-dystroglycan at its C-terminus and to actin filaments via its N-terminus. A second sub-complex of the DAPC comprises a group of related transmembrane proteins called sarcoglycans; a third is based on syntrophin proteins and nitric oxide synthase, and several further proteins are known to associate with the complex. The DAPC is found at the membrane of skeletal muscle fibres and several other cell types in the body, while a similar complex in which dystrophin is replaced by the related protein utrophin is distributed widely throughout the body.

Dystrophin is the largest product of the DMD or Duchenne muscular dystrophy gene, which is responsible for a spectrum of X-linked conditions including Duchenne and the milder Becker muscular dystrophies, cardiomyopathies and mental retardation (7,8). In Duchenne muscular dystrophy the sarcolemma is torn during muscle contraction, leading to a cycle of death and replacement of muscle fibres which results in an accumulation of scar tissue in the muscle and a gradual loss of function and eventually death. Many other muscular dystrophies and congenital myopathies are caused by mutations affecting other components of the complex, such as a congenital dystrophy linked to laminin-α2, and type-2 limb girdle muscular dystrophies (LGMD2), some of which are linked to sarcoglycans, calpain 3, caveolin 3 and dysferlin (9–13).

The distribution of dystrophin- and utrophin-containing DAPC within muscle fibres and the consequences of loss of dystrophin for the integrity of the sarcolemma and ultimately the survival of the muscle fibre have been well documented in studies of both patient biopsies and the dystrophin-deficient mdx mouse. It is thought that, in Duchenne patients, remaining structural proteins in the sarcolemma such as spectrin and integrin complexes are insufficient to protect non-specialized areas of the membrane from forces generated during exercise. This leads to the appearance of tears along the length of the fibre, and eventually to the replacement of dead muscle fibres with scar tissue (14). The specialized neuromuscular junction is unaffected because it contains utrophin, which despite some up-regulation is insufficient to compensate for dystrophin loss over the whole fibre (15–17). By contrast to Duchenne patients, the mdx mouse is only mildly affected, perhaps (18–20) because of efficient regeneration and effective compensation by
utrophin (21,22). This last observation has lead to attempts to identify ways to increase utrophin production in the hope of allowing a therapeutic level of compensation (23).

**DAPC IN ZEBRAFISH**

Homologues of dystrophin and other DAPC components have now been identified in all of the common genetic model organisms, and have been shown to have a necessary function in muscle in animals as diverged from humans as the nematode and *Drosophila*, indicating that the complex is evolutionarily ancient (24–30). Given that muscle fibres are similar in animals and essentially the same in all vertebrates, gene functions can be studied in muscle using a range of organisms. Taking the gene-first route, several groups have therefore begun to address the function of known DAPC components and muscular dystrophy loci in zebrafish. Their work has shown that orthologues of dystrophin, dystroglycan and the sarcoglycans show conserved sequence and expression between humans and zebrafish, with the additional discovery that in embryonic zebrafish the DAPC is localized initially at a distinct specialization of the sarcolemma, the end attachment point.

Bolanos-Jimenez *et al.* (31,32) first described the embryonic expression of mRNA produced from the zebrafish DMD gene orthologue. They found that muscle cells contain mRNA dystrophin and a shorter product Dp71 that is localized to the ends of fibres. The possible conservation of this mechanism and its functional significance remain to be investigated.

A complementary set of findings was made by Parsons *et al.* (33), who investigated the distribution and role in zebrafish of the lamin receptor dystroglycan, to which dystrophin binds directly. Having identified laminin mutations that disrupt notochord development, they were seeking to identify the laminin receptors important in this process when they described the localization of laminin and dystrophin proteins at the ends of embryonic muscle fibres. Furthermore, they used morpholinos, modified antisense oligonucleotides, to knock down levels of dystroglycan protein in wild-type embryos. Using this method they found that, as in the mouse, dystroglycan is essential for the normal differentiation of muscle fibres, too early to cause muscular dystrophy. Dystroglycan itself has not been demonstrated to be the mutated gene in any muscular dystrophy as yet, but several diseases including Fukuyama type muscular dystrophy result from defects in proteins responsible for modifying it (34–36). Dystroglycan removal from zebrafish results in muscle fibres lacking the ability to form the regular array of the sarcomeric proteins that are the engine of contraction. This phenotype and the large number of mutations in unidentified zebrafish genes that cause failure of muscle differentiation, may represent models of congenital myopathies (37–39).

The similarity between human and zebrafish muscle was further emphasized by Donald Love's group, who have carried out an immunohistochemical analysis of dystrophin in adult zebrafish muscle that was designed to reproduce the type of data that is familiar to physicians and those working with mouse models (40,41). By labelling transverse sections of adult skeletal muscle with anti-dystrophin, they observed characteristic rings of signal, indicating that the expected localization in non-specialized sarcolemma is conserved as a later phase of expression. They also further confirmed the conservation of dystrophin gene structure and expression by showing in western blots, as did Bolanos-Jimenez *et al.*, that the same range of isoforms are present as in mammalian muscle. This group has also identified zebrafish orthologues of the sarcoglycan family of transmembrane proteins, which form a sub-complex of the DAPC. Mutations in these loci lead to human type-2 limb girdle muscular dystrophies, and Chambers *et al.* (41) have shown that these proteins localize to the sarcolemma and to the ends of muscle fibres in zebrafish. They are currently applying gene-silencing RNAs to DAPC components in the zebrafish with the aim of developing this technology for the rapid creation of genetic models for a variety of diseases. This approach may well eventually deliver the ability to create stable inducible transgenic lines in which a gene of interest can be down-regulated with both spatial and temporal control, hugely increasing the scope of the zebrafish as a system for studying monogenic disorders.

Most recently, Guyon *et al.* (42) have used similar techniques to identify zebrafish sarcoglycan homologues, and have used the morpholino knockdown technique to begin to address the essential issue of how far the function of the DAPC is conserved in the zebrafish. They used morpholinos to the reduce levels of dystrophin protein in embryos and showed that this in turn affected the levels of other co-localized DAPC components, a good indication that the composition of the complex is conserved, and therefore that the function may well be too. However, this did not reveal any insights to the role the DAPC plays in zebrafish in maintaining muscle cell integrity.

In combination, these data show us that there is a highly-conserved dystrophin-based form of the DAPC present in zebrafish muscle and that it shifts from being localized at fibre ends to the more familiar pattern of sarcolemmal distribution as development proceeds. However, the question of whether loss of the DAPC causes a muscular dystrophy in the zebrafish has recently been answered using a mutation that causes exactly the right kind of phenotype (3).

**A NOVEL MECHANISM OF PATHOLOGY IN A MODEL OF MUSCULAR DYSTROPHY**

Large-scale genetic screens in zebrafish have identified a large number of mutants that affect muscle formation, with one class showing a very specific degeneration of skeletal muscle. Preliminary investigation during the screening process revealed that mutations at several independent loci share the broad phenotype of developing visible lesions in the somitic trunk muscle during the second day of development once movement begins, which gradually accumulate until the animals die before reaching adulthood, a phenotype superficially similar to muscular dystrophy in humans. This class of mutations is now being examined by the authors, and, in particular, one specific locus, *sapfe (sap)*, has been identified as a model of a human muscular dystrophy that reveals a novel pathological mechanism that we suggest may be of clinical relevance (3).

We have found that dystrophin is lost from the end of muscle fibres in *sap* mutant zebrafish embryos, confirming the class of muscle degeneration mutants may represent muscular...
dystrophy phenotypes (Fig. 2A and B). Histological examination and confocal imaging of skeletal muscle expressing green fluorescent protein (GFP) showed that the lesions occur where the ends of sap mutant muscle fibres become separated from their attachment sites (Fig. 2C–F). Many fibres are seen to detach at one end and contract to a fraction of their original length, showing compression or even collapse of the sarcomeric banding. Furthermore, these cells show nuclear condensations under electron microscopy that indicate cell death that are not seen in intact neighbouring cells. A subset of these detached fibres takes up the vital dye Evans Blue, which only enters cells with compromised plasma membranes, indicating that some tearing of the membrane does occur (Fig. 2G and H). Thus, despite sharing the phenotype of muscle degeneration at the whole organism level at the cellular level, the pathology of sap mutant zebrafish is apparently different to that of human muscular dystrophies, where membrane damage occurs along the length of the fibre. The DAPC is localized embryonically in zebrafish to the ends of muscle fibres before it becomes detectable at the sarcolemma, suggesting that loss of the complex might compromise muscle attachments and possibly allowing detachment of the kind seen in sap. This is a surprising finding because this has not been reported in any human muscular dystrophy and, even in mouse mutants that show ultrastructural abnormalities of the cytoskeleton at the MTJ, there have been no reports of actual failure occurring in vivo (43–47).

We have now shown that sap corresponds to the dmd gene, the zebrafish orthologue of the human Duchenne muscular dystrophy locus, and thus revealed a novel functional requirement for the DAPC in the stability of muscle attachments. We have identified a nonsense mutation towards the N-terminal actin-binding domain of dystrophin that removes the large muscle-specific isoform and causes a progressive fatal muscle degeneration. sap is a far more severe phenotype than the mouse dystrophin mutant mdx, showing the same progression to early lethality as the human disease, perhaps because both human and zebrafish lack the regenerative capacity and compensatory levels of utrophin that are thought to protect mdx mice. Urophin is not found at embryonic muscle attachments in zebrafish, and is absent from the non-specialized sarcolemma along the length of muscle fibres during development, but is present in the skin and pronephros. This lack of either dystrophin or utrophin in muscle perhaps makes sap mutant embryos most similar to mouse models thought to lack any functional DAPC link, notably the laminin-2 (dy), dystroglycan and mdx/utrophin double mutants (21,22,48–50). In these animals, the MTJ lacks folding almost completely and may be weaker than in mdx animals, but it is unclear whether it ever fails completely. Only very slight folding of the sarcolemma is present in wild-type zebrafish muscle (51), indicating that sap resembles these mouse models in its anatomical details, and that the complex involvements of the mammalian MTJ may have evolved to withstand the rigours of life on land. A similarly severe loss of the mechanical function might occur in the merosin-2 deficient congenital muscular dystrophies (6), a group of very severe early muscle disorders, raising the possibility that such diseases might involve MTJ defects in human muscle. Within some mammalian muscles, dystrophin is also enriched at specialized myomuscular junctions (MMJs) that also transmit force between the ends of muscle fibres (52). These occur as either intrafascicular fibre terminations, connecting single fibres into networks both end-to-end and end-to-side (53), or as fibrous sheets called tendinous intersections that separate segmented blocks of non-overlapping fibres. These in particular bear a striking structural resemblance to the dystrophin-dependent attachments between somites in zebrafish (53,54). If the failure of MMJs failure were a significant factor in mammalian muscle disease, their differential deployment might contribute to the observed variations in pathology between individual muscles, and between humans and the different dystrophic animal models.

The sap mutation may only affect a subset of the products of the dmd gene, as do many of the known human disease-causing
mutations. The phenotype of sap mutant embryos may be less severe than that of embryos injected with a morpholino designed to block dystrophin translation (42), as it does not entail the same body curvature, perhaps because these two situations disrupt different exons and therefore different isoforms of dystrophin. To date no other mutations are known in the zebrafish dmd gene, but it will be of great interest to examine the effects of mutations at different points in the gene that affect different gene products. This type of information is, however, more likely to be provided in the near future by the use of silencing RNAs and morpholinos directed to different exons, which will hopefully begin to model the full spectrum of dystrophinopathies.

As well as accurately representing the progressive nature and fatality of DMD, sap closely resembles known Duchenne-causing nonsense mutations in the N-terminal, making sap a new model of the disease and raising the possibility that muscle attachment failure contributes to the pathology of either Duchenne or other muscular dystrophies. The known localization of the DAPC to this site is consistent with this, but it seems possible that such pathology might so far have been overlooked, as muscle biopsies are often deliberately taken from sites at a distance from the tendon in order to simplify histological examinations. It remains to be seen to what extent this novel requirement for the DAPC for the stability of muscle attachments might contribute to human muscle diseases, but it might be prudent to re-examine these structures, especially myomuscular junctions.

THE FUTURE OF ZEBRAFISH MUSCLE DISEASE RESEARCH

The zebrafish is rapidly being developed as a system for the study of muscle disease, with exciting results, and it is to be hoped that this trend continues. In future there will be more laboratories beginning to dedicate a proportion of their time to this approach; both existing muscular dystrophy and more laboratories beginning to dedicate a proportion of their time to this approach; both existing muscular dystrophy and dystrophinopathies. Cardiology, 99, 1–19.


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