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Review

Beyond mice: Emerging and transdisciplinary models for the study of early-onset myopathies

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ABSTRACT

The use of the adapted models to decipher patho-physiological mechanisms of human diseases is always a great challenge. This is of particular importance for early-onset myopathies, in which pathological mutations often impact not only on muscle structure and function but also on developmental processes. Mice are currently the main animal model used to study neuromuscular disorders including the early-onset myopathies. However strategies based on simple animal models and on transdisciplinary approaches exploring mechanical muscle cell properties emerge as attractive, non-exclusive alternatives. These new ways provide valuable opportunities to improve our knowledge on how mechanical, biochemical, and genetic/epigenetic cues modulate the formation, organization and function of muscle tissues. Here we provide an overview of how single cell and micro-tissue engineering in parallel to non-mammalian, *Drosophila* and zebrafish models could contribute to filling gaps in our understanding of pathogenic mechanisms underlying early-onset myopathies. We also discuss their potential impact on designing new diagnostic and therapeutic strategies.

Contents

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1. Introduction

The early-onset myopathies represent a large and heterogeneous group of genetic skeletal muscle diseases with an onset generally at birth or early infancy (for review 1). Thus this group includes numerous pathologies due to mutations in multiple different genes, and with multiple modes of inheritance or severity. Despite their genetic heterogeneity and the resulting wide range of symptoms and molecular mechanisms, the early-onset myopathies display common mechanical defects – such as muscle weakness, joint contractures, respiratory and cardiac complications, which can also be associated with other muscular disorders. Several murine models of early-onset myopathies have been developed; however because of technical and ethical issues they cannot be applied to address all questions that remain, leaving these disabling diseases without adapted treatments. Alternative cellular or animal models, both mammalian and non-mammalian, are thus essential to improve our knowledge on physiological and pathological processes muscle mechanisms. In this review, we highlight the emerging field of single myoblast and in vitro generated skeletal muscle tissue analyses that could provide cues into mechanical defects of diseased muscle and in particular muscle weakness. We also discuss insights gained from alternative simple animal models, zebrafish and *Drosophila*, and how these alternative approaches could pave the way to the development of clinical therapies or for the examination of the functional effects of patient-specific mutations.

2. Single cell models for the analysis of mechanical properties

Physical forces play essential roles in muscle functioning. In fact muscle contraction takes place under spatial constraints and such forces are able to modulate cell metabolism and properties. Moreover, while the main symptom in early-onset myopathies as in other myopathies is muscular weakness, muscle stiffness can also be present. In a search to unravel the very early mechanisms involved in the appearance of the disease, an emerging approach is to characterize the mechanical properties of isolated satellite cells and myoblasts. The concept of mechanical properties includes three different aspects: first how a cell deforms under applied mechanical stress – rheological properties; second, its ability to develop forces against its substrate – contractility; and finally how a cell adapts to an applied mechanical stress – mechanotransduction.

2.1. Rheological properties of myoblasts

Biophysicists have designed numerous cell rheometers in which the deformation of a cell under a controlled applied mechanical stress is measured. The mechanical stress can be applied either locally, via an AFM tip [2] or via micro-sized beads coated with an adhesive protein such as fibronectin and actuated with optical [3] or magnetic tweezers [4], (Fig. 1A, B). A mechanical stress can also be applied globally to a cell, for instance by aspiring it in a micropipette [5] or by deforming it between two parallel microplates [6] (Fig. 1C). The deformation of the cell is then inferred either by the measured displacement of the probe (AFM tip of bead) or by a direct measurement of the cell deformation. The measured rheological parameter is the creep function when a constant stress is applied to the cell, or the visco-elastic modulus as a function of frequency when the stress is periodic in time. It is widely recognized that the key players of the cellular mechanics are actin filaments and their associated proteins, including myosin [6,7], which are often mutated in congenital myopathies [1]. Furthermore, it has been recently shown that Intermediate Filaments (IF) are central actors of the cytoplasmic visco-elastic modulus [8]. This was demonstrated for vimentin in fibroblasts [9] and for desmin in C2C12 myoblasts [10]. Vimentin depleted fibroblasts show a cytoplasmic rigidity divided by two as compared to WT. Similarly, C2C12 myoblasts over-expressing WT desmin exhibit an increased overall rigidity whereas this rigidity is unchanged under the expression of a mutated desmin, which is a signature of the loss of function of the latter. It was also shown that primary human myoblasts from patients with a particular desminopathy appear stiffer than healthy ones [4]. In conclusion it seems that there is a signature of myopathies on the mechanical properties of isolated myoblasts, even though it is not ubiquitous. It would probably be useful to generalize to other congenital myopathies this type of approach, which has only been used so far for another type of pathology, namely epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) [11].

2.2. Force generation by isolated myoblasts

The force developed by an isolated cell on its substrate has also been measured on various cellular types. In Traction Force Microscopy (TFM), cells are seeded on a soft hydrogel substrate embedded with fluorescent microspheres (typically latex beads less than 1 μm in diameter, Fig. 1 D); displacements of the beads caused by cells are tracked by microscopy and converted to traction stress field [12]. The substrate may also be composed of micropillar arrays and cellular traction forces are directly inferred from the deflection of these micropillars [13]. The force developed by an entire cell on two parallel microplates can also be measured [14,15] (Fig. 1E). All these techniques have highlighted the ability of cells to adapt their contractility to the stiffness of their substrate [13–15]. In the context of diseases, some TFM experiments revealed an abnormal traction behavior for pathological cells [12]. In the context of myopathies, it was recently demonstrated that the expression of a mutant desmin can alter the traction forces generation of single myoblasts [10]. It would be interesting to develop this type of approaches for congenital myopathies, so far unexplored. As already mentioned in the previous paragraph, this kind of single cell approach is promising, and it would be useful to look for a signature of other congenital myopathies on the ability of myoblasts to develop forces.

2.3. Mechanotransduction of myoblasts or myotubes

The ability of cultured cells to sense mechanical cues in their surrounding environment and to transduce them into biochemical signals can also be characterized in vitro by a variety of techniques. A cyclic mechanical stress may be applied to cultured cells, either

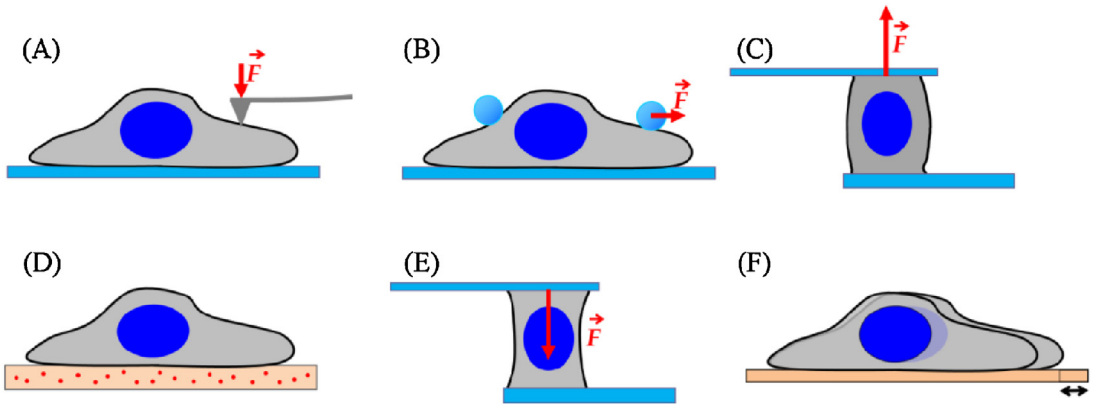

Fig 1. Several tools used to characterize the mechanical properties of cultured cells.
(A) An AFM tip is used to apply a controlled force. (B) Micron-sized bead coated with an adhesion protein are actuated by optical tweezers or magnetic tweezers; in magnetic twisting cytometry, a magnetic torque can alternatively be applied to the beads. (C) Single cell rheometer, in which a uni-axial stress is applied to a cell by two parallel micro-plates. (D) In Traction Force Microscopy, the deformation of the substrate by cell contractions is measured. (E) The Single Cell Rheometer can also measure the traction force developed by an entire cell. (F) Stretchable substrates can be used to apply cyclic strains on cells.

locally, using for instance micron-sized beads coated with a receptor for integrins and actuated by optical or magnetic tweezers [3], or globally, by seeding cells on stretchable substrates [4,16,17]. The response to cyclic stretch is of particular interest in the context of muscle and muscular disease. Indeed it was shown that some mutations implicated in myopathies altered the reorientation dynamics of myoblasts subjected to cyclic stretch [16,17]. This probably impairs their ability to align in order to fuse and form parallel multinucleated myotubes. This could play a role in the mis-organization of the contractile apparatus often observed in myopathies.

Cells may also be subjected to different mechanical cues by culturing them on substrates of varied stiffness [18]. It was indeed demonstrated that myoblasts differentiate optimally into myotubes on substrates with tissue-like stiffness [18]. It was also shown that the expression of lamins A/C, which directly or indirectly regulate many proteins, correlates with the stiffness of the substrate [19]. This could be of importance in the understanding of the LMNA-related form of congenital muscular dystrophy (LMNA-CMD), but also of Emery-Dreifuss muscular dystrophy and dilated cardiomyopathy, all caused by mutations in the gene encoding lamins A/C.

2.4. A tool with potential for congenital myopathies?

Single cell approaches are an alternative to expensive and time-consuming animal models. The search for a signature of myopathies in mechanical properties of cultured cells, either their visco-elastic moduli or their ability to develop forces against their substrates, is thus a promising approach [20], in particular for nemaline myopathy, often due to mutations in genes encoding thin filaments, or for LMNA-CMD. Such studies should help to unravel the different steps that lead from a gene mutation to progressive muscle weakness, which are still largely unclear. The development of such pertinent in vitro models also opens the way to a fast screening of chemicals or gene therapies for myopathies. Yet one has to be very careful of the need to work on soft substrates, with a stiffness similar to the muscle's one, rather than on stiff ones, to recapitulate the physiological properties of muscle cells [21].

3. In vitro skeletal muscle tissue engineering

The ability to recreate in vitro skeletal muscle tissues that resemble the structural and functional properties of native muscle would enable design of accurate in vitro models for studies of muscle physiology and development. Furthermore, these models could serve for drug-screening applications or examinations of the functional effects of patient-specific mutations, which are common in congenital myopathies and some forms of congenital muscular dystrophy. Since the late 1970s, muscle tissues have thus been engineered mostly to serve as tissue models that complement the conventional two-dimensional (2D) cell cultures and animal models. Here we will focus on the recent developments in skeletal muscle tissue engineering, with an emphasis on 3D culture systems for early onset myopathies. The main goal of these developments was to bypass the existing limitations in traditional 2D culture assays. Indeed, muscle cells in culture dishes naturally form randomly organized cell sheets, on very stiff substrates, far from the highly organized architecture of their native, soft environment. Moreover, the culture of mature myotubes is particularly difficult, as more mature myotubes readily detach from tissue-culture surface when contracting [22]. To address these issues, and thanks to the development of silicone-based lithography (i.e. soft lithography) in the late 90's, a large number of microdevices were developed over the past two decades, mostly based on microfluidics, microelectromechanical systems and 3D printing.

3.1. 2D muscle tissue engineering

Several groups thus developed approaches for aligning myotubes on soft materials while limiting cell detachment. By micropatterning Extracellular Matrix (ECM) proteins such as laminin, collagen or fibronectin on the surface of dishes or soft polyacrylamide gels, researchers demonstrated the strong impact of the geometrical and mechanical properties of the substrate on the alignment, fusion and maturation of myotubes [18,23–25]. Similarly, authors microfabricated microgrooves in synthetic or natural polymers to guide linear cellular alignment and showed that these microscale topographic features regulated cell and cytoskeleton alignment, myoblast proliferation, myotube assembly and striation [26–30]. Recently, Yang et al. cultured primary murine myoblasts on nanopatterned substrates to generate aligned and mature myotube patches [31]. When transplanted into *mdx* mice, the genetic homologue of Duchenne muscular dystrophy (DMD), the myotube patches led to the formation of a significantly greater number of dystrophin-positive muscle fibres, highlighting a possible potential for such approach in the treatment of muscle disease. Only few studies using these approaches are reported on human myoblast differentiation in vitro, and even fewer on human myoblasts carrying genetic diseases. Sen-

gupta et al. evaluated the behavior of C2C12 and human primary myoblasts on micropatterned substrates and found that the geometry of these protein stripes needed to be adapted to the cell model [32]. Others guided the differentiation of human healthy and dystrophic myoblasts into myotubes with polyacrylamide hydrogels presenting physiological-like mechanical properties and micropatterned parallel lanes of three ECM proteins [33]. Myotubes exhibited a high degree of sarcomeric maturity, with an optimum onto a 15 kPa elastic hydrogel and a matrigel patterning. In addition, healthy myotubes cultured in these conditions showed a significant membrane-localized dystrophin expression. These approaches thus allow a maturation of the myotubes sufficient for quantitatively studying how genetic cues and defects modulate the differentiation and maturation of human myoblasts.

3.2. 3D muscle tissue engineering

An alternative to these 2D approaches is the formation of 3D skeletal muscle tissues. In addition to their closer resemblance to the native muscle, 3D constructs favor a high degree of cell-cell contact, thus excluding myotube detachment. While early models of 3D skeletal muscle constructs were scaffold-free [34–37], most approaches since the 2000's rely on the encapsulation of muscle cells within a natural matrix such as collagen, fibrin or Matrigel (Fig. 2). As cells tend to align along the lines of tension, the constraint of cell-laden matrices by uniaxial anchoring points promotes the maturation and the mechanically-induced alignment of the myotubes [38–40]. Moreover, these 3D approaches also allow for force measurement, by using rigid strain gauges or flexible levers as anchoring points. Different strategies have been developed for providing stable anchoring points such as Velcro [41,42], suture [34,43] or flexible polydimethylsiloxane (PDMS) anchors [40,44,45]. These 3D muscle constructs were shown to be contractile upon electrical stimulation [42,46–48] or when co-cultured with motor neurons [49]. Recently, Juhas et al. used this approach to engineer and study highly biomimetic murine skeletal muscle tissues with functional satellite cells capable of supporting myogenic and self-regenerative events characteristic of native muscle [50]. When implanted within the dorsal skin-fold of mice, these predifferentiated engineered muscle tissues exhibited continued myogenesis and improved contractile function when compared to undifferentiated engineered muscle tissues. In addition, they underwent robust vascularization and perfusion. Sharples et al. used a similar approach to study multiple population-doubled (MPD) murine myoblasts, previously shown to have an aged phenotype in monolayer cultures [51]. 3D constructs incorporating MPD cells presented reduced myotube size and diameter, associated with reduced peak force development, and a generally impaired differentiation/regenerative potential [52]. Madden et al. were the first ones to report engineering of functional human muscle tissues able to contract in response to electrical and chemical stimuli [53]. Interestingly, their human muscle constructs exhibited a high degree of similarity with native muscle, such as a higher sensitivity to cerivastatin than lovastatin, the induction of autophagic myopathy upon exposure to chloroquine, or hypertrophy and increased contractile strength at low clenbuterol doses followed by muscle weakness at higher doses. Overall, these recent results highlight the potential for this approach to provide a future test bed for screening therapeutics or patient-specific mutations in vitro.

3.3. High throughput platforms

Nevertheless, although these centimeter scale systems can recapitulate many developmental processes, their scale limits nutrient and oxygen diffusion and often necessitates large quantities of cells. Moreover, their scale often requires histological sectioning to visualize fine cellular structures and protein distributions within the construct, thus limiting their throughput. To address these issues, a major effort was made in the late 2000's to miniaturize 3D muscle constructs. Vandenburgh et al. were pioneers in the field with a 96-well assay system containing two flexible PDMS pillars in each well [54]. When a suspension of cell laden collagen/Matrigel was added to the wells, the cells spread inside the matrix, formed cell-cell contacts, and spontaneously compacted the matrix. The PDMS pillars anchored the contracting matrix, constraining the contraction of the collagen/Matrigel matrix to form a 4 mm linear tissue that spanned across the top of the pair of pillars. This resulted in a large amount of muscle constructs at once, with a direct access to the cell-generated forces by measuring the bending of the pillars. Thanks to this technique, Vandenburgh et al. are currently the only ones who engineered 3D diseased muscle tissues [55]. They used their platform to generate muscle tissues from *mdx* murine myoblasts and tested thirty-one compounds of interest as potential treatments for patients with DMD at different concentrations. They demonstrated that eleven of the tested compounds significantly increased tetanic force and identified beneficial as well as deleterious compound interactions. Altogether, their results pave the way to the production of high-quality functional muscle tissues, opening an exciting avenue for high-throughput, low volume screening of new potential treatments for early onset myopathies. In an attempt to further improve the throughput of such approach, the device was later adapted and miniaturized by the Chen's group who used microfabrication techniques to generate 500 μm microtissues wrapped around flexible micropillars [56]. This technique allowed for the generation of hundreds of microtissues per square centimeter from very low cell quantities and was used to study the mechanical interplay between cellular contractility, ECM mechanics, and tissue organization within 3D microtissues composed of murine fibroblasts [56], rat cardiomyocytes [57], human airway smooth muscle cells [58], murine C2C12 myoblasts [59] or human induced pluripotent stem cell–derived cardiomyocytes from patients suffering from dilated cardiomyopathy due to mutations of the *TTN* gene encoding titin, which can also cause congenital muscle disease [60].

3.4. Future challenges in skeletal muscle tissue engineering

In conclusion, despite the considerable recent progress in generating skeletal muscle tissues in vitro, there is still a need to develop more advanced, integrated models for use in the development of clinical therapies or for the examination of the functional effects of patient-specific mutations. Indeed, muscle cells never function independently *in vivo*, and a few studies started investigating the engineering of muscle tissue by co-culturing muscle cells with fibroblasts [22,61–63], endothelial cells [64,65] or neurons [49,65]. These co-cultures could act synergistically to provide support during development, homeostasis, and repair. A major aim for muscle constructs should be to better reproduce normal *in vivo* contractility levels, likely involving constructs with innervation and vascularization. As we emphasized above, there is also a very strong need for developing skeletal muscle tissues from human myoblasts, and especially from patient-derived cells to model functional deficits observed in different muscle pathologies. The ability to create such constructs would likely provide valuable opportunities to elucidate how mechanical, biochemical, and genetic cues modulate the formation, organization and function of healthy and diseased muscle tissues. Moreover, these 3D skeletal muscle tissues could provide a pre-clinical assay for improved predictive pharmacological testing and a potential alternative to costly animal studies.

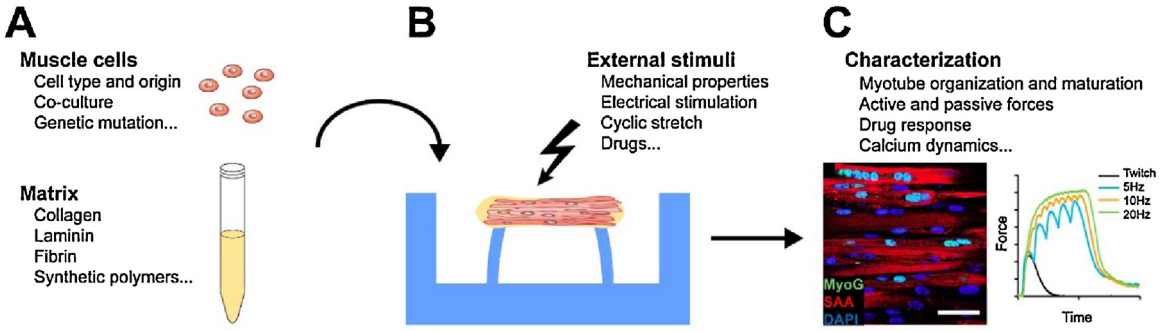

Fig. 2. Schematic illustration of the concept of 3D skeletal muscle tissue engineering. (A) Muscle cells encapsulated in a matrix self-assemble into a muscle tissue between two anchoring points. (B) The engineered muscle tissue can be subjected to physical or chemical stimulations before (C) characterizing its structural and functional properties. Image and graph in C adapted from [53].

4. Alternative animal models

Among the non-mammalian model organisms, zebrafish and *Drosophila* have been successfully used to evaluate significance of gene variants and pathological mutations associated with muscular dystrophies [66–68] some of them being applied to design and test therapeutic strategies [69,70]. Nowadays, growing number of new *Drosophila* and zebrafish models of muscular diseases including early-onset myopathies is being created and made available for research and various medical applications [67,71]

Several reasons make *Drosophila* and zebrafish attractive and well adapted when elaborating models of human myopathies with one of particular importance – their muscles share many structural, histological and functional similarities with human muscle [67]. Below we provide some instances of how *Drosophila* and zebrafish were applied to gain insights into early-onset myopathies with special focus on the most severe congenital muscle diseases and discuss specific strengths and limitations of each model (Fig. 3).

4.1. *Modeling early-onset myopathies in* Drosophila *and zebrafish*

Modeling the severe early-onset forms of human myopathies in animal allows assessing embryonic muscle defects and testing their modifiers. Here, simple animal models are particularly well suited, because they provide easy access to externally developing and, in the case of zebrafish, fully transparent embryos.

4.1.1. *Walker-Warburg syndrome*

Walker-Warburg syndrome (WWS) is thought to be the most severe form of congenital muscular dystrophy (CMD) and is due to mutations in different genes which eventually lead to defective O-glycosylation of α-Dystroglycan (α-DG), a protein of the dystrophin-associated complex. Particularly, mutations in O-mannosyl transferase 1 (encoded by *POMT1*), have been identified with high frequency in WWS patients [72,73]. Functional analyses of *POMT1* orthologue in *Drosophila* known as *rotated abdomen* (rt) or *dPOMT1* [72] have led to better understanding the pathophysiological defects underlying WWS and to finding that *dPOMT1* and dystroglycan act in the same pathway. It has been shown that defective dystroglycan glycosylation in *dPOMT1* mutants decrease the efficacy of synaptic transmission and leads to changes in the subunit composition of post-synaptic glutamate receptors at neuromuscular junctions (NMJs). In addition, synaptic strength was impaired in *dPOMT1* mutant larvae due to a decrease in the number of synaptic vesicles released from the presynaptic motoneurons [72]. These NMJs defects provide a cue to the muscle weakness observed in WWS patients. On the other hand, the observed synaptic abnormalities are also a plausible cause of mental retardation, a major WWS symptom [72]. Further analyses of knockdown flies for *POMT1* and *POMT2* orthologues [74] also revealed an increased apoptosis that could contribute to muscle defects occurring in WWS.

In parallel, the zebrafish model has been extensively used to test the impact of mutations in genes encoding WWS-causing glycotransferases. The zebrafish expresses orthologues of the α-DG modifying glycosyltransferases with strong homology to the human forms, including the orthologue of the gene encoding fukutin-related protein (FKRP), originally identified as the defective protein in Fukuyama Congenital Muscular Dystrophy (FCMD) [75]. Using a morpholino against FKRP, the resulting knocked down morphants showed an abnormal formation of muscle and eye [76] but also defects in muscle organization, affected locomotor activity and reduction in α-DG glycosylation analogous to the human diseases [77]. Similarly, morpholino knockdown of zebrafish *ISPD* encoding isoprenoid synthase domain protein recapitulated pathological WWS defects with apparent hypoglycosylation of α-DG and disrupted sarcolemma integrity [78]. This study provided evidence for ISPD to be one of major actors in WWS pathogenesis in humans. Moreover, mutations in GTDC2 [79], B3GNT1 [80] and B3GALNT2 [81] glycosyltransferase, recently identified in consanguineous WWS-affected families have been assayed in zebrafish revealing that their knockdowns replicate all the WWS features (hydrocephalus, ocular defects, and muscular dystrophy), providing further evidence that zebrafish represents an accurate model to test WWS-associated mutations.

4.1.2. *X-linked centronuclear myopathy*

X-linked centronuclear myopathy (XLCNM or myotubular myopathy) is the most severe form of CNM and is due to mutations in the *MTM1* gene (for review see [82]. MTM1 plays an important role in endocytosis and membrane trafficking by maintaining the spatial and temporal equilibrium of phosphoinositides (PIs). However how the disruption of the balance that regulate PIs levels may lead to neuromuscular diseases remains poorly understood. Some insights into XLCNM pathogenesis have been obtained using *Drosophila* models. Studies of *Drosophila myotubularin* (*mtm1*) showed that *mtm1* is central to endolysosomal function, cortical actin remodeling and is also involved in integrin-mediated myofiber attachment [83]. Integrin accumulated with PI3P on endosomal vesicles when mtm1 was depleted, suggesting that *mtm1* is required for intracellular integrin trafficking and recycling to the sarcolemma [84]. As integrin localization defects have also been observed in CNM patients [83], this finding provided a cue to cell pathways affected in XLCNM. It has also been found that mtm1/PI3 K equilibrium is essential to coordinate integrin trafficking and recycling to the plasma membrane [84] suggesting that modulation of PI3 K levels may constitute a potential therapeutic strategy for XLCNM.

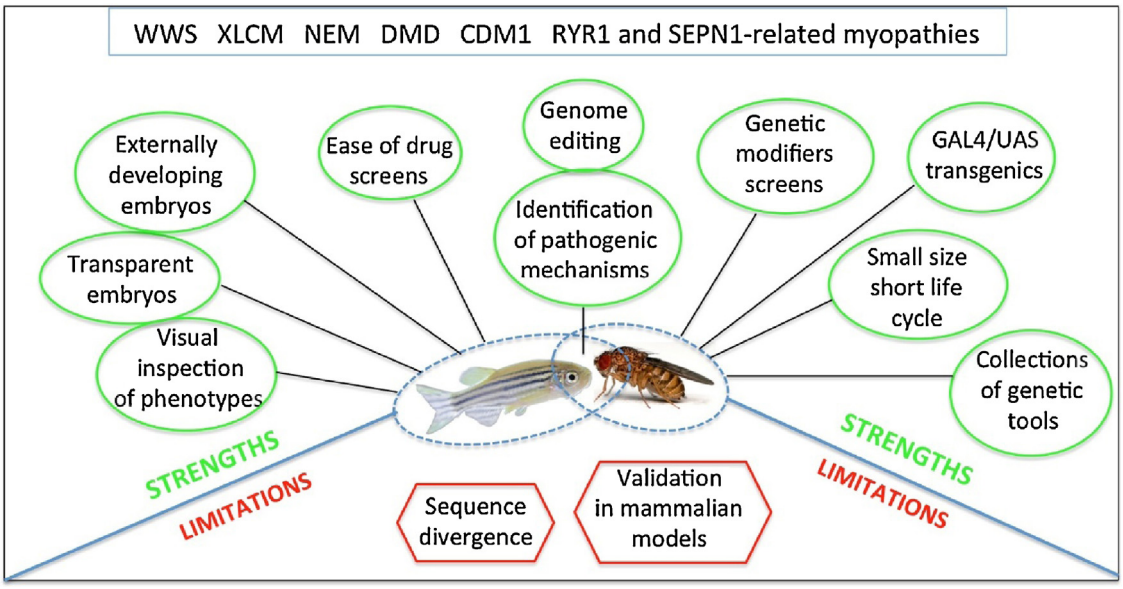

Fig. 3. A schematic representing examples of early-onset myopathies for which Drosophila and zebrafish models have been generated. Particular strengths (in green circles) and limitations (in red hexagons) of each model are listed. Notice that genome editing approaches could now be applied to both model organisms and represent an important advantage

The role of MTM1 as the primary PI3P phosphatase in skeletal muscle development has been determined in the zebrafish model of XLCNM [85]. It has been found that *mtm1* morphants display an increase of PI3P in the skeletal muscles but not in the whole embryos. Muscle from the *mtm1* morphants displayed centrally located nuclei, affected neuromuscular junctions (NMJs) and skeletal muscle hypotrophy, mimicking the human disease [85]. Interestingly, the skeletal muscle phenotype of the mtm1morphants was rescued by delivery of *mtmr1* and *mtmr2*, the closest *mtm1* paralogs, indicating that the primary defects in the morphants were due to a lack of compensation by other phosphoinositide phosphatases [85]. Reduction of myotubularin in skeletal muscle affected also the organization and the morphology of the T-tubules and sarcoplasmic reticulum (SR), suggesting that the regulation of PIs phosphorylation is essential for the excitation-contraction coupling machinery. These defects in internal structures of myofibers concomitantly with affected NMJs are potentially at the origin of diminished touch-evoked escape behavior observed in zebrafish model [85] and may thus represent a main cause of muscle weakness in XLCNMs patients.

4.1.3. *RYR1-related myopathies*

Mutations in the skeletal muscle ryanodine receptor gene (*RYR1*), encoding a calcium channel and a key regulator of calcium homeostasis, lead to defective excitation-contraction coupling and are at the origin of the most common group of congenital muscular diseases in childhood [86]. Disease manifestations include impaired ambulation, muscle weakness, eye movement paralysis, joint contractures, progressive scoliosis and in some cases respiratory failure, which can lead to early mortality [87]. To date no adapted treatments have been developed to *RYR1*-involving myopathies highlighting a need to characterize affected cellular pathways and underlying gene deregulations. To address this issue, a zebrafish model of *RYR1*-related myopathies termed *relatively relaxed* (*ryr*) [88] has been applied for transcriptional profiling. Several cellular pathways including oxidative stress have been found deregulated. Importantly, it has been reported [89] that patient myotubes also displayed aberrant oxidative stress, suggesting that anti-oxidant treatment could represent a therapeutic strategy. Indeed, using the anti-oxidant N-acetylcysteine resulted in restoration of *ryr* zebrafish motor function to the level of control fish [89]. Thus, this animal model-based approach lead to the identification of a relevant pathophysiological mechanism in *RYR1*-related myopathies and indicated a promising therapeutic strategy.

4.1.4. *ACTA1-associated nemaline myopathy (NEM)*

NEM is characterized by muscle weakness often associated with hypotonia, and the presence of the rod-like (nemaline) bodies within the muscle fibres. Nemaline rods are electron-dense bodies that show structural similarity to the sarcomeric Z-discs and were found to contain Z-discs proteins such as skeletal muscle α-actin (ACTA1) and α-actinin (ACTN). The most severe forms of NEMs result in death within the first few months of life. Mutations in ACTA1 but also in genes encoding ACTA1 binding proteins have been identified to cause the disease; however, the mechanism underlying skeletal muscle weakness remains elusive, as no correlation exists between the frequency of nemaline bodies and disease severity [90]. To better understand the pathogenesis of this devastating muscular disease, fruitfly and zebrafish models of NEMs have been generated [91,92].

High sequence conservation of actin between *Drosophila* and human made it possible to reproduce some of the *ACTA1* nemaline mutations within the fruitfly Act88F actin gene specific to flight muscle [91]. As most human NEM causing mutations are dominant, the corresponding Act88F mutations were examined in a heterozygous context. All of them resulted in flightless phenotypes, showed poor myofibrillar structure and electron dense Z disc related « zebra bodies », thus producing dominant disruption of muscle structure and function, a common NEM feature [91]. Because of their localization within the actin monomer, NEM causing mutations are likely to disrupt actin–actin interactions. Among them, the G268D mutation, believed to affect intra strand-associations in the actin helix, has been found to influence flight muscles formation leading to highly disorganized myofibrils with areas characterized by aberrant actin filaments accumulation. Similarly, impaired actin incorporation and localization in the sarcomeres has been found in ACTA1D286G-EGFP transgenic zebrafish in which GFP signal was used to follow mutated ACTA1 patterns [92]. Intriguingly, the different NEM causing mutations modelled in *Drosophila* flight muscles [91] did not lead to the formation of typical nemaline rods. Instead,

Z-disc-like « zebra bodies » have been observed that could represent a similar structure. Overall, the fly Act88F NEM models being viable are well suited to study developmental, structural and functional muscle defects occurring in nemaline myopathies.

To follow the formation of nemaline bodies and get insights into their role in NEM pathogenesis, dedicated transgenic zebrafish ACTA1-EGFP mutant models have been generated [92]. As an outcome, three different subtypes of nemaline bodies exhibiting distinct stability and different subcellular origins have been identified. One subtype, with actin accumulation in the sarcoplasm, was found to be highly dynamic and associated with muscle weakness. Another nemaline body subtype formed more stable cytoplasmic rods and was characterized by a reduced sarcomeric actin staining. Finally, the third type associated with myofibrillar disorganization originated from the Z discs and formed mainly in their vicinity [92]. Thus, the in vivo examination of nemaline body formation and progression in zebrafish demonstrated that they could by highly dynamic and transitory in nature, providing explanation to the fact that their frequency is not always correlated with the NEM severity.

4.1.5. Duchenne muscular dystrophy

DMD is due to loss of dystrophin, a key component of the dystrophin-associated glycoprotein complex (DGC). Knowledge gained so far on dystrophin function suggests that it plays a double role: first, it maintains muscle membrane integrity during contraction by linking the sarcolemmal α-DG to the actin cytoskeleton; second, it constitutes a scaffold for signal transduction in contracting muscle [93]. However, despite the three decades of research on mouse model of DMD, the mechanisms whereby lack of dystrophin causes muscle degeneration remain incompletely characterized and no effective cure has been developed to treat DMD. This is in part due to a profound muscle regeneration response, leading to a non-lethal phenotype in the commonly used *mdx* mouse model, thus indicating that alternative animal models could provide further insights into DMD.

The conservation of genes forming the DGC complex including a single, highly conserved dystrophin orthologue makes the fly a well-adapted model to study DMD [93]. Indeed, muscle-specific RNAi knockdown of dystrophin or of α-DG leads to severe muscle degeneration and progressive climbing deficits of adult flies [94]. Furthermore, mutations in fly dystrophin also cause heart defects with age-dependent disruption of cardiac myofibrillar architecture, alterations in cardiac performance and development of dilated cardiomyopathy as seen in DMD patients [95]. *Drosophila* dystrophin and α-DG are also specifically expressed at the postsynaptic neuromuscular junctions (NMJ). Interestingly, muscle-specific RNAi-mediated knockdown of a dystrophin isoform (DLP2) that localizes to the NMJs leads to an increase of neurotransmitter release, a phenotype that is rescued by postsynaptic expression of DLP2 [96]. Thus, this phenotype suggests that dystrophin modulates a retrograde signaling pathway from muscles to motor neuron terminals.

Dystrophin deficiency has also been modelled by morpholino knockdown and by using identified null mutations in zebrafish [97]. A null *dystrophin* allele called *sapje* was identified in a forward genetic screen and found to closely resemble the human DMD condition both in disease severity and progression [98–100]. Importantly, and in contrast to mouse *mdx* model, the zebrafish *dystrophin* mutants displayed abundant necrotic myofibers, extensive fibrosis and insufficient proliferation of muscle progenitors unable to compensate for muscle loss [93]. The zebrafish *sapje* mutants were employed in a small-molecule screen that identified several compounds with positive effect on the dystrophic phenotype [101]. Moreover the exon skipping therapeutic strategy based on administration of antisense oligonucleotides that alter splicing has been tested in zebrafish *dystrophin* mutants. Altogether, these experiments demonstrated the value of zebrafish model in elaborating and testing drug and exon skipping based therapies for DMD.

4.2. Strengths and limitations of Drosophila and zebrafish models

The examples discussed above, but also *Drosophila* and zebrafish models of other myopathies such as congenital myotonic dystrophy 1 [102] demonstrate their value in assessing pathogenic mechanisms of human muscular diseases. When compared to vertebrate models, the clear advantage of *Drosophila* is the abundance of genetic tools that allow rapid investigation of gene function in vivo (Fig. 3). Among them, different transposable elements, including the most common P element, have been "hopped" throughout the genome to provide insertions in the majority of genes. The transposon-carrying vectors are also used for the generation of transgenic flies and in particular those designed for binary GAL4/UAS targeted expression system [103] equivalent to the Cre/Lox system in mice. Considering the power of this system, the UAS-RNAi [104] and UAS-ORF [105] line collections have been generated, allowing tissue-specific gene knockdown or overexpression, respectively. However, analyzing particular human disease mutations in the *Drosophila* model is to some extent limited by the divergence of sequences and/or exon/intron structures even if the mutated gene is conserved (Fig. 3).

Another strength of the fly model is the speed and relative ease in performing large-scale genetic screens that allow identifying suppressors or enhancers of disease phenotypes. This is due to the small size of animals, large numbers of progeny and short life cycle taking only 10 days from embryo to fertile adult. One particularly powerful screening scheme, involving the GAL4/UAS system, is based on the effect of gene expression in the fly eye. Because the eye is not required for viability, otherwise lethal mutations can be readily studied when targeted to the eye [106]. Importantly, the *Drosophila* eye, built of about 800 ommatidia, displays a highly repetitive structure allowing amplification of what might otherwise be subtle phenotypes. The macroscopic aspect of fly eye also facilitates inspection, making it adapted for a high-throughput scale. Alternatively, large-scale loss or gain of gene function screens could be designed to identify genetic modifiers able to rescue/improve mobility defects [107].

Both eye morphology and mobility-based screenings could be applied for testing drugs/small molecules in *Drosophila*. However, for large-scale screening of molecules zebrafish model appears better adapted than *Drosophila*. This is mainly due to the ease of drug administration, embryo transparency and visual assessment of muscle structure and mobility phenotypes (Fig. 3) [71,77]. Genome sequence of zebrafish revealed that it conserves as much as 84% of genes known to be associated with human diseases, with high degrees of synteny between the conserved genes [108]. The zebrafish offers another advantage particularly important for modeling early-onset diseases: it can quickly produce a high number of externally developing, diploid embryos allowing real-time imaging of all developmental stages [109]. To generate disease models, three main ways are used in zebrafish: morpholino-mediated knockdown [110], N-ethyl-N-nitrosourea (ENU) mutagenesis, which was widely used to identify new genes involved in muscles diseases [98,100] and more recently genome-editing technologies [111] based on TAL effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) associated with Cas9 endonuclease (CRISPR/Cas9) methods.

The main limitation in using fly, but also to some extent zebrafish, to model human diseases is related to the fact that all findings need to be validated in mammalian systems before they are applied to the clinic. Validation step is crucial especially in the con-

text of drug screenings where doses and physiological effects could substantially differ between non-mammalian models and humans.

4.3. Future challenges –disease models engineered by genome editing

The possibility of introducing specific DNA elements by homologous recombination using the TALEN [112,113] or the CRISPR/Cas9 system [114–116] provide means to test the impact of non-null mutations and to generate fly and zebrafish models with particular gene variants mimicking pathological condition in Human (Fig. 3). Thus, genome-editing in simple animal models, and in particular highly efficient CRISPR/Cas9 technology opens new avenues to generate patient-specific models with hope to bridging gaps between patient genotyping and phenotyping [117,118]. Combining engineered zebrafish models carrying particular human mutations with *in vivo* compound screens could allow identifying new drugs and treatment schemes.

The availability of simple non-mammalian models dramatically improved our understanding of the molecular underpinnings and pathogenesis of early onset muscular dystrophies. Aside from the zebrafish and fruit fly discussed here, CRISPR/Cas9 is currently being employed to engineer human disease-associated mutations in larger animals, including pigs and non-human primates [119]. It is likely that a fully integrative approach, with extensive use of a variety of novel genome-edited animal models, will be required for major steps forward and for translating therapeutic targets to human patients.

5. Conclusion

Assessing mechanical features of single muscle cells represents a promising way to identify common molecular pathways underpinning muscle weakness and/or stiffness in early-onset myopathies, in a variety of mutations. The mechanical force-based approaches could help a better characterization and selection of pertinent cellular models. In parallel, the development of engineered microtissues and simple non mammalian animal models opens the way for efficient drug screening environments to develop new therapeutic strategies. In conclusion all the tools and models highlighted here will likely provide valuable opportunities to elucidate how mechanical, biochemical, and genetic/epigenetic cues modulate muscle formation, architecture and function, opening an exciting avenue for further understanding of muscle weakness in early-onset myopathies.

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