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Analysing regenerative potential in zebrafish models of congenital muscular dystrophy☆

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ABSTRACT

The congenital muscular dystrophies (CMDs) are a clinically and genetically heterogeneous group of muscle disorders. Clinically hypotonia is present from birth, with progressive muscle weakness and wasting through development. For the most part, CMDs can mechanistically be attributed to failure of basement membrane protein laminin-α2 sufficiently binding with correctly glycosylated α-dystroglycan. The majority of CMDs therefore arise as the result of either a deficiency of laminin-α2 (MDC1A) or hypoglycosylation of α-dystroglycan (dystroglycanopathy). Here we consider whether by filling a regenerative medicine niche, the zebrafish model can address the present challenge of delivering novel therapeutic solutions for CMD. In the first instance the readiness and appropriateness of the zebrafish as a model organism for pioneering regenerative medicine therapies in CMD is analysed, in particular for MDC1A and the dystroglycanopathies. Despite the recent rapid progress made in gene editing technology, these approaches have yet to yield any novel zebrafish models of CMD. Currently the most genetically relevant zebrafish models to the field of CMD, have all been created by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. Once genetically relevant models have been established the zebrafish has several important facets for investigating the mechanistic cause of CMD, including rapid ex vivo development, optical transparency up to the larval stages of development and relative ease in creating transgenic reporter lines. Together, these tools are well suited for use in *live*-imaging studies such as in vivo modelling of muscle fibre detachment. Secondly, the zebrafish's contribution to progress in effective treatment of CMD was analysed. Two approaches were identified in which zebrafish could potentially contribute to effective therapies. The first hinges on the augmentation of functional redundancy within the system, such as upregulating alternative laminin chains in the *candyfloss* fish, a model of MDC1A. Secondly high-throughput small molecule screens not only provide effective therapies, but also an alternative strategy for investigating CMD in zebrafish. In this instance insight into disease mechanism is derived in reverse. Zebrafish models are therefore clearly of critical importance in the advancement of regenerative medicine strategies in CMD.

This article is part of a Directed Issue entitled: Regenerative Medicine: The challenge of translation.

Introduction

Since the inception of zebrafish as a model system for studying developmental biology, there has been a constant progression in their use and scope within biological research. One of the most exciting recent developments for the zebrafish is their growing presence in the field of regenerative medicine. Much of this growth is owed to advances in the range of tools available for investigating genetics in zebrafish. Of particular importance to the toolbox is the ability to generate genetically relevant models of diseases of interest, a sticking point in the progress of zebrafish research for some time (Lieschke and Currie, 2007). Traditional site-directed DNA recombination methodology used to create knock-out mouse models unfortunately has had limited success in zebrafish, due to a lack of established zebrafish embryonic stem cell culture techniques (Dong and Stuart, 2004; Fan and Collodi, 2006; Fan et al., 2006). Serendipitously, a plethora of methods for specifically targeting the zebrafish genome has arrived almost simultaneously with exomic sequencing, a technique that has identified numerous novel monogenic disease alleles in humans (Huang et al., 2011; Foley et al., 2009; Hwang et al., 2013). When combined with properties such as the rapid *ex-utero* development and the high level of genetic conservation between humans and zebrafish, the system becomes an astute choice for modelling newly discovered disease alleles (Clark

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et al., 2011; Catchen et al., 2011). Zebrafish are also one of the few vertebrate models systems capable of taking advantage of *live*-imaging, based on their optical transparency during development and the relative ease in creating transgenic fluorescent reporter lines. In vivo imaging is an essential component of an approach that aims to elucidate novel aspects of disease mechanisms, occurring early in embryonic development. It is therefore perhaps unsurprising that there is an increase in the adoption of the zebrafish as a model system to study disorders of development, such as congenital muscular dystrophy (CMD) (Bassett and Currie, 2003; Kimmel and Warga, 1987; Siegel et al., 2013; Rodrigues et al., 2012). With no effective therapies currently available for any of the CMDs, a disease modelling approach that encompasses aspects of regenerative medicine in zebrafish may prove a novel route to therapy development. This review will therefore examine the most relevant regenerative therapy pathways currently being investigated in zebrafish models of CMD.

Congenital muscular dystrophy

CMDs are comprised of a heterogeneous group of typically autosomal recessive maladies and are broadly characterised as having the following clinical features: an onset at birth or infancy, delayed motor milestones, hypotonia, muscle weakness and wasting (Sparks and Escolar, 2011). In health, muscle fibre adherence at the myotendinous junction (MTJ) stabilises the contractile apparatus during force transmission. Conversely in CMD, the pathology is nearly always associated with a deficiency in one of the components responsible for maintaining the interaction between muscle fibres and the MTJ (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992; Petrof et al., 1993). CMD is very similar in its presentation clinically to congenital myopathy (North, 2011; Nance et al., 2012). However, it is the load that causes the fibres to detach in CMD, whereas conditions that arise as a result of fibres failing to attach at the MTJ during development forms the mechanistic basis of congenital myopathy. Ultimately it is cycles of degeneration and regeneration that ensue in CMD, evident on biopsy, but not observed in congenital myopathy, which is important for distinguishing the two diseases clinically (Bertini et al., 2011). The CMDs can essentially be divided into three groups: Abnormality of endoplasmic reticulum (ER) proteins, disorders of dystrophin associated glycoprotein complex (DGC), and disorders of the basal lamina (Mendell et al., 2006; Rocha and Hoffman, 2010). This review will not address ER CMD i.e. rigid spine syndrome and the laminopathies (Liu et al., 2005; Flanigan et al., 2000), instead focusing on the disorders that more directly relate to maintenance of myofiber adherence at the MTJ. The attention will therefore be on the disorders zebrafish have made the most significant contributions to: the lamininopathies, in particular MDC1A, and the dystroglycanopathies.

Current approaches to modelling CMD in zebrafish

Until recently, mice have been the organism of choice in which to model CMD. There are, however, several limitations of the mouse model that have proven critical in the success of the zebrafish finding a niche in the CMD field. Perhaps most importantly, zebrafish do not require a Reichert's membrane to develop and can therefore bypass the early embryonic lethality of dystroglycan and laminin CMD mouse models (Williamson et al., 1997; Xu et al., 1994). Because of the difficulty in generating viable dystroglycan and laminin deficient mice, particularly of the primary dystroglycanopathy, often only one feature such as muscle weakness or brain abnormalities is modelled in any given mouse strain using tissue specific promoters, with the exception of the MORE-DG null mouse which has features analogous to each aspect of muscle-eye-brain disease (MEB) (Cote et al., 1999; Cohn et al., 2002; Moore et al., 2002; Satz et al., 2008, 2009). For zebrafish to truly occupy a regenerative medicine niche in CMD, the models generated must in the first instance be genetically, biochemically and pathologically relevant to the disease of interest. Therefore here the status of forward, reverse and transient genetic approaches to modelling CMD genes in zebrafish is assessed.

Forward genetic approaches are the oldest strategy discussed here for generating relevant CMD mutant zebrafish lines. The chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) produces a mutation in approximately 1:700 gametes making it an attractive system for mutation screens (Justice et al., 1997). However, the method is random and this lack of specificity means that a large number of animals must be screened, making this approach difficult to justify for small labs (Knapik, 2000). The method is phenotype driven and therefore the screening technique is highly dependent on screening technique of choice. Subtle phenotypes can therefore be easily missed. Nonetheless several CMD lines for laminin, integrin and primary dystroglycanopathy have been identified using this approach. The dag1, patchytail (Gupta et al., 2011), dag1$^{hu3072-/-}$ (Lin et al., 2011) and *lama2* mutant zebrafish *candyfloss* lines have irregular vertical myosepta borders and a collapsed sarcomere phenotype (Hall et al., 2007; Jacoby et al., 2009). In contrast, the mutants *laminin-α1 bashful*, *laminin-β1 grumpy* and *laminin-γ1 sleepy* did not have the same muscle basement membrane pathology indicating the dystroglycan laminin-α2 interaction is specific for maintaining muscle fibre attachment at the MTJ (Parsons et al., 2002b; Pollard et al., 2006). Using a reverse genetic approach, the zebrafish mutation project aims to create a nonsense mutation in every protein-coding gene in the zebrafish genome, potentially delivering mutant lines for the remaining CMD genes. The process remains random but by creating a genotyping pipeline a less biased coverage of genes will be achieved. The exomic DNA of F1 generation fish with phenotypes are sequenced following enrichment with the Agilent Sure Select system on an Illumina Hi-seq platform. Specific alleles are then sequenced in the generated F2 by KASpar (allele specific amplification, developed by KBiosciences) to confirm any mutations (Clark et al., 2011; Kettleborough et al., 2011, 2013). Despite some success in generating relevant CMD zebrafish models with reverse genetics, there are some major drawbacks, including patchy coverage in terms of CMD genes mutated, Table 1. It is therefore clear that a more targeted approach is required, to gain insights into yet to be mutated genes.

Targeted transient approaches offer the fastest method to gain preliminary data for CMD genes of interest. However, off target effects are common with such strategies and often hard to delineate from genuine findings. Transient approaches have been very popular in CMD since there are currently no stable mutant (i.e. an effective knockout of the gene of interest) zebrafish lines for any of the secondary dystroglycanopathies, although the new technologies becoming available, lead to the expectation that mutant lines will be published in the near future. All studies investigating secondary dystroglycanopathies to date have been carried out using morpholino antisense-oligonucleotides (MOs). Efficient knockdown can be achieved with MOs since they bind to native nucleic acids with high affinity, but are resistant to degradation and therefore persistent in vivo (Summerton, 2007; Nasevicius and Ekker, 2000). Most recently MOs have been used to rapidly assess whether novel Walker–Warburg Syndrome disease genes such as *B3GALNT2* and *B3GNT1* recapitulate the respective clinical phenotype (Stevens et al., 2013; Buysse et al., 2013). As a natural progression of this approach, MOs can be used to knockdown potential CMD genes, for example *col22a1* in zebrafish (*COLXXII* in humans) (Charvet et al., 2013). MOs have also importantly been gaining ground in some proof of principle therapeutic studies. The