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Skeletal muscle α -actin diseases - “actinopathies”

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Abstract

Mutations in the skeletal muscle α -actin gene (*ACTA1*) cause a range of congenital myopathies characterised by muscle weakness and specific skeletal muscle structural lesions. Actin accumulations, nemaline and intranuclear bodies, fibre type disproportion, cores, caps, dystrophic features and zebra bodies have all been seen in biopsies from patients with *ACTA1* disease, with patients frequently presenting with multiple pathologies. Therefore increasingly it is considered that these entities may represent a continuum of structural abnormalities arising due to *ACTA1* mutations. Recently an *ACTA1* mutation has also been associated with a hypertonic clinical presentation with nemaline bodies. While multiple genes are known to cause many of the pathologies associated with *ACTA1* mutations, to date actin aggregates, intranuclear rods and zebra bodies have solely been attributed to *ACTA1* mutations. Approximately 200 different *ACTA1* mutations have been identified, with 90% resulting in dominant disease and 10% resulting in recessive disease. Despite extensive research into normal actin function and the functional consequences of *ACTA1* mutations in cell culture, animal models and patient tissue, the mechanisms underlying muscle weakness and the formation of structural lesions remains largely unknown. Whilst precise mechanisms are being grappled with, headway is being made in terms of developing therapeutics for *ACTA1* disease, with gene therapy (specifically reducing the amount of mutant skeletal muscle α -actin protein) and pharmacological agents showing promising results in animal models and patient muscle. The use of small molecules to sensitise the contractile apparatus to Ca^{2+} is a promising therapeutic for patients with various neuromuscular disorders, including *ACTA1* disease.

4-6 Keywords:

Skeletal muscle α -gene (*ACTA1*) diseases

Clinical phenotype

Genetics

Pathology

Pathobiology

Future directions

General Introduction

The existence of skeletal muscle α -actin disease was suggested in two publications in 1995: the paper describing slow α -tropomyosin (*TPM3*) as the first known nemaline myopathy gene [42] and a review of sarcomeric protein diseases [39]. The review stated: *“It is difficult to imagine the phenotype of skeletal muscle α -actin disease. However, although some mutations might not be compatible with life, there will probably be a spectrum of disease severity associated with different mutations. It is perhaps safe to assume that someone, somewhere in the world is suffering from ‘actin’ disease.”*

It is one thing to hypothesise that a disease might exist, another to prove it. The crucial paper that paved the way to the identification of the skeletal muscle α -actin diseases was the paper by Goebel et al describing three unrelated patients with similar muscle pathology [21]. One child died of cardiorespiratory insufficiency at 4 months, another at 3 months when respiratory support was withdrawn, and the third child was still alive at 4.5 years of age. Two patients had heart disease - one cardiomyopathy, the other cardiomegaly [21].

Strikingly, the muscle biopsies from the three patients showed many myofibres with large areas devoid of sarcomeres containing homogenous material, consisting of dense masses of thin filaments arranged haphazardly. These areas did not stain for myosin adenosine triphosphatase or for oxidative enzymes, so were not hyaline bodies (myosin storage myopathy) or ragged red fibres [21]. The large masses of thin filaments were shown by immunological electron microscopy to contain striated muscle actin. The muscle biopsies from all three patients also showed variation in fibre sizes [21], whilst two had intranuclear rods and two sarcoplasmic rods.

Due to these findings, Goebel et al [21] used the terms “congenital myopathy with excess of thin myofilaments” or “actin myopathy” to describe the disease in the three patients (OMIM #102610). They believed that at that time only two such cases had previously been described: one briefly [38] and another in more depth by [5]. Later the terms “actin aggregate myopathy” [67] or “actin filament aggregate myopathy” [22] have been used.

Overall, the muscle pathology suggested the skeletal muscle α -actin gene (*ACTA1*), which codes for the predominant actin in postnatal skeletal muscle, as the candidate gene for the disease in the three children. Equally, likely candidates appeared to be factors involved in actin biology for example in actin synthesis and normal folding, insertion into the muscle thin filament, breakdown of the thin filament or recycling. However, Nowak and colleagues [50] later identified mutations in *ACTA1* in all three patients and in 11 other patients with nemaline myopathy, or intranuclear rod myopathy.

Our purpose here is to review and update the clinical aspects of the “*ACTA1* diseases”, the muscle pathologies, genetics and pathophysiology and suggest future perspectives for research and therapy.

Clinical aspects

Skeletal muscle α -actin is the major protein component of the skeletal muscle thin filament. It is essential for muscle contraction and therefore movement and breathing since myosin, through binding actin, generates the force of muscle contraction [30]. *ACTA1* mutations therefore are likely to have a significant effect on muscle contraction. In fact, the most frequent clinical presentation of *ACTA1* diseases is that of a severe congenital myopathy phenotype, of hypotonia and weakness at birth (possibly no spontaneous movements), a myopathic face, high arched palate, no spontaneous respiration or poor respiratory function, and a poor suck. Death frequently results before one year of age [41], while some of those patients who do survive are severely incapacitated and may require constant mechanical ventilation [71]. However *ACTA1* diseases, as hypothesised, range in severity. Some of the even more severely affected patients are diagnosed with foetal akinesia and thus have onset early *in utero* [60, 64], whilst others have adult onset [1]. As will be discussed later in the section on muscle biopsy findings, multiple different muscle pathologies may be seen in association with *ACTA1* mutations, but, in general, the clinical phenotypic spectrum seems to be the same despite variable muscle pathology. Patients with the same *ACTA1* mutation, including those from the same family, can however exhibit varying disease severities [29,32,41], suggesting “modifying factors” for disease severity, either genetic or environmental (see below).

Electromyography in the severe cases may be “neurogenic” [50], as may occur in any severe congenital myopathy [83], and patients may therefore receive a clinical diagnosis of spinal muscular atrophy [50].

The heart is not involved in the vast majority of *ACTA1* disease patients [41], but hypertrophic cardiomyopathy may occur with some mutations, for example [36], and may be sufficiently severe to be fatal [12]. The extraocular muscles are spared in all cases, so that even in severely affected infants, there is no ophthalmoplegia.

The range of clinical phenotypes associated with *ACTA1* mutations was recently significantly expanded, with the identification of an *ACTA1* mutation in a patient with muscle stiffness, hypertonicity and hypertrophy who showed episodes of paroxysmal muscle contraction [34]. The muscle biopsy showed nemaline bodies, which was discordant with the clinical phenotype. *ACTA1* mutations have also recently been suggested to have extramuscular effects in the central nervous system and skeleton formation [60].

Therapeutic aspects

There is currently no curative therapy for *ACTA1* diseases. The most important aspect in the care of patients is therefore management of the symptomatology and recently comprehensive standards of care for the congenital myopathies were published [85]. The most important part of symptomatic care is respiratory intervention, particularly nocturnal ventilation [83]. Respiratory insufficiency, especially nocturnal respiratory insufficiency, may be insidious but nevertheless life-threatening, even if the disease is otherwise mild [35]. Cardiac function should also be monitored. There should also be intervention for speech and swallowing difficulties, scoliosis, provision of orthotics and physiotherapy is always warranted.

The benefit of long-distance cycling has been documented for one patient with an *ACTA1* mutation [32]. There has also been an anecdotal report [37] and a small clinical study [59] of the benefits of dietary L-tyrosine for nemaline myopathy patients.

Until effective therapies are developed and perhaps even afterwards, depending on the choice of each affected family, accurate genetic counseling, preimplantation and prenatal diagnoses are effective and practical solutions for each family, despite the difficult choices involved.

Muscle biopsy findings

ACTA1 mutations cause nine different muscle pathologies (Figure 1). From A to Z these are: actin filament aggregates [50], caps [28], core-like areas [36,84], dystrophic features [80,82], fibre type disproportion [40], intranuclear rods [50], minimal change [36], nemaline bodies [50] and zebra bodies [66]. The muscle biopsy from any one patient may demonstrate admixtures of the muscle pathologies such as nemaline bodies and core-like areas [35], or actin filament aggregates, intranuclear rods and nemaline bodies [64]. This overlap in pathologies, even within one biopsy, has led to the concept of a spectrum of pathologies associated with *ACTA1* mutations, which makes the boundaries between traditional and novel entities less clear.

The pathological features associated with *ACTA1* disease can also result from defects in more than one gene, adding other complexity. For example, aside from *ACTA1*, caps can be caused by β -tropomyosin (*TPM2*) [44,73] and α -tropomyosin (*TPM3*) [13,51] mutations. Nemaline bodies may also result from mutations in α -tropomyosin_{SLOW}, beta-tropomyosin, nebulin, muscle specific cofilin, troponinT_{SLOW} and, the latest, BTB (POZ) domain containing 13 [84]. With so much clinical and pathological overlap, it is rarely possible to identify the causative gene from the pathological features. Notably however, no genes other than *ACTA1* have been shown to cause frequent intranuclear rods or actin aggregates, although some patients with these pathologies have not been found to have *ACTA1* mutations using the current screening protocol (NG. Laing, unpublished observations), suggesting other genetic causes, or an as yet unidentified mutation affecting *ACTA1*.

We shall briefly comment on each muscle pathology associated with *ACTA1* mutations.

Actin filament aggregates

Actin filament aggregates, as described above, were the first pathology to suggest *ACTA1* disease existed and led directly to the identification of the first mutations identified in *ACTA1* [50].

Caps

Caps are sub-sarcolemmal structures comprised mainly of disorganised thin filaments. They react strongly for nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), actin and α -actinin; moderately for other thin filament or Z-band related proteins such as desmin; and show reduced staining for myosin and reduced activity for myosin adenosine triphosphatase (ATPase) [20,28].

There is only one reported case of caps caused by an *ACTA1* mutation (M49V) [28]. Of note is that a biopsy taken from a young age showed non-specific myopathic features, a subsequent biopsy at an older age displayed caps [28], highlighting a need for repeated biopsies to establish accurate diagnosis for some patients. Most caps are sub-sarcolemmal and whilst peripherally located, extend to central regions, however there were none found to be only internal [28]. The caps weakly stain by SDH, ATPase and Gomori trichrome stains, whilst by NADH they stain strongly [28]. Caps consist of loosely arranged, abnormal thin filaments by electron microscopy, and in some cases, expanded Z bands [28].

Core-like areas

Cores, core lesions or core-like areas, are focused areas without oxidative enzyme activity, and can be further sub-classified as central cores (large, singular cores in the centre and along the entire length of a myofibre) or minimicores (multiple, smaller cores). Different types of cores can be seen within the same muscle biopsy, or separately in different biopsies within the same patient, or from affected members belonging to one family [68].

Currently only two mutations in *ACTA1* have been described as resulting in cores, and these produced what the authors describe as a “core only” myopathy, due to the absence of other striking features in all affected individuals (except type I fiber predominance) [36]. Amongst affected individuals, the appearance of cores, their size and prevalence differed. The D1Y mutation mainly produced singular, unstructured core lesions, which were central or eccentric, and poorly circumscribed [36]. Although these cores were broad, they were not found along the whole long axis of muscle fibers [36]. Actin immunostaining was normal Whilst also poorly circumscribed, central or eccentric, cores associated with the E334A mutation were very small, and multiple.

Dystrophic changes

Rather than showing a pattern of pathology consistent with a congenital myopathy, the muscle pathology may be remarkably dystrophic with certain *ACTA1* mutations. Mutations of the *ACTA1* stop codon lead to translation of part of the 3' untranslated region, resulting in a larger than normal ACTA1 protein [80]. One patient compound heterozygous for a missense, and single nucleotide deletion mutations, was reported to have a non-specific congenital myopathy, with the biopsy exhibiting some dystrophic features [82].

Fibre type disproportion

Fibre type disproportion is atrophy or hypotrophy of type I fibres, and hypertrophy of type II muscle fibres. It is known that such a histological pattern can be detected as a secondary finding in a wide range of disorders (eg dystrophies [79], syndromes [24] and also in association with other features, for example along with nemaline bodies [1]. The three original cases of fibre type disproportion caused by *ACTA1* mutations all had severe congenital weakness and respiratory failure [40]. Their diagnosis was “congenital fibre type disproportion” (CFTD), which is used when patients show clinical features of a congenital myopathy, fibre type disproportion >25% between fibre types and the absence of any other pathological features [7]. Interestingly, the authors noted that none of these *ACTA1* patients had ophthalmoplegia [40], which is seen in about 50% of patients with severe CFTD [7].

Intranuclear rods

Intranuclear rods are electron-dense, rod-like structures found within the nucleus, immunoreactive for actinin like cytoplasmic nemaline bodies [24]. Intranuclear rods are often much larger than sarcoplasmic rods, and may stretch the length of several sarcomeres [25]. Interestingly, there have been no reports of intranuclear rods lying over the nuclear envelope, ie. “entering” or “leaving” the nuclei of cells, suggesting that they are formed inside the nucleus rather than being imported into the nucleus as a rod-like structure. Some patients have intranuclear rods in only a few muscle fibres, yet others have been shown to have rods in the majority of their myofibre nuclei [25,29]. A nucleus may contain single, or multiple intranuclear rod bodies [25].

The number of muscle fibres affected with intranuclear rods is thought to correlate with the severity of disease [25]. In Goebel & Warlo’s series of reviewed cases, the eldest patient was alive at 25 years and had intranuclear rods in only 2% of his muscle fibres, whereas the most severe patients (1 died at 6 days, another at 2 months) had 80% of muscle nuclei with rods [25].

Minimal change

For some patients with muscle weakness, there is nothing remarkable on muscle biopsy, only minimal, nonspecific changes, and no obvious abnormalities by electron microscopy. Such “minimal change myopathy” [47] or non-specific changes may be seen in *ACTA1* [28,36].

Nemaline bodies

Nemaline bodies are the commonest pathology associated with *ACTA1* mutations, causing approximately 20% of all nemaline myopathy cases, and about 50% of the severe presentations [32,82]. It is generally accepted that nemaline myopathy was first described, nearly 40 years ago, by two separate groups [8,70]. However, the first documented description of a patient with nemaline myopathy was probably by the Australian Dr Douglas Reye who in 1958 described a 4-year old child with a “rod myopathy” [63]. This patient was shown over 40 years later to have an *ACTA1* mutation (I136M; patient 4, Ilkovski *et al.* 2001 [32]).

In Greek, “nema” means “thread”, and nemaline bodies are threadlike structures that often occur in the sarcoplasm as sub-sarcolemmal structures. Nemaline bodies are not obvious with paraffin-embedded, haematoxylin and eosin stained muscle biopsies. They can however be visualised by the modified Gomori trichrome staining but are best visualised by electron microscopy [70].

Ultrastructurally, nemaline bodies are electron dense structures, with a density similar to that of the sarcomeric Z-line. They occasionally appear in close proximity to areas of Z-line widening and can often be seen in continuity (“lateral extensions”) with the Z lines of adjacent sarcomeres [18]. Rods are filament-like and contain a double striation when they are sectioned parallel to their longitudinal axes. For these reasons it was hypothesised that nemaline bodies originate in the Z-line and would consist of Z-line proteins. Immunofluorescence techniques have revealed that indeed Z-line-related proteins are present in nemaline bodies, namely α -actinin [72], myotilin [65], desmin [62], and actin [86].

In many cases of nemaline myopathy, muscle biopsies contain a predominance of type I fibres, with these fibres being atrophied [23]. Nemaline bodies can however be associated with other myopathies, and are also seen in ageing muscle, extraocular muscles, and at the myotendinous junction [18].

The relationship between the presence of nemaline rods and the muscle weakness in nemaline myopathy is unknown. No correlation between the number of nemaline rods present and muscle weakness was found in sequential samples from patients [81].

Zebra bodies

Zebra bodies are striped, rod-shaped bodies visible by electron microscopy. They can be present near disorganised regions, but also in relatively normal looking muscle [43,56]. The cases reported by Lake & Wilson [43] and Reyes *et al.* [56] had nemaline as well as zebra bodies. The Lake & Wilson patient [43] was subsequently identified as having an *ACTA1* mutation [66]. On examination of a second biopsy taken from this patient at the age of 29 years, multiple features were noted, such as fewer zebra bodies than the original biopsy, ring fibres, type I fibre predominance and a wide variation in fibre size, excess internal nuclei, actin accumulation, cytoplasmic bodies, fibrosis, rods and core-like areas [43]. Patients with homozygous *ACTA1* null mutations, leading to absence of ACTA1 protein, also had zebra bodies [49].

Genetics

Affected gene and its structure

The *ACTA1* gene at chromosome 1q42 consists of 7 exons, 6 of which (exons 2-7) code for the skeletal muscle α -actin protein (ACTA1) [74]. Mature skeletal muscle α -actin protein is 375 amino acids, created by cleavage of the N-terminal 2 amino acid residues from the precursor protein [57].

Since *ACTA1* is such a small gene (less than 4 kb total genomic length), it is relatively easy to sequence using Sanger sequencing. Many diagnostic laboratories in multiple countries now screen the *ACTA1* gene for mutations and we would urge all laboratories analyzing the gene to submit definitive mutations, polymorphisms and variants of uncertain effect to the *ACTA1* locus-specific database (http://www.dmd.nl/nmdb2/home.php?select_db=ACTA1).

Mutation spectrum

There are now nearly 200 known *ACTA1* mutations, curated in the *ACTA1* locus-specific database. Whilst most of the *ACTA1* mutations are missense mutations, there are also nonsense mutations, splice site mutations, frameshift mutations, a two amino acid duplication, mutations of the stop codon, missense mutation of two consecutive amino acid residues and insertion of an amino acid residue. The mutations are distributed throughout most of the peptide sequence of the ACTA1 protein (Figure 2). No non-synonymous missense polymorphisms have been definitively identified [41]. Thus, one can almost be certain that any amino acid change identified in *ACTA1* will be disease-causing. This relates to the high level of conservation of the amino acid sequence of actin isoforms.

Dominant *ACTA1* disease mutations

90% of *ACTA1* mutations cause dominant disease. These are mostly missense mutations, but also include amino acid duplication and insertion mutations and the stop codon mutations [41]. The dominant mutant ACTA1 proteins are therefore abnormal proteins and it is hypothesised that they interfere (as “poison peptides”) with the function of the normal ACTA1 expressed from the normal allele in patients [71]. The majority of patients with dominant *ACTA1* disease have *de novo* dominant mutations not present in the peripheral blood DNA of either parent [41]. This means that the family structure looks like recessive disease (affected child, unaffected parents) but the disease in the affected child is in fact dominant. Families with dominantly transmitted *ACTA1* mutations tend, obviously, to have milder disease, since the patient has to reach adulthood in order to be able to transmit the disease.

Recessive *ACTA1* disease mutations

Around 10% of *ACTA1* mutations cause recessive disease [41], and occur more frequently in consanguineous communities [49]. Most of these are genetic null mutations (nonsense, frameshift, splice-site mutations) and lead to a total absence of ACTA1 protein, or a lack of functional ACTA1 protein [41,49]. However, certain missense mutations are associated with recessive disease [41,49] and these have been shown to be functional null mutations in that they prevent the normal folding of the actin protein, so that no mature protein is produced [9].

***De novo ACTA1* mutations**

The fact that a large proportion of patients with *ACTA1* mutations do not reach adulthood means that *ACTA1* mutations are generally genetically lethal. In the 2009 review of *ACTA1* mutations [41], 84% of sporadic patients where parental DNA was available had *de novo* dominant mutations, not recessive mutations, suggesting a high new mutation rate. This indicates that this is the mechanism by which the disease retains its prevalence worldwide.

Somatic and gonadal mosaic families

The high *de novo* mutation rate found in *ACTA1* is also associated with somatic or gonadal mosaicism in families [41,50]. In somatic mosaic patients, only a proportion of myonuclei are carrying the *ACTA1* mutation and therefore the proportion of mutant ACTA1 protein is less, suggesting that one modifying factor in dominant disease is the amount of mutant protein (Figure 3). In gonadal mosaicism, the *ACTA1* mutation is not present in the peripheral blood cell nuclei, but is present at an unknown level in the ovaries or testes of the parent and thus the parent may have multiple affected children. The level of gonadal mosaicism determines the recurrence risk for subsequent pregnancies, which may be anything from zero (if the level of gonadal mosaicism is zero), to 50% (if 50% of the sperm or eggs carry the mutation).

Pathophysiology

Actin thin filaments interdigitate myosin thick filaments, and the interaction between myosin heads in the thick filament and the actin monomers in the thin filament produces the force needed to slide the thick and thin filaments past each other, shortening the sarcomere in muscle contraction in what is referred to as the sliding filament model of muscle contraction [30]. Reflecting its major role in skeletal muscle, actin accounts for more than 20% of muscle protein [69].

Given the fundamental role of actin in skeletal muscle function, and how intensely actin has been studied one might think that the effect of *ACTA1* disease-causing mutations on muscle function should be obvious and simple to determine. However, the precise pathobiology of the *ACTA1* disease mutations remains largely unclear, despite intensive research. One thing is certain, different *ACTA1* mutations have different effects on function.

As well as binding myosin, actin interacts with an estimated >100 other actin-binding proteins [16]. Since it is a relatively small globular protein, most of its surface is functionally involved. This multifunctionality must relate to the very high conservation in amino acid sequences between actin isoforms and across species, to the extent of 87% identity at the amino acid level between rice and human actins [69], and 99% identity between human skeletal muscle and cardiac α -actins [75]. The high conservation of actin proteins through phylogeny [76] means that the study of actin function in almost any species is relevant to *ACTA1* diseases.

Inferences about pathophysiology from studying recessive and dominant ACTA1 disease patients

The pathophysiology of recessive *ACTA1* disease seems clear, in that the disease is caused by genetic or functional null mutations [49]. Nemaline bodies form in recessive cases lacking mutant Acta1 protein, and therefore nemaline bodies in these patients are not due to the presence of a mutated protein.

The pathomechanism of dominant *ACTA1* diseases is less clear apart from that they involve poison peptides. It might also be inferred that the nemaline bodies in the hypercontractile patient must form in a different way to those in patients with nemaline myopathy phenotype of hypotonia and weakness. In keeping with this, expression in tissue culture of the K326N mutant protein identified in the patient with the hypercontractile phenotype, did not show rod-shaped structures like all the mutant ACTA1 proteins associated with hypotonic nemaline myopathy that have been tested previously in tissue culture.

Epigenetic factors

Autosomal recessive *ACTA1* disease patients retain high levels of cardiac α -actin protein (ACTC) in their skeletal muscles [1,49]. ACTC is the predominant actin isoform expressed in foetal skeletal muscle and the adult heart [31]. The severity of recessive *ACTA1* disease correlates at least in part with the amount of retained ACTC [49].

There is anecdotal [31,50,52] and more recently, experimental evidence [53,54], that the amount of mutant ACTA1 protein within muscle influences dominant *ACTA1* disease severity. In all cases of *ACTA1* disease the extraocular muscles are spared, even in severe cases in which the patients experience almost complete paralysis. This is likely due to the co-expression of ACTC in extraocular muscles [52] resulting in a dilution of the proportion of mutant ACTA1 present. Similarly, in most cases the heart is spared, despite ~30% of the actin pool comprising ACTA1 [31]. Thus the relative proportion of mutant ACTA1 to the total actin pool is thought to modify the dominant-negative effects of mutant ACTA1. Further anecdotal evidence comes from studies of somatic mosaic families (as mentioned above) [41,50].

Experimentally, the amount of mutant ACTA1 expressed in a transgenic mouse model harbouring the D286G mutation correlates with disease severity, with the phenotype becoming much more severe with an increase from ~25% mutant actin to ~45% mutant actin [53,54].

Theoretical analysis of actin mutations

Some of the first analyses performed on the *ACTA1* mutations were theoretical analyses of the likely effects of the mutations on actin function, based on the position of the mutated amino acid in the actin molecule and prior knowledge of the functions of the different regions of the actin protein [71]. This suggested clustering of the mutants associated

with at least some of the mutations causing particular phenotypes in the monomer, for example clustering of mutations associated with actin filament aggregates and intranuclear rods around the nucleotide binding cleft and hinge region, while the majority of congenital fibre type disproportion mutations are situated on the outer surface of the monomer and are exposed in the polymer [71]. Mutations associated with the formation of nemaline bodies were on the other hand distributed throughout the actin molecule [19,71].

Tissue culture models

Early experimental studies of the effects of *ACTA1* mutations on function were performed in tissue culture. These studies demonstrated that mutations that caused for example, intranuclear rods in human patients, also caused intranuclear rods in culture, though other mutations, not associated with intranuclear rods in patients also caused intranuclear rods in culture [33]. Thus there was some consistency in the effect of mutant proteins between patients and tissue culture, but the association was not complete.

Biochemical analysis

Other studies investigated the biochemical properties of the mutant *ACTA1* proteins. These biochemical studies demonstrated that some of the mutant actins failed to fold properly and were therefore non-functional proteins [9]. These *ACTA1* proteins that failed to fold properly were associated with recessive *ACTA1* disease, confirming that the recessive mutations are functional null mutations. Other mutant *ACTA1* proteins failed to polymerise properly, whilst others showed a greater affinity for cyclase-associated protein indicating folding instability.

Serum response factor (SRF) regulates a transcriptional program crucial for muscle development and maintenance that is directly controlled by the balance between actin assembly and disassembly [78]. A study of 12 *ACTA1* mutants in cell culture, found that most showed altered SRF signaling and lower nuclear MAL (a cofactor for SRF activation and globular-actin binding partner) accumulation in response to serum [78]. These mutants also mostly showed actin co-polymerisation defects [9]. SRF activity in some of these mutants could be significantly increased by treatment with drugs that induce nuclear localization of MAL and the authors proposed that drugs that enhance SRF pathways might be of therapeutic benefit [78].

In depth analysis of the V163M intranuclear rod mutation in patient tissues and cell culture showed that the mutant *ACTA1* accounted for a small proportion of the total *ACTA1* pool, with levels of ~20% in one patient, only ~3-6% in another and undetectable levels in a third patient from the same family [15]. The amount of mutant *ACTA1* present correlated with the age of onset of muscle weakness in these patients [29]. Patient muscle also showed signs of regeneration (a feature not typically associated with nemaline myopathy, despite microarray evidence to the contrary [61]) and it was proposed that increased regeneration and degradation of the mutant *ACTA1* likely contributes to the decreased mutant *ACTA1* load and clinical severity. The V163M protein preferentially aggregated within the nuclei of cultured cells, rather than incorporating into cytoplasmic microfilaments. It was further proposed that the intranuclear aggregation of mutant *ACTA1* protein within the patient muscle might further contribute to the relatively mild phenotype, since the normal sarcomeric register can be maintained in the absence of mutant *ACTA1*.

In further, elegant, studies Domazetovska and colleagues showed that intranuclear rods formed by V163M and V163L mutant *ACTA1* arose within the nucleus (rather than entering from the cytoplasm), and that these rods were highly motile and dynamic [14]. In addition, they showed that the mechanism of rod formation by wild-type *ACTA1* in response to cellular stress was similar, indicating a common pathogenic mechanism for intranuclear rod formation. Intranuclear rods displace DNA and result in a decreased mitotic index, it is likely that these effects contribute to muscle weakness in patients with pure intranuclear rod myopathy, since the sarcomeric structure is often relatively well preserved [14,15].

In vitro motility assay studies

On the whole, there is little correlation between the mutation, pathological phenotype and the effect of the mutant *ACTA1* on parameters measured by the *in vitro* motility assay [19]. This may be due to a relatively poor understanding of the dynamic actin structure-function relationships [19]. Furthermore, mutations are likely to affect several functions together making it difficult to dissect out clear genotype-phenotype relationships.

The *in vitro* motility assay has however definitively clarified the pathobiology of two *ACTA1* mutants. The D292V mutant associated with CFTD was found to lock the thin filament in the “off” position [6]. A similar defect was observed with the insect actin E93K mutation [4,55]. Conversely the K326N mutation associated with the novel hypertonic phenotype [34] moves the thin filament equilibrium towards the “on” position.

Study of patient myofibres

The function of myofibres from one patient with *ACTA1* disease has been studied. Counter intuitively, this study showed that in single myofibres from a patient harbouring the F352S *ACTA1* mutation (with mild nemaline myopathy) greater strain was produced per individual crossbridge [45]. The *in vitro* motility assay has also shown increased force

for three other ACTA1 mutants (V132M; D268G; E336K), associated with nemaline myopathy of differing disease severities [19,71].

Animal models

Drosophila

Prior to *ACTA1* mutations being associated with human muscle diseases, a whole body of research had already been performed on the effect of missense mutations on *Act88F*, the only actin expressed in the indirect flight muscles of *Drosophila*. Numerous studies have demonstrated that some *Act88F* mutants are antimorphic (positively destroying muscle architecture and/or function) while others are hypomorphic (eg. loss of function *KM88* line) [26]. Interestingly, unlike recessive *ACTA1* disease and *Acta1*^{+/-} mice, hemizygous *KM88* flies are flightless and have defective myofibrils [3]. The extent to which *Act88F* mutants are antimorphic varies, with mutants falling into different classes. Some mutants at a ratio of 1:1 to wild-type produce partially flighted flies and at a ratio of 1:2 produce flies with almost normal flight ability. While other mutants give only a partial recovery of flight at a ratio of 1:2, and another group remain flightless at a ratio of 1:2 [11,17]. Some mutants cause only slight alterations in indirect flight muscle structure, despite functional defects, while others show a lack of sarcomeric structure, disorganised thick filaments, aberrant Z-disc structures and zebra bodies. Typical electron-dense nemaline bodies have not been reported with any *Act88F* mutants [26].

Mouse models

Mouse models of both recessive and dominant *ACTA1* diseases have been created.

Autosomal recessive *ACTA1* disease mouse model

Homozygous *Acta1* knock-out mice (*Acta1*^{-/-}) present with failure to thrive from approximately postnatal day 3 onwards, and all die by, or on postnatal day 9 [10,48]. These mice mimic the severely affected recessive *ACTA1* disease patients.

Autosomal dominant *ACTA1* disease mouse models

Tg(ACTA1)^{D286G}

The transgenic *Tg(ACTA1)*^{D286G} line has a mild disease. The mice have a normal lifespan and show no overt signs of disease, however their skeletal muscles produce reduced specific force and are less active than wild-type mice [53].

These *Tg(ACTA1)*^{D286G} mice were bred with hemizygous *Acta1*^{+/-} mice to produce *Tg(ACTA1)*^{D286G}.*Acta1*^{+/-} mice. Approximately 75% of these mice present with a severe phenotype of paralysed hindlimbs and reduced hindlimb musculature between postnatal day 10 and 17 [53]. However, at birth and during the very early neonatal period the *Tg(ACTA1)*^{Dor+/+}.*Acta1*^{+/-} pups are indistinguishable from littermate controls. This line models the severe dominant disease seen in most patients with dominant *ACTA1* disease mutations [41]. It was found that the dramatic shift in phenotype was associated with an increase in the amount of mutant ACTA1 protein from ~25% in the *Tg(ACTA1)*^{D286G} mice to ~45% in the *Tg(ACTA1)*^{D286G}.*Acta1*^{+/-} pups. Subsequently it was found that surviving *Tg(ACTA1)*^{D286G}.*Acta1*^{+/-} mice had slightly reduced levels of mutant ACTA1 protein (~35%). Thus, a small reduction in the proportion of mutant protein is coupled with a significant improvement in phenotype [53].

Tg(ACTA1)^{D286G.EGFP}

This transgenic model harbours the D286G ACTA1 mutant fused with enhanced green fluorescent protein (EGFP), reproduces the structural lesions seen in patient muscle (Figure 1) and exhibits muscle weakness [54].

KI(Acta1)^{H40Y}

Mice expressing the H40Y mutation [46] exhibit skeletal muscle pathology reminiscent of human patients (actin aggregates, cytoplasmic and intranuclear rods; Figure 4). However, whilst ~50% of *KI(Acta1)*^{H40Y} male mice die suddenly by 9 weeks of age, and a further 25% die by 5 months of age, female mice have a normal lifespan. There is no known association between disease severity and sex in human patients with *ACTA1* disease, and the discrepancy between severity in the male and female *KI(Acta1)*^{H40Y} mice remains an anomaly [46]. The male mice from this line model the severe dominant disease seen in many patients [41].

Muscle from adult mice expressing the D286G mutation (with and without the EGFP tag) and the H40Y mutation (see below) show ringbinden fibres (Figure 4), a feature rarely reported in patients with *ACTA1* mutations [66].

Future perspectives for research and therapy

Future directions in gene discovery

ACTA1 mutations have not been identified in all patients with actin aggregates (NG Laing, unpublished observations). It is unlikely that the other mutations causing actin aggregate myopathy lie in control regions for the *ACTA1* gene, since the patients with recessive *ACTA1* disease (who lack *ACTA1* protein), do not have actin aggregates [49]. This suggests that mutations in other genes, or environmental factors, can cause actin filament aggregates. The other mutated genes might code for any of the >100 known actin binding proteins, or other proteins indirectly involved in thin filament

turnover in skeletal muscle. Next generation DNA sequencing technology should facilitate discovering other genes causing actin aggregates.

Future directions in therapy

Unraveling pathobiology

Though much can be done to improve the quality of life of patients with *ACTA1* mutations, there are currently no curative therapies, and therefore there is a strong imperative that they are developed. The effectiveness of potential therapies under investigation may be effective for only certain *ACTA1* mutations, depending on the precise pathobiology of each. A great deal of further research, particularly by cell and protein biologists, is required to fully understand the disease mechanisms of *ACTA1* mutations.

Exercise and muscle fibre hypertrophy

One patient with a I136M *ACTA1* mutation was a competitive long-distance cyclist at age 45 despite having nemaline bodies in 98% of his muscle fibres (patient 4, [32]). The majority of this patient's muscle fibres were hypertrophied, suggesting that both exercise and muscle fibre hypertrophy may be beneficial for patients with certain *ACTA1* mutations.

Recently Nguyen *et al.* [46] demonstrated that two factors which induce hypertrophy of muscle fibres, FHL1 and IGF1, improved function in the *Acta1*H40Y knock-in mouse model of actinopathy, indicating that such factors are targets for *ACTA1* disease therapies.

L-tyrosine

In the same paper, oral L-tyrosine also improved muscle fibre function [46]. This is experimental confirmation of the effectiveness of L-tyrosine reported in patients [37,59]. The enticing evidence available on L-tyrosine makes a full clinical trial an imperative.

Actin replacement therapies

Replacing ACTA1 protein in a recessive *ACTA1* disease patient who lacks ACTA1, may induce an immune response. Evidence suggests that ACTC can effectively replace ACTA1 in skeletal muscle. The known best functioning *ACTA1* null patient has high levels of ACTC [49], and transgenic expression of ACTC in postnatal skeletal muscles of *Acta1* knock-out mice can rescue the mice from death by 9 days postnatal to a full adult life [48]. Upregulation of ACTC is therefore a promising therapeutic target for recessive *ACTA1* diseases. Understanding the transcription factors responsible for down-regulating ACTC expression in skeletal muscle before birth may help facilitate therapy, just as the recent identification of *BCL11A* as the major factor in switching foetal haemoglobin expression has generated new possibilities for haemoglobinopathy therapies [2].

The somatic mosaic parents, the lack of ophthalmoplegia and the absence of cardiac effects in the vast majority of *ACTA1* patients, and the results of our transgenic mouse experiments, all indicate that the proportion of mutant poison protein in dominant *ACTA1* disease is one factor that determines disease severity. Therefore, any intervention that decreases the proportion of the poison protein, and/or increases the proportion of normal, wild-type actin protein, should be beneficial to patients with dominant *ACTA1* mutations.

The somatic mosaic families indicate which *ACTA1* mutations can be effectively diluted to have a significant clinical benefit. There are currently only four such families in the *ACTA1* database. It is important for clinicians and all diagnostic laboratories analyzing *ACTA1* to continually search for these families and to submit their findings to the *ACTA1* Database. Each piece of information gained from these "accident of nature" families helps us understand more about the *ACTA1* diseases.

Therapies involving muscle stem cells [77] should be effective in treating many of the *ACTA1* mutations, since incorporation of normal stem cells into muscle fibres would lead to a similar effect to that in the somatic mosaic parents, where only a proportion of their muscle nuclei are carrying the mutation.

Certain *ACTA1* mutations may be insurmountable. End-capping mutations, mutants that block further extension of the actin polymer within the filament [27] may be very difficult to treat. Such mutants might have to be very considerably diluted before seeing any therapeutic effect.

Mutations at the actin-tropomyosin interface alter the activation state of the muscle thin filament. As described earlier the alteration may be to either decrease activation [6], or increase activation [34]. Pharmaceuticals that alter actin myosin interactions, calcium kinetics or thin filament responsiveness [58], may be able to act as therapies for these particular *ACTA1* mutations.

Summary

Since the identification of the first *ACTA1* disease-causing mutations in 1999 [50], the number of *ACTA1* mutations, and associated phenotypes and pathologies, have continually increased. We still do not understand the pathobiology of

all of the mutations, but we do have insight into the pathobiology of some. Although curative therapies have not been developed, each patient with an *ACTA1* mutation can be helped by interventions currently available. Above all at present, the fact that *ACTA1* mutations are now sought and identified in patients empowers the family through knowledge of the exact genetic cause of their child's disease. The future will surely see better understanding of the pathobiology and more effective treatments.

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Figure Legends

Figure 1: Images of muscle biopsies showing the range and variety of pathological features that can be seen in patients with *ACTA1* mutations. Biopsies A and B are both stained for NADH-TR, C – E with Gomori trichrome, F for ATPase at pH4.3, and G – J are all electron micrographs. (A) A population of small dark type 1 fibres and larger type 2 fibres, several of which show core-like areas (arrow). (B) Fibre uniformity and no distinction of fibre types, and unevenness of stain or core-like areas in some fibres (arrow). (C) From a severely affected neonate, showing moderate variation in fibre size and large clusters of red staining rods in several fibres (arrow). (D) From a severely affected neonate with a null mutation in *ACTA1*, showing a population of smaller fibres and a population of larger fibres. The rods are very small, but give a reddish colour to the small fibres (arrow). (E) Biopsy from a 7 year old infant showing large clusters of rods that are often peripheral in many fibres that show only a little variation in size. (F) Predominance and hypotrophy of type 1 fibres (stained dark) constituting fibre type disproportion. (G) Typical rods (arrows) in a longitudinal section. (H) A singular intranuclear rod. (I) A large peripheral accumulation of actin thin filaments (*) and rod like structures in an adjacent fibre. (J) A zebra body (arrow). All images (except for H) kindly provided by Prof Caroline Sewry. Image H is from Dr Komala Pillay.

Figure 2: Distribution of missense mutated amino acid residues in the peptide sequence of skeletal muscle α -actin. Amino acid residues that have been found to be mutated are indicated in red.

Figure 3: Schematic diagram of the thin filament, showing the double-stranded filamentous actin (F-actin) in two shades of pink, nebulin in black, the troponin complex in purple, and tropomyosin in turquoise. The top image contains only wildtype *ACTA1* protein. The middle image represents the scenario of a somatic mosaic parent, where only a proportion of their muscle cell nuclei contain the *ACTA1* mutation, and thus only a percentage (less than 50%) of the *ACTA1* protein (grey spheres) is mutant. The bottom image shows when a person has a dominant, heterozygous *ACTA1* mutation, which is expressed at ~50% (grey spheres) of the total *ACTA1* protein.

Figure 4: Pathology seen in gastrocnemius muscle of *KI(Acta1)H40Y* mice. Nemaline bodies seen by Gomori trichrome (A, C). Actin lakes (*) obvious by hematoxylin and eosin (B), and Gomori trichrome (C) staining. These features are also evident at the ultrastructural level (E, F). Additionally a ringbinden fibre (^) is apparent in (C). In (D), phalloidin-FITC staining (green) shows lack of uniformity for staining of filamentous actin, Hoechst (blue) shows central nuclei, whilst α -sarcoglycan (red) demarcates the myofibre membrane.

Figure 1
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1 MCDEDETTALVCDNGSGLVKAGFAGDDAPR
31 AVFPSIVGRPRHQGVVMVGMGQKDSYVGDEA
61 QSKRGILTLYPIEHGIITNWDDMEKIWHH
91 TFYNELRVAPEEHPTLLTEAPLNPKANREK
121 MTQIMFETFNVPAMYVAIQAVLSLYASGRT
151 TGIVLDSGDGVTHNVPIYEGYALPHAIMRL
181 DLAGRDLTDYLMKILTERGYSFVTTAEREI
211 VRDIKEKLCYVALDFENEMATAASSSSLEK
241 SYELPDGQVITIGNERFRCPETLFQPSFIG
271 MESAGIHETTYSIMKCDIDIRKDLYANNV
301 MSGGTTMYPGIADRMQKEITALAPSTMKIK
331 IIAPPERKYSVWIGGSILASLSTFQQMWIT
361 KQEYDEAGPSIVHRKCFX

Figure 3
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