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A Two Stage Model of Skeletal Muscle Necrosis in Muscular Dystrophy – The Role of Fiber Branching in the Terminal Stage

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1. Introduction

Branched fibers are a well-documented phenomenon of regenerating dystrophic skeletal muscle. They are found in the muscles of boys with Duchenne muscular dystrophy (DMD) and in the muscles of *mdx* "aged" mice, an animal model of DMD. However, only a handful of studies have investigated how the contractile properties of these morphologically deformed fibers differ from those of normal fibers in aged muscle. These studies have found an association between the extent of fiber branching and susceptibility to damage from eccentric contractions. They have also found that branched muscle fibers cannot sustain maximal contractions and that branch points are sites of increased mechanical stress. New imaging techniques like second harmonic imaging have revealed that the sub-cellular myofibrillar structure is greatly disturbed at branch points. These findings have important implications for understanding the function of dystrophin. It is commonly thought that dystrophin's role is to mechanically stabilise the sarcolemma, as numerous studies have shown that eccentric contractions damage dystrophic muscle more than normal muscle. However, the finding that branched fibers are mechanically weakened raises the question: Is it the lack of dystrophin, or is it the fiber branching, that leads to the vulnerability of dystrophic muscle to contractile damage? The other question is how the presence of these branched fibers alters the contractile properties of "aged" dystrophic muscle. Throughout this chapter I will use the term branched to describe the malformed fibers. Most earlier studies use the terminology "split fiber", but because it conjures up images of a Y-shaped bifurcation, with one adult fiber giving rise to two daughter fibers it is somewhat misleading as we now know many fibers, if not most display a complex syncytia of interconnecting branches. Some branches do not originate from the main fiber but are results of incomplete regeneration with myotubes fusing to repair the damaged adult fiber. Branching, I feel, is a more accurate description of the malformed fiber morphology. With respect to the branching terminology I am following the lead of Ontell & Feng, 1981 where they state "the term branched has been preferred because it describes an existing condition while the term split implies a mode formation. Unfortunately there is no immediate apparent substitute the terms parent and daughter". Readers can make up their own mind on the split verse branched question by examining morphology of fibers in the following chapter.

2. Evolutionary aspects of fiber branching

2.1 Fiber branching in crustacean proprioceptors

Even though in mammalian skeletal muscle fiber branching occurs during the regenerative process, there are a group of muscles that normally show fiber branching. These are the intrafusal muscle fibers found in muscle proprioceptors, muscle fibers specialized for their role of proprioception. In crustaceans, using confocal laser scanning and conventional light microscopy, the morphology and organization of the muscle fibers in a proprioceptor, the thoracic coxal muscle receptor organ (TCMRO), and the associated 'extrafusal' promoter muscle were investigated in two species of decapod crustacean, the crayfish *Cherax destructor* and the mud crab *Scylla serrata*. The diameter of the TCMROs was shown to increase distally, with an increase up to 350% recorded for the crayfish. The tapered shape of the crayfish TCMRO was demonstrated to amplify movements mechanically at the transducer region where the afferent nerves attach. Serial sectioning of the TCMROs, showed that the fiber number increased in the proximal to distal direction from 14 to 30 fibers in the crayfish and from 7 to 20 in the crab. Optical sectioning with laser scanning confocal microscope revealed that the increase in fiber numbers was the result of muscle fibers branching in the distal third section of the TCMRO Fig. 1.

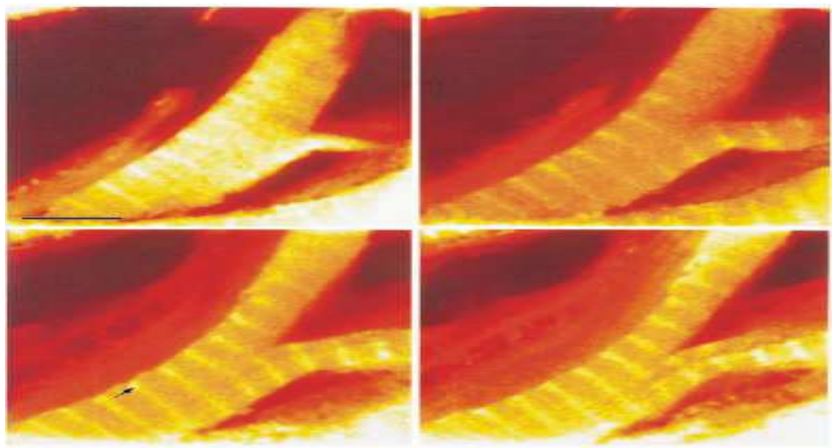


Fig. 1. Laser scanning confocal longitudinal sections of TCMROs from *Cherax destructor*. Fibers were stained with the F-actin binding dye phalloidin, conjugated with rhodamine, BODIPY, or fluorescein. —A. Preparation of a macerated crayfish TCMRO, showing a series of sections from the top of a single branched fiber stained with phalloidin/rhodamine through to the bottom section of the branch. Modified from Parkinson et al., 2001.

2.2 Fiber branching in mammalian proprioceptors

In the mammalian muscle spindle the intrafusal muscle fibers also exhibit branching, although it should be stressed that the evidence for this is not as strong as the case for crustacean proprioceptors. Vertebrate muscle spindle intrafusal fibers have been demonstrated to have the unique morphologies of the nuclear bag and nuclear chain fibers (Boyd, 1962). They are striated except for their central regions and branching has been reported in the central regions close to the area where the afferent nerve fibers originate (Barker & Gidumal, 1961). In the

muscle spinal the branching of a small or intermediate fibers takes place over a distance of 60-90 micrometers. This largely occurs in the proximal pole or in the proximal part of the equatorial region of the muscle spindle. The two fibers produced may either taper off, or reunite in the distal pole or distal part of the equatorial region. The process of branching or reuniting is distinct from the condition where one fiber branches into two over a length of several hundred microns. Fig. 2 is a serial section from a mammalian muscle spindle and it clearly shows one fiber branching into two.

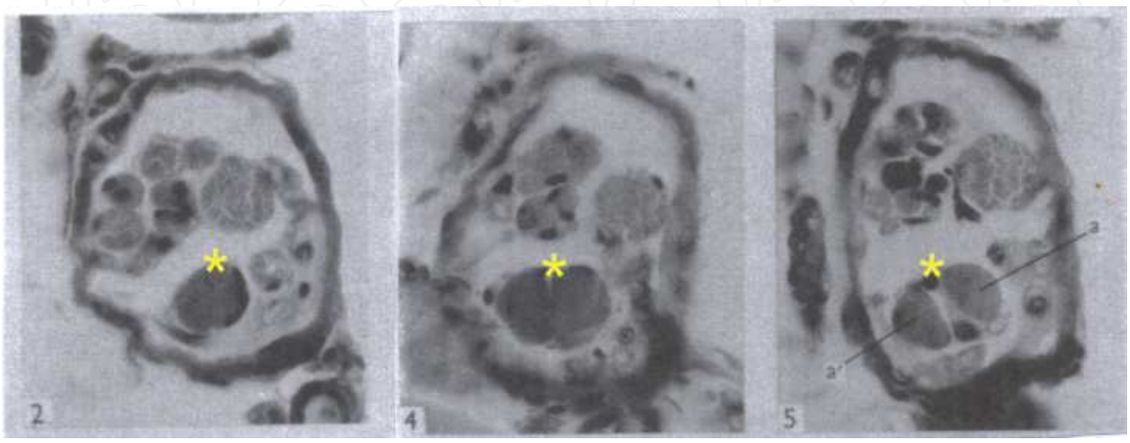


Fig. 2. Serial section through a single spindle from cat rectus femoris, the yellow * marks the position of a single intrafusal fiber as it branches into 2, in the first panel the diameter of the fiber is 11 micrometers. Serial transverse 12 micrometer paraffin sections stained with haematoxylin and eosin; numbers in the bottom left indicate the interval between segments. Modified from plate 2 in Barker & Gidumal, 1961.

2.2.1 What is the role of fiber branching in these systems?

In the crustacean receptor the tapered shape that results from fiber branching serves to amplify small movements, fine tuning the proprireceptor to respond to minor perturbations of its leg. However, overall it is tempting to speculate that fiber branching is protective and helps to protect the proprocepor intrafusal muscles from eccentric damages that occurs as a result of repeated eccentric length changes during locomotion.

3. What is a branched fiber?

A branched fiber is a skeletal muscle fiber composed of two or more cytoplasmically continuous strands. Some examples are shown in Fig.3 *B–F* and Fig.4. Branched fibers are demonstrable either by enzymatic muscle digestion (Head *et al.* 1990) or by reconstruction of serial cross-sections (Isaacs *et al.* 1973). Branching patterns vary greatly (Blaivas&Carlson, 1991;Tamaki *et al.* 1993), ranging from simple bifurcations (e.g. Fig. 3B) to complex, intertwining syncytia (e.g. Fig. 3E and Fig.4). Branched fibers have been found in muscular dystrophy (Swash & Schwartz, 1977; Ontell & Feng, 1981), in whole muscle transplants Fig.4 (Bourke & Ontell, 1984; Blaivas & Carlson, 1991), in muscles subjected to chemical or physical injury (Sadeh *et al.* 1985; Guti´errez *et al.* 1991) and in overloaded muscles undergoing hypertrophy (Hall-Craggs, 1970; Eriksson *et al.* 2006).

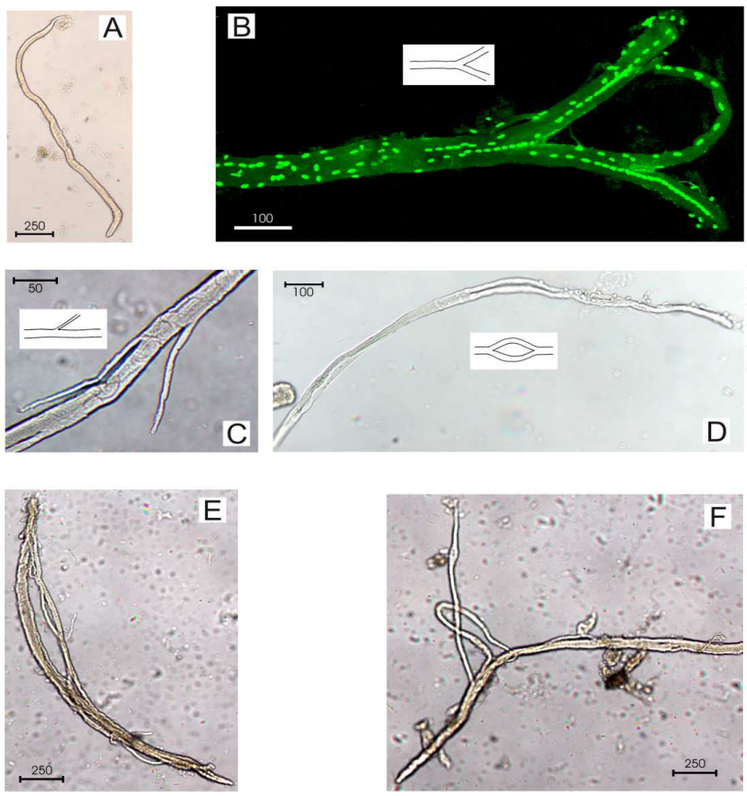
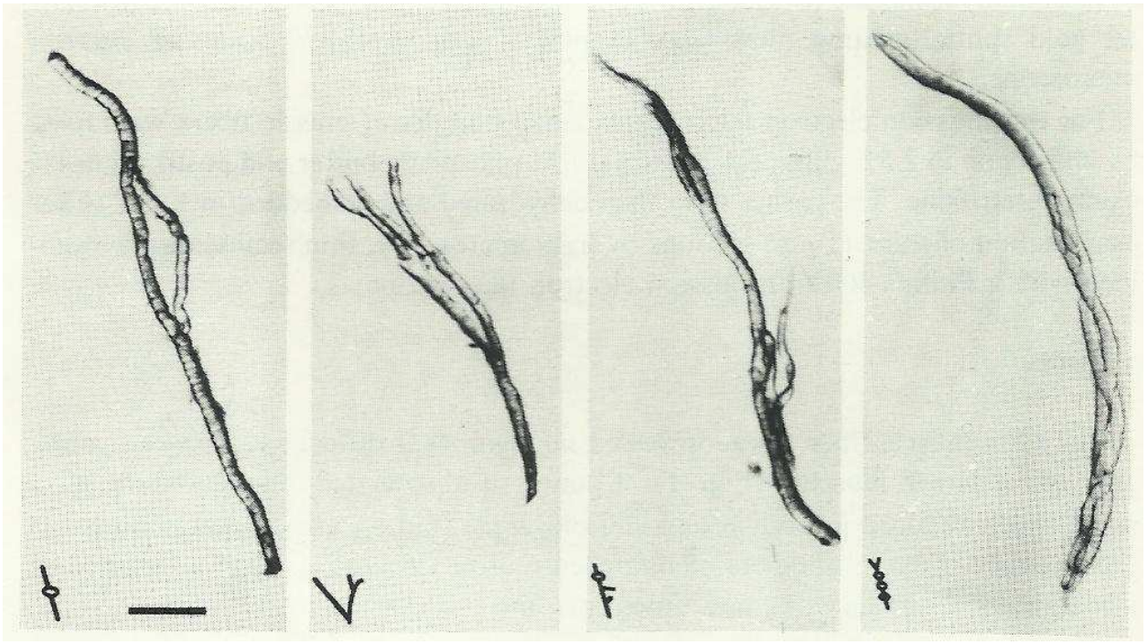


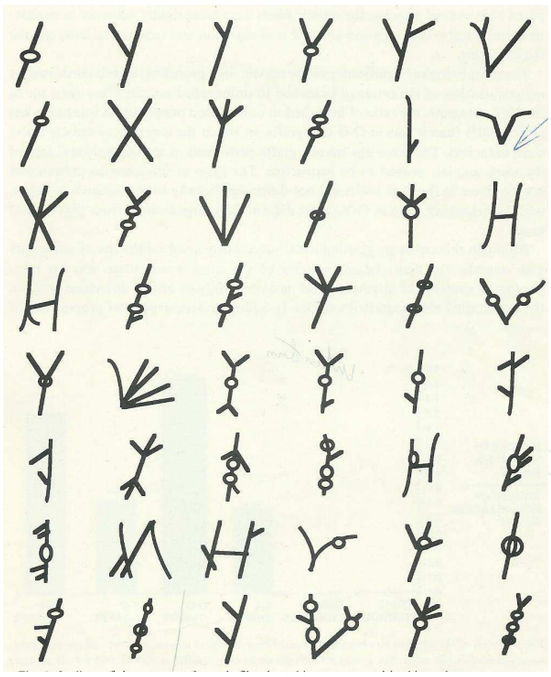
Fig. 3. **Examples of branched fibers.** Low power images of enzymatically dispersed single muscle fibers from EDL muscles of *mdx* mice. A, a morphologically normal, unbranched fiber. B, a branched fiber with bifurcations, imaged with confocal laser scanning microscopy and stained with ethidium bromide to highlight nuclei. Note the centrally located nuclei within the branches. C, a fiber with two small branches. D, a fiber that branches, then recombines. E, a fiber with highly complex branching patterns, forming an intertwining syncytium. F, another fiber with complex branching. Scale bar units are in microns. Modified from Chan *et al*.,2007.

3.1 How does branching occur?

Branching is most likely to result from the imperfect fusion of myogenic cells as they attempt to regenerate a fiber segment or complete fiber that has become necrotic (Schmalbruch, 1976; Ontell *et al.*1982). The association of branching with regeneration is evidenced by the frequent occurrence of centrally located nuclei in branched fibers (e.g. Fig. 3*B*; and see Schmalbruch, 1976; Ontell *et al.* 1982). Regenerating muscle has different functional characteristics from uninjured muscle. During regeneration, muscles display contractile differences such as reduced isometric force (Beitzel *et al.* 2004; Stupka *et al.* 2007; Iwata *et al.* 2010), longer twitch contraction and relaxation times (Beitzel *et al.* 2004; Stupka *et al.* 2007) and a dependence of contractility upon the extracellular Ca^{2+} concentration (Louboutin *et al.* 1996). Although branched fibers are a well-documented phenomenon of regenerating muscle, only a handful of studies have examined how their physiological properties may differ from those of morphologically normal fibers (Head *et al.* 1990, 1992, 2004; Chan *et al.* 2007; Lovering *et al.* 2009; Friedrich *et al.* 2010; Head, 2010).



(a)



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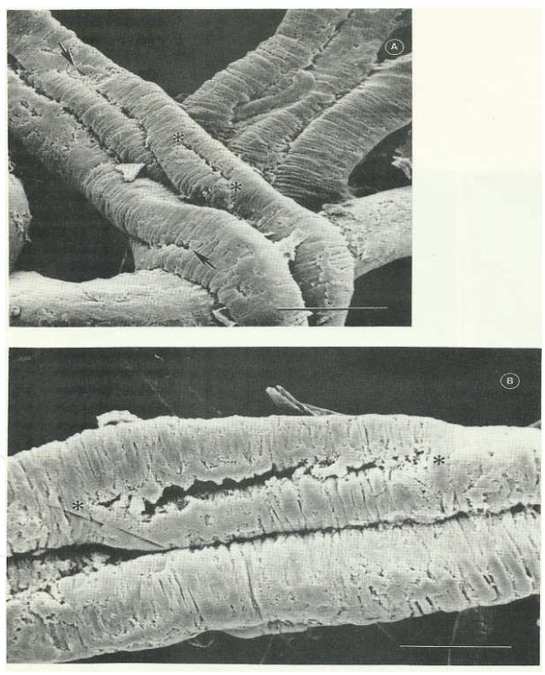

(c)

Fig. 4 A. LM pictures of dystrophin positive branched fibers form grafted rat muscle, the stick drawing in the bottom left of each panel represents the branch pattern. scale bar 100 micrometers. B. Patterns of branching encountered in the dystrophin positive grafted muscle. C. SEM of branched fibers, top panel arrows indicates branch points, * show a loop scale bar 100 micrometers. Bottom panel high magnification of loop shown by * in both panels Scale bar 50 micrometeres. Taken from Blaivas & Carlson, 1991.

3.1.1 Assessing intracellular continuity of branched fibers

It is essential to determine the actual boundaries of a single functional fiber to establish that these complex structures were not simply strong structural associations between more than one discrete cells. Intracellular continuity between the main body of the fibers and various appendages was assessed by a number of physiological techniques. The fluorescent dye, Lucifer Yellow, was ionophoresed into the intracellular environment of fibers (n = 9) of varying complexity at a single focal point. As is apparent in Fig. 5, Lucifer Yellow was able to diffuse from the point of injection to occupy the cytoplasm of all appendages of deformed fibers indicating that no barrier existed, at any junction within the fiber, to the internal diffusion of this dye. It was also possible to measure the resting membrane potential with an intracellular microelectrode at a number of locations in branches of deformed fibers. All values recorded from individual segments of a single, complex fiber were within a few millivolts, fiber depolarization, which was initiated with the impalement electrode, always lead to the contraction of all branches which constituted part of a single fiber. It was apparent, however, that the contraction of individual branches within a single fiber, as detected by video frame-by-frame analysis, was often unsynchronized, with some branches distal to the impalement electrode shortening before proximal branches (Head et al., 1990).

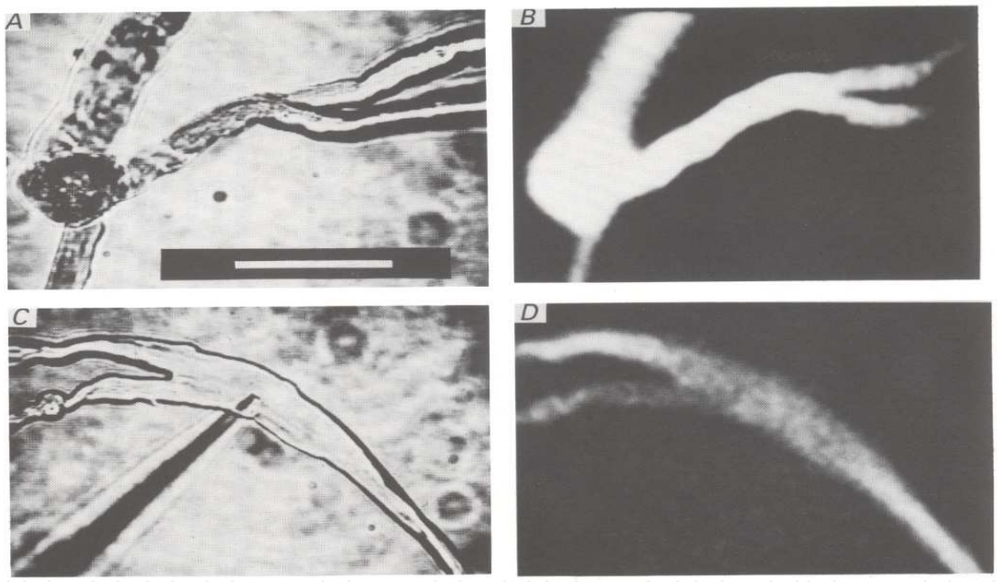

Fig. 5. Fluorescence images (B and D) of single intact dystrophic soleus muscle fibers (A and C). In each case in A & C an intracellular microelectrode was used to inject the dye just above the branch point, In C the tapering shadow of the electrode can be seen delivering dye. Scale bar 80 micrometers. From Head et al., 1990.

3.2 Do branched fibers exist in the intact muscle?

The early evidence for the presence of branched fibers within the skeletal muscle relied on reconstructing serial sections. In recent times collagenase has been used to digest the muscle so that entire isolated single fibers can be viewed, Head *et al.,* 1990 were among the first groups to apply this to mouse muscle, where it is now a key physiological tool worldwide. This has advanced our understanding of branched fiber physiology because it has allowed the

visualisation from tendon to tendon of the entire extent of branching in a fiber. It has also enabled contractile physiology experiments to be carried out on branched fibers. It has been suggested that fiber branching may be an artefact of the enzyme digestion technique, although it is hard to explain why we do not see branching in normal muscle! Given the importance of this point a study was undertaken in my laboratory using confocal laser scanning microscopy to examine branched fibers *in situ*, as they lay within the muscle. This involves fixing the muscle in formalin and then dehydrating the muscle in an alcohol series, before finally clearing the tissue with methyl salicylate. Before fixation a micro electrode had been used to fill 2 to 3 fibers within the muscle with the marker dye Lucifer yellow. The auto-fluorescence of the fibers was such that it was possible to imaging all muscle fibers *in situ* in the muscle Fig 6. Interestingly in fibers with simple Y-shaped bifurcating branches, both branches are aligned along the longitudinal axis of the muscle Fig. 6 B&D. These *in situ* pictures of dystrophic muscle fibers are also interesting because with they have caught branched fibers which have been damaged and are undergoing necrosis Fig 6. E&F. (from Head et al., 1992)

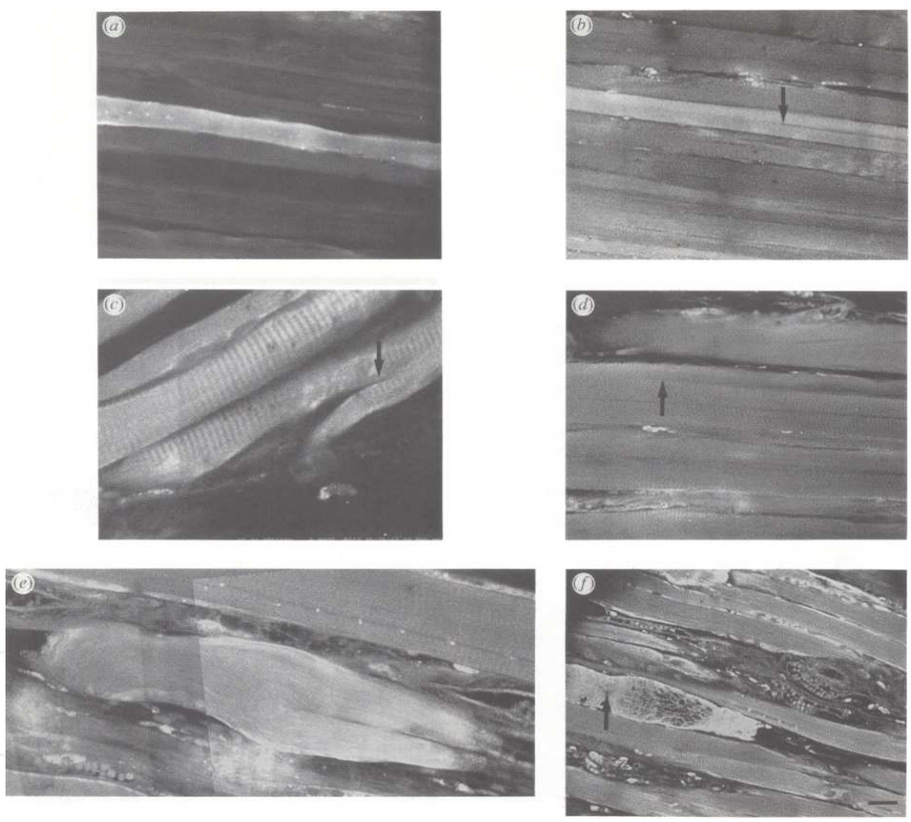

Fig. 6. Confocal laser scanning micrographs showing muscle fibers in the muscle. (a). A single EDL fiber filled with Lucifer Yellow, note the surrounding fibers are visible because they are auto-fluorescence. (b) Further along this same fiber a clear branch is evident (arrow). (c) Auto-fluorescence image from an FDB muscle showing a fiber branching at the at the attachment to the tendon(arrow). (d). A soleus fiber which branches into 2 asymmetrically with a large and small daughter branch (arrow). (e) A deformed EDL muscle fiber which is in the process of pathologically hyper-contracting and has detached from the tendons.(f) a necrotic soleus muscle fiber. Scale bar (all are scaled in relation to the bar bottom right on panel (f)) in micrometers: (a,b), 30; (c), 10; (d), 15; (e),15; (f), 30. From Head et al., 1992.

3.2.1 Myofibrillar micro branching within a muscle fiber

Friedrichs group has utilised a novel biological imaging technique which allows the microarchitecture of the myofibers in single muscle fibers to be visualised. Using their recently developed technique of second harmonic imaging they have elegantly demonstrated that in old *mdx* mice the myofibrils within muscle fibers are deformed, bifurcating into Y shaped branches which they term verniers Fig.7. Even in dystrophic fibers which appear to be macroscopically unbranched i.e. are straight fibers similar to those seen in control animals, the myofibrils branch into Y shaped verniers which are misaligned with the longitudinal axis of the fiber. The myofibril malformations in branched fibers are even more marked. Friedrich *et al.* 2010 have calculated that this alteration in the microarchitecture of the dystrophic fibers has a deleterious effect on the force output. From a biophysical standpoint because neighbouring sarcomere activation would be unsynchronized, myofibril misorientations in single mdx fibers can partly explain the decreased force in *mdx* muscle. Their image analysis provides the first quantitative estimate of an ultrastructure relate force deficit in *mdx* fibers. This deficit will vary among individual fibers, depending on the degree of myofibril twisting and local angle deviations. The force deficit is also inhomogeneous within fibers, depending on whether additional branches are present or not. They found normalized cosine angle deviations of up to 20% from the long axis that would not contribute to force output from the dystrophic muscle fiber (Friedrich et al., 2010).

3.2.2 Friedrich summarised

In the dystrophinopathies it is apparent that all the myofibrils are not pulling together and this worsens as the animal ages. As the force vectors are not aligned along the longitudinal axis the tendon doesn't get all the tension you would expect from the cross section of myofibrils. In effect the muscle is wasting energy because the cross bridges are working, burning up ATP, but the force is not being transmitted to the tendons.

4. Do branched fibers occur in human muscle disease especially DMD?

In recent years my laboratory and several others around the world, have used the *mdx* mouse model of DMD to demonstrate the importance of branched muscle fibers in the pathophysiology of dystrophic muscle function. One of the critiques of this work has been "YES, but does this phenomenon occur in boys with DMD, and also does it occur in other human myopathies?" The answer to these questions is a resounding "YES"!

4.1 Evidence for branched fibers in human dystrophy

In the 1970s and 1980s there was a large amount of detailed histological work carried out on human tissue. This work demonstrated unequivocally that branched fibers are present in DMD and other human myopathies. In fact the importance of fiber branching in human muscle diseases was the subject of an editorial in the Lancet in March 25, 1978 pp.646; *Muscle Fiber Splitting_ A Reappraisal*. Because of the importance of this question, i.e. does the *mdx* mouse model of fiber branching makes a good model for human DMD, in this section I am going to review the evidence that demonstrates that fiber branching is a major factor in DMD and also other human myopathies.

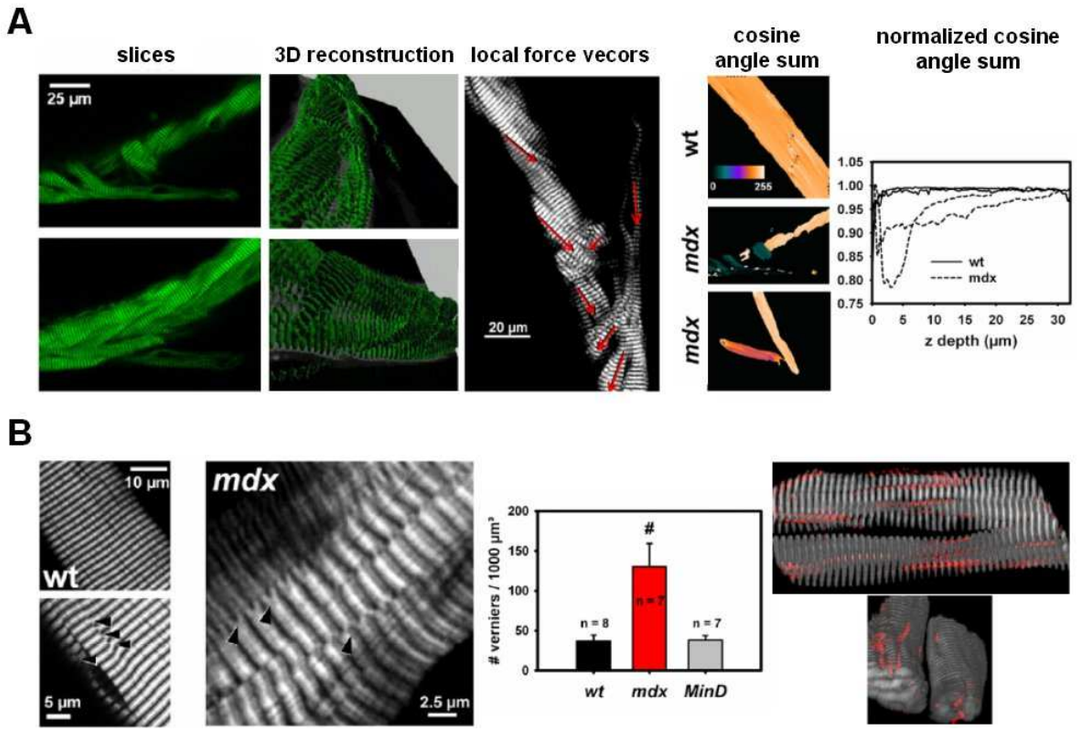

Fig. 7. SHG-microscopy reveals vastly altered sarcomere ultrastructure in intact single mdx fibers. **A,** example slices and 3D reconstruction of an *mdx* fiber (12 mo) show tilted myofibril geometry suggesting misorientated local force vectors. The degree of force drop from the geometry was quantified with gradient filter masks. The cosine angle sum is close to unity within wt single fibers (myofibrils run parallel) and reduced ~20 % in *mdx* fibers. **B,** magnified images show local disruptions of the sarcomere pattern ('verniers'). Their number is vastly increased in *mdx* but close to wt levels in minidystrophin (MinD) fibers. In *mdx* fibers, verniers run in streaks through the fiber centre (From Friedrich et al., 2010.).

4.1.1 The long history of branched fiber reports in human dystrophies

In humans fiber branching was first reported in boys with DMD by Erb in 1891, closely followed by Krosing in 1892. In the 20th century there have been reports by Greenfield *et al.* 1957; Adams *et al.* 1962; Pearce & Walton 1962; Bell and Conen 1968; Schwartz *et al.* 1976; Swash *et al.* 1977; Schmalbruch 1984 and Hamida *et al.* 1992.

4.2 Regenerated fibers in DMD: A serial section study by Schmalbruch (1984)

Fig. 8 shows a cross-section from two patients with Duchenne muscular dystrophy. Due to the importance of this result the 2 figures have been scanned and retain their orininal figure numbers and legend. They will be referred to as Figure1'and Figure 2'. In each case a sample cross-section from each patient is shown in the first panel while the second panel shows a reconstruction 6 mm long and from these cross-sections. In order to produce this very detailed reconstruction, 1200 sections were obtained from the 6 mm biopsy. Then every 10th section was photographed at X350. From these prints he reconstructed the fibers shown in the second panels. This rigorous approach allows the reconstruction of branched fibers and shows how they positioned *in situ* in the human muscle. This elegant study was one of the

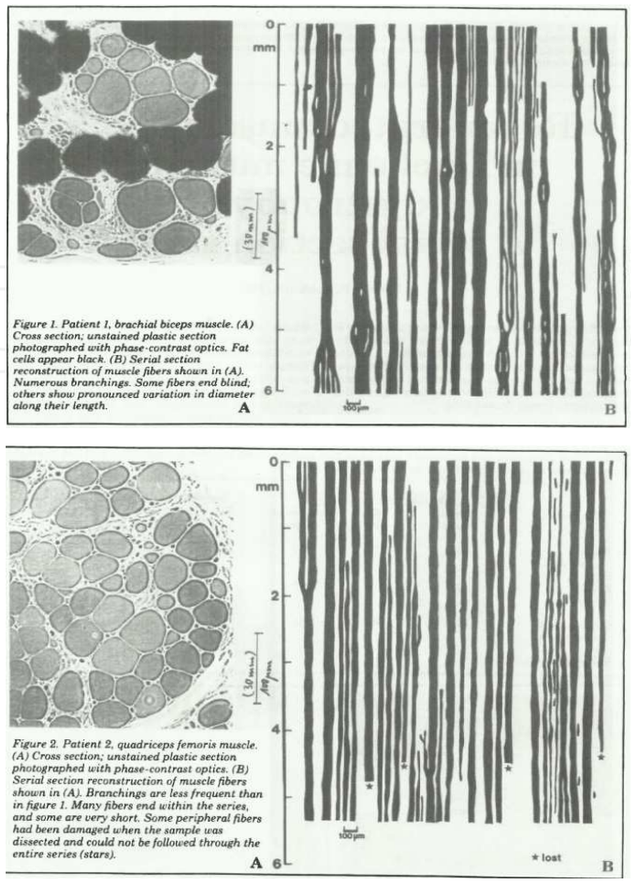

Fig. 8. Copied from Schmalbruch (1984) Figure 1 & 2.

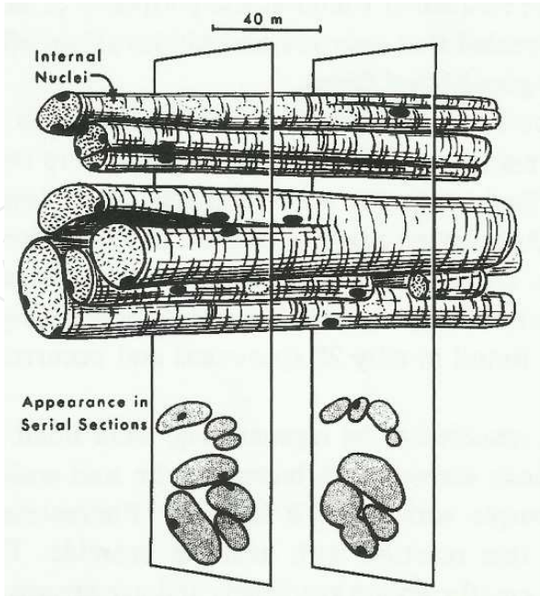

Fig. 8. A three-dimensional reconstruction from serial sections of branched fibers in DMD copied from Bell & Conen 1968.

first to link the degree of fiber branching to the severity of the disease. Patient one Figure 1′ was clinically more severely affected than patient two Figure 2′, the creating kinase level was higher and the biopsy specimen was heavily infiltrated with fat. As can clearly be seen patient one Figure 1B′had a much higher degree of fiber branching.

4.2.1 Branched fibers in samples from 84 boys with Ducehenne muscular dystrophy

In another landmark study Bell & Conen 1968, obtained muscle biopsies from 84 DMD patients and control samples from a further 72 children. All of the control fibers showed predominantly normal histological features. All of the samples from the 84 DMD boys showed branching. Fig.8. illustrates branched fibers serially reconstructed from cross-sections.

4.2.2 branched fibers occur in other human muscle diseases

It's not only in DMD that we see branched fibers, they are present in many other myopathies (Swash & Schwartz 1977). demonstrates. Fig. 9 is taken from that paper to show some serial sections taken from the quadriceps of a patient suffering from Kugelberg-Welander disease of at least 10 years duration. This is a very nice illustration in humans of a large fiber separating into three apparently separate fibers, but of course we know that the three separate "daughter" fibers actually branch from a common trunk. Interestingly this panel also illustrates a commonly observed phenomena associated with fiber branching that is the presence of a "sentinel" nuclei near the membrane invagination at the stem of the branch

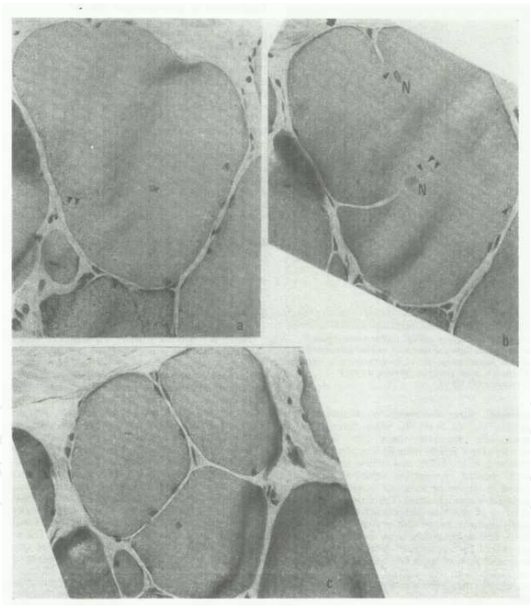

Fig. 9. Serial section of quadriceps. Arrows=branch formation, Kugelberg-Welander disease H&E X 560.(a) One complete large fiber 250 micrometer in diameter.(b) 22 micrometers from (a) two clefts associated with central nuclei (N); (C) 70 micrometers from (a) the fiber has branched into three daughter fibers. From Swash & Schwartz 1977.

In this paper they also demonstrated fiber branching in Charcot-Marie-Tooth syndrome. In humans fiber branching has also been reported in poliomyelitis, motor neuron disease and limb girdle dystrophies.

4.2.3 Is fiber branching a compensatory respose to muscle loss?

Swash and Schwartz 1977 proposed that fiber branching represents a compensatory mechanism whereby the number of fibers and the overall mass of the diseased muscle is increased. This is a very teleological explanation and nowadays one I think that can be discounted in light of new information we have regarding the fact that fiber branching represents misguided skeletal muscle regeneration.

4.2.4 Some observations on branch formation in human myopahties

The paper by Schwartz *et al*., 1976, throws further light on the role of the nucleus in fiber branching. They used the electron microscope to look at early phase fiber branching and found that a zone of separation of myofibrils was often in close relation to a central nucleus. This zone consisted of granular cytoplasm containing glycogen granules and myofibrillar debris. Present, in nearly all cases, near the boundary of the dividing edge were large mitochondria and pinocytotic vesicles. In fact the membrane forming the dividing branch point seemed to be derived from the membrane formed during the extrusion of material by these pinocytotic vesicles. These observations require further investigation in order to understand the mechanisms involved.

4.2.5 Is there a fiber type bias for branching?

Several of these human studies have noted that the branching appears to affect type slow I fibers more commonly than their fast type II cousins. Studies on the *mdx* mouse also show that fiber branching is most prevalent in slow muscle (Head *et al*.,1992). This is an area which merits more investigation.

4.3 Microneurography studies on single human dystrophic muscle fibers

In humans branched fibers have complicated the interpretation of microneurography results obtained from patients with dystrophinopathies. Studies in Duchenne and Becker dystrophy by Stalberg 1977 and Hilton-Brown & Stalberg, 1983 have shown substantially increased fiber density. While typical multiple spike potentials have greatly increased duration there is also an increase in the duration of the mean interspike interval, these findings reflect an increased fiber size variation. Also there are occurrences of simultaneous blocking of two or more single muscle fiber action potentials in a multiple spike potential which is thought to be due to transmission failure at a neuromuscular junction of a muscle fiber branch. The increased fiber density reported in DMD and Becker dystrophy is at first site an apparent contradiction to the known loss of muscle fibers from the motor units in these conditions. However, the contradiction is resolved because the increased fiber density was interpreted to arise as a result of fiber branching. Each branch of a multiply branched fiber gives rise to a separate action potential, indistinguishable from true single muscle fiber action potentials; however, they are identified by a low jitter between action potentials. Thus the split fibers may account for a significant part of the increase fiber density in muscular dystrophy (Hilton-Brown et al., 1985).

5. Whole muscle and skinned fibers experiments show the susceptibility of branch points to damage

Enzymatically digesting soleus and EDL muscles from old *mdx* mice revealed that > 90% of fibers had some degree of branching, confirming numerous previous studies on dystrophic

muscle (Isaacs 1973; Ontell & Feng, 1981; Bourke & Ontell, 1984; Schmalbruch 1984; Head et al 1990;1992; Tamaki et al. 1993; Pastoret & Sebille1995; Lefaucheur et al. 1995; Schafer et al. 2005; Bockhold et al. 1998; Chan et al. 2007; Lovering et al. 2009; Friedrich et al. 2010, Head, 2010). It seems clear that these branches occur as part of the regenerative process in *mdx* muscles (Blaveri et al 1999). If the enzymatically isolated branched fibers are suspended in a high relaxing solution (50mM EGTA) then they can be manipulated under a dissecting microscope so that they can be attached to a force transducer in various configurations; i.e. with or without a branch between the attachment points. It is also possible to liberate single fibers from eccentrically damaged muscle and probe them with the membrane impermeable dye, Evans blue, to see where the membrane integrity has been compromised. In skinned fiber experiments the absence of the sarcolemma removes any contribution played by the presence or absence of dystrophin to the stability of the surface sarcolemma. This is because dystrophin, if present on the inner surface of the sarcolemma, is removed with the membrane. When a fiber was activated (>50% of max) with a branch point between the attachment points, in the majority of cases it broke: when the unbranched portion of the same fiber was reattached to the transducer, the unbranched segment could generate a normal force/pCa curve Fig. 10Ai/ii. (and see; Head et al.,1990; 2010)

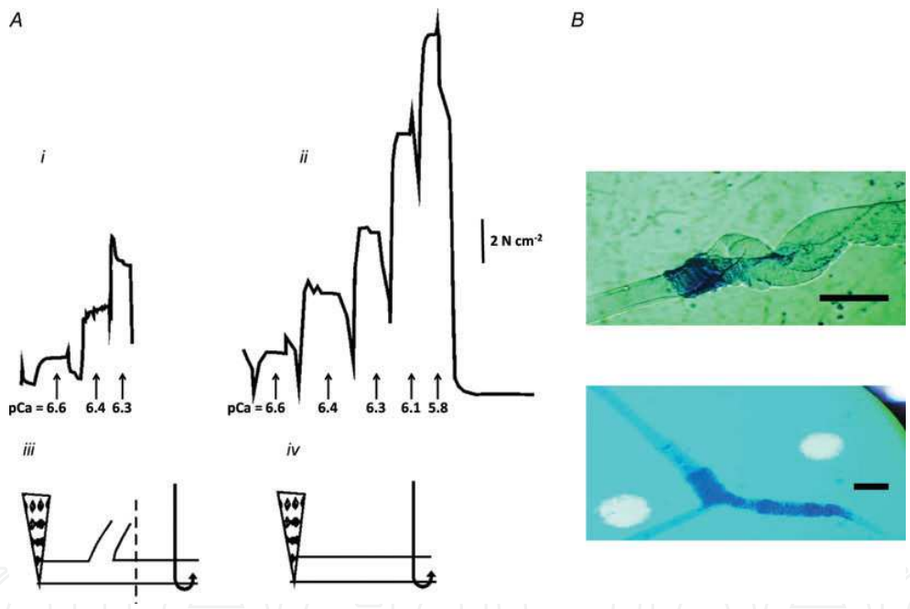

Fig. 10. *A*, generation of force–pCa curves in a branched EDL fiber from an *mdx* mouse. Top traces are the force–pCa curves, with each peak representing the force developed by the fiber at a certain value of pCa (i.e. −log10[Ca^{2+}]). The [Ca^{2+}] was progressively increased until maximal force was reached or until the fiber broke. The schematic drawings below each force–pCa curve indicate how the fiber was tied when generating that curve. When the fiber was tied as shown in *Aiii*, with a branch point between the sites of attachment, the fiber broke before reaching maximal activation (*Ai*). When the main trunk was retied as shown in *Aiv*, with no intervening branches, a full force–pCa curve was obtained (*Aii*). *B*, two fibers from eccentrically contracted EDL muscle from an *mdx* mouse, showing uptake of Evans Blue dye at branch points. Scale bar represents 50 μm in upper picture, 30 μm in lower picture.(From Head, 2010).

When old *mdx* muscles were subjected to a moderate eccentric contraction (a contraction which caused no damage in age matched controls or young mdx with less than 10% branched

fibers) the branched fibers were damaged at a branch point, fig. 10B shows examples of two branched enzymatically liberated fibers from the eccentrically contracted muscle. Evans blue will only penetrate damaged membrane and it is clear in this figure that the Evans blue uptake is in the vicinity of the branch points. These experiments support the hypothesis that it is the mechanical architecture of the fiber branches which weakens the fiber Fig. 10.

6. Reduced life span of mdx mice

Surprisingly there has been a misrepresentation in the literature to the effect that *mdx* mice have a normal lifespan. It's time to put this myth to rest, there have been several publications clearly demonstrating that *mdx* mice have a significantly reduced lifespan (Pastoret & Sebille 1995a,b; Lefaucheur, *et al.,* 1995). Most recently Chamberlain *et al.,* 2007 looking very old mice demonstrated a significantly reduced lifespan in both male and female *mdx* mice Fig. 11.

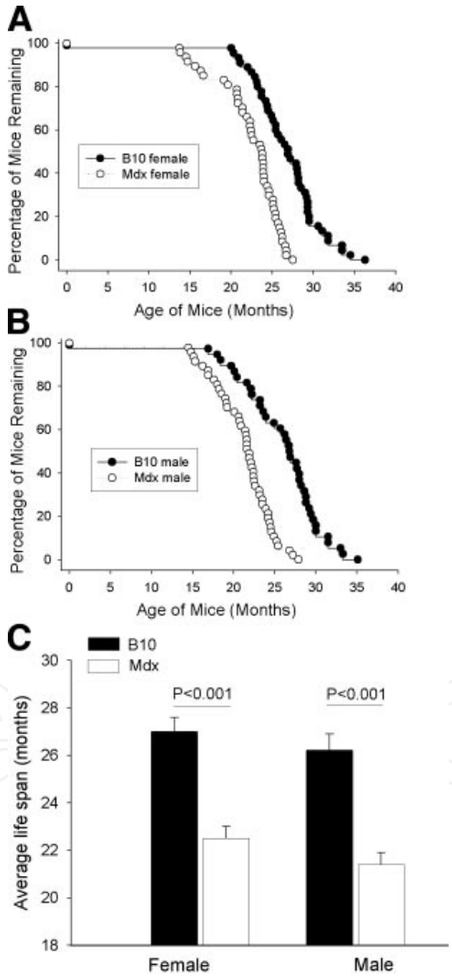

Fig. 11. Life span analysis for wild-type and mdx male and female mice. Graphs showing the age at death for the female (A) and male (B) mice. Circles denote the age at which each animal died. C) Histogram showing the average age at death of male and female wild-type (C57BL/10) and *mdx* mice. The average life span between wild-type and *mdx* males and between wild-type and *mdx* females was highly significant, as shown. From Chamberlain *et al.,* 2007.

They also noted as might be expected, that 24 month wild-type diaphragm muscles displayed no morphological abnormalities while aged matched *mdx* diaphragm showed a large degree of fibrotic infiltration and loss of muscle fibers. It is commonly accepted in the field that the diaphragm is the muscle with clinical features most similar to DMD. However, importantly and in direct contradiction to publications reporting that *mdx* muscle limb muscle do not display a DMD morphology, they noted they the *mdx* muscles displayed typical dystrophic features at each age examined. The dystrophic features included centrally located myofibers, necrotic fibers, small calibre regenerating fibers, moderate amounts of fibrosis, and some fatty infiltration. By 26 months of age fibrosis and fatty ill filtration were extreme. For example, they note increased necrosis fibrosis and adipocyte accumulation in the 26 months soleus compared with the same muscle group at 4 months. Interestingly they highlight the slow-twitch soleus as the most morphologically dystrophic limb muscle in the *mdx* mouse at any age. This correlates with early findings from my laboratory that the soleus muscle is the first to display extensive branching in the *mdx* mouse and reports (see section 4.2.5) that it is type I slow-twitch muscle fibers in humans which are most likely to be branched.

6.1 Aged mdx mice are an excellent model for Duchenne muscular dystrophy

Aged *mdx* mice represent the most advanced dystrophic condition that can be generated as a result of dystrophin deficiency in a small mammal model. The aged mouse phenotype resembles late stage DMD (Gregorevic et al., 2008). As detailed in this section the assumption that *mdx* skeletal muscle does not show dystrophic changes is because most of these studies were carried out on young animals of less than three months of age, when the dystrophic phenotype has not fully developed.

6.2 The main age related dystrophic change in the *mdx* mouse is the formation of branched fibers

The major skeletal muscle changes that you see with age in the *mdx* mouse is an increase in the number of branched fibers, both in the number of branched fibers present within the muscle and the number of branches on an individual muscle fiber (Head *et al.,* 1992; Tamaki *et al.,* 1993; Bockhold *et al.,* 1998; Chan *et al.,* 2007; Lovering *et al.,* 2009; Friedrich *et al.,* 2010; Head 2010). Since branched mature myofibers are not present at the time of onset of clinical symptoms, they must be formed as a secondary consequence of the absence of dystrophin Fig. 12.

6.2.1 Contractile abnormalities of aged muscles with branched fibers

Numerous studies have shown that old *mdx* dystrophin deficient skeletal muscles resembles the DMD phenotype, due to space constraints only a selection is given here, (Pastoret & Sebille,1995; Lefaucheur *et al.,* 1995; Lynch *et al.,* 2001; Chan *et al.*, 2007; Claflin & Brooks, 2008; Lovering *et al.,* 2009; Friedrich *et al.,* 2010; Head,2010; Mouisel *et al.*, 2010; Wooddell *et al.,* 2010; Hakim *et al.,* 2011). Old *mdx* muscles generate less specific force and are more easily damaged by mild eccentric contractions when compared with young *mdx* dystrophin deficient muscles.

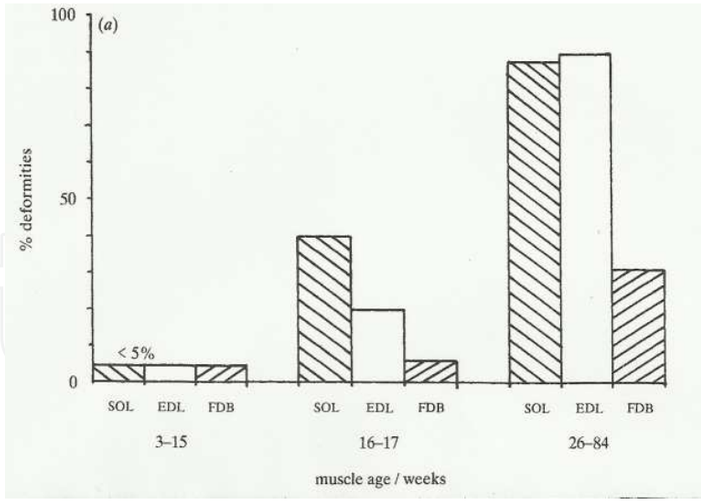

Fig. 12. Percentage of fibers with branched in different age groups of mdx mice, From Head et al., 1992.

6.2.2 Both young and old mdx muscle lack dystrophin so why should old mdx be more susceptible to damage? We know its not aging per se

So the primary question is; given dystrophin is absent from both young and old muscles, why is absence of dystrophin linked to major membrane damage only in old muscles? My laboratory has carried out a key study in this field (Chan *et al.,* 2007). We developed a mild eccentric contraction protocol which had no significant effect on normal muscle. When this contraction protocol was carried out on young *mdx*, there was no difference compared to young age matched controls. When the same contraction protocol was given to aged *mdx* where over 80% of the muscle fibers were extensively branched then there was a massive 60% drop in force. My thesis and the theme of this chapter is: The drop of force is not due to an absence of dystrophin, but is due to the presence of branched fibers. Wooddell *et al.,* 2010 exercised young and old *mdx* mice and compare the degree of muscle damage by the use of the membrane impermeable dye Evans blue. Evans blue is only taken up by damaged cells. There was significant threefold increase of dye uptake only in old *mdx* mice 12 to 19 month of age. Young *mdx* mice were not affected and there was little dye uptake. They also confirm this finding by measuring the creating kinase levels (a marker of muscle damage) which was only significantly elevated in old *mdx* mice. Once again it has been shown branched fibers are mechanically weak and more easily damaged when they contract (Chan *et al.,* 2007; Friedrich *et al.,* 2010; Head *et al.,* 1990,1992; Head 2010; Lovering *et al*., 2009;). So it is reasonable to conclude that Wooddell *et al.,* 2010 results are explained by the presence of branched fibers in the old *mdx*. It must be emphasised that other factors such as age and absence of dystriophin have been taken into account and do not play a major role.

6.2.3 Even when not damaged old mdx muscles generate less force and power

Lynch *et al*., 2001 showed differences in the effect of age on structure-function relationships of limb muscles of *mdx* mice compared to control mice. They demonstrated that limb muscles from 24- to 28-month-old *mdx* mice are smaller and weaker with lower normalised force and power; once again age matched studies showed that this was not an ageing

phenomenon but was due to the disease process. These findings were supported and extended by Mouisel *et al.,* 2010. Given that both young and old *mdx* lack dystrophin I propose that the force and power deficits are due to the presence of branched fibers. It has been shown that the architecture of the branched fiber compromises the coordinated activation of the fiber in the longitudinal axis of the muscle fiber (Friedrich et al., 2010; Head, 2010)

7. Branched fibers in other systems are also susceptible to damage: Lamininopathies and regeneration in normal tissue

The129/ReJ dy/dy mouse lacks laminin-alpha-2 and has a severe muscular dystrophy phenotype. It shares three fundamental characteristics with Duchenne muscular dystrophy; progressive and severe muscle weakness, progressive degeneration and disappearance of skeletal muscle with a massive degree of fibrosis and increased serum activity of sarcoplasmic enzymes (Rowland, 1985). In some respects it is a superior functional mouse model for DMD. By three months of age animals are unable to use their hind legs. Enzyme digests have demonstrated the presence of extensive complexed fiber branching in limb muscles in the laminopathies (Head *et al.,* 1990, 2004).

7.1 Skinned fiber studies in the dy/dy mouse show branched fiber weakness

In an early study we utilised the skin fiber technique to tie up single branched fibers on the force transducer, such that a branch was between the connection points, the large majority of cases the these fibers broke at a branch point. Importantly the muscle fiber was not itself intrinsically weak in itself because when the same broken fiber was retied with no branch between the points of attachment fibers could sustain maximal force development (Head et al., 1990).

7.1.1 Intact dy/dy muscles cannot sustain isometric stimulation and the subsequent force drop is directly connected to the number of branched fibers lost

When these branched fibers were present in the intact muscle and the isolated muscle stimulated repeatedly with maximal isometric contractions then the muscle force loss was around 35% (after allowing for the effects of fatigue and in comparison to age matched controls). Because of large amounts of connective tissue present in the dy/dy mouse enzyme digests were particularly successful and it proved possible to view almost the entire population of fibers after digestion. This facilitated the very important discovery that after repeated isometric stimulation there was a 35% drop of force and this force loss was correlated with a 40% loss of branched fibers (Head et al., 1990)!

8. Passive properties of mdx mice, if you stretch an old mdx muscle it pulls apart

In the most recent publication on the effect of age on the *mdx* mouse Hakim *et al.,* 2011 looked at the passive properties of fast-twitch edl muscle in two month old mice compared with 20 months old *mdx* mice. What they did to the EDL was to passively stretch it, i.e. the muscle was not contracting actively, and look at the effect on muscle integrity. Surprisingly when they gave the muscle a very large stretch from 110% Lo to 160% Lo; in 2 month old

mdx mice a partial tear was observed only at the proximal end of the muscle, at 6 months of age there was some separation across the entire muscle belly, although there were still substantial attachment and finally in 14 and 20 month old *mdx* mice the muscle simply pulled apart. The important point here is that the damage in age matched control was minimal! It is instructive to compare this finding with results from my laboratory which show a strong correlation in the degree of eccentric contraction damage and the age related increase in branched fibers and the increased complexity of the branching (Chan *et al.* 2007). Once again it is important to bear in mind that both the young and old *mdx* mouse lack dystrophin, and the age matched control animals demonstrated age in itself is not a significant factor. So logically it is reasonable to infer that some other disease process, which increases with age, is causing the muscle weakness in the aged dystrophin deficient *mdx* mouse. The branched fibers are the obvious, and I would suggest, the only candidate to account for the age related increase in susceptibility to damage in dystrophinopathies.

9. Young muscles are not unaffected by the absence of dystrophin

This chapter focuses on old *mdx* muscle and branched fibers, however, I do not wish to imply that young dystrophin deficient non-branched muscle fibers are normal. As I detail in the final section of this chapter the hypothesis is that muscular dystrophy is a two-stage process. In the first stage in the dystrophinopathies, around the time when the animal starts to use their skeletal muscles, there is a rise in cytosolic calcium which acts as a trigger for fiber necrosis. This starts the cycle of degeneration and regeneration which produces the branched fiber phenotype. Initially it was thought absence of dystrophin weakened the sarcolemma and when the muscle was activated micro-tears appeared allowing a pathological influx of calcium. Most current research shows that this explanation was too simplistic and not supported by experimental data. It now seems clear that dystrophin plays a role in organizing and aggregating ion channels in the membrane.

9.1 The role of free radicals, stretched activated ion channels and calcium in dystrophic damage

Eccentric contraction studies on non-branched dystrophic fibers showed that there was a long time delay between the contraction and subsequent increase in intracellular calcium (see Allen & Whitehead, 2010 for a review). If the muscle membrane simply ripped then the calcium would go up immediately, as is the case in branched fibers, where the contraction induced damage leads to an almost instantaneous explosive rise in intracellular calcium (Head, 2010). A similar argument applies to the proposal that the dystrophic membrane contains population of abnormal stretch activated channels, in this case you would predict that the calcium would rise as soon as the muscle is stretched, the significant delay allows this mechanism to be discounted. However, blocking the stretch activated channel does prevent he delayed rise in intracellular calcium, so clearly they do have a role to play (see Allen *et al.,* 2010 for a review). So what's happening? It seems stretching the dystrophic muscle causes a higher than normal free radical oxygen production and it is these free ROS molecules that activated the stretch activated channels allowing the delayed influx of calcium to occur (Whitehead *et al.,* 2006). This calcium triggers fiber necrosis, followed by regeneration producing branched fibers. The branched fibers are mechanically weak and as mentioned before strong activation will directly allow calcium to rush in through membrane ruptures.

10. Dystrophinopatheis a 2 stage pathology: The importance of branched fibers in the aetiology of muscular dystrophy

I propose that the skeletal muscle pathophysiology of the dystrophinopathies is a twofold process see Fig. 13. Initially, before the animal becomes mobile the muscle fibers are normal in appearance, although the plasma creating kinase is elevated. There are several studies on the *mdx* mouse which show that if muscles are immobilised either by denervation or mechanically using rods or a cast, dystrophic changes do not occur in the muscle (see for example; Mokhtarian et al., 1999). So the absence of dystrophin is thought to either weaken the membrane and/or alter the activity of membrane ion channels. The alteration of stretch activated ion channels is mediated by abnormal free radical formation that occurs during dystrophic muscle contraction. This first stage leads to muscle fiber degeneration and initiates a cycle of degeneration and regeneration which results in the accumulation branched fibers. Branched fibers are mechanically compromised and cannot sustain the normal stresses and strains of everyday contraction. It is the presence of these branched fibers which terminally weaken the muscle.

10.1 Implications for the pathogenesis of Duchenne muscular dystrophy

The fact that fiber branching in itself can increase muscle damage during eccentric contractions challenges our current understanding of dystrophin's function as a mechanical and ion channel stabiliser of the sarcolemma. It has important implications for our understanding of the progression of dystrophin-deficient muscular dystrophy.

10.2 The branch initiating dystrophic event in straight fibers

The initiating event that triggers fiber necrosis in *mdx* mice must occur while the fibers are still unbranched, as branching results from regeneration following necrosis. Hence, to ascertain the function of dystrophin, it is necessary to study fibers before they become branched. The studies mentioned in this chapter which found greater force deficits for *mdx* mice compared with wild-types all used mice which were older than 6-8 weeks, at which age it is known that 17% of fibers are already branched (Chan *et al.,* 2007). Thus not clear whether they are examining the primary pathological event, or the downstream consequences, resulting from a loss of dystrophin. Any supposed mechanical weakness resulting from an absence of dystrophin is confounded by the mechanical weakness resulting from fiber branching. It is just as likely that the initiating event is an influx of Ca^{2+} through malfunctioning ion channels (Carlson, 1998), rather than contraction-induced damage to a mechanically compromised sarcolemma. Several classes of ion channels, reviewed in Allard, 2006, have been observed to function abnormally in mdx muscle fibers. It has been proposed that Ca^{2+} entering through these channels activates enzyme mediated cell damage pathways, leading to fiber necrosis. Another class of ion channel that may allow excessive Ca^{2+} influx is stretch-activated channels, which may be abnormally activated in *mdx* fibers through mechanisms involving reactive oxygen species (ROS) (Allen *et al.,* 2010; Allen & Whitehead, 2010).

My argument may be summarised in the flowchart in Fig. 13. We envisage dystrophinopathy in the *mdx* mouse as a two-stage process. The immediate consequences of losing dystrophin's normal functions might be referred to for simplicity as a "primary stage" in which the loss of

dystrophin initiates muscle damage, in the absence of any pre-existing fiber deformity. The regenerated fibers formed during the primary stage are branched. It is the progressive increase of branching which initiates the secondary stage. In the secondary stage the branched fibers are mechanically weak and damage in this terminal phase enters into a positive feedback loop.

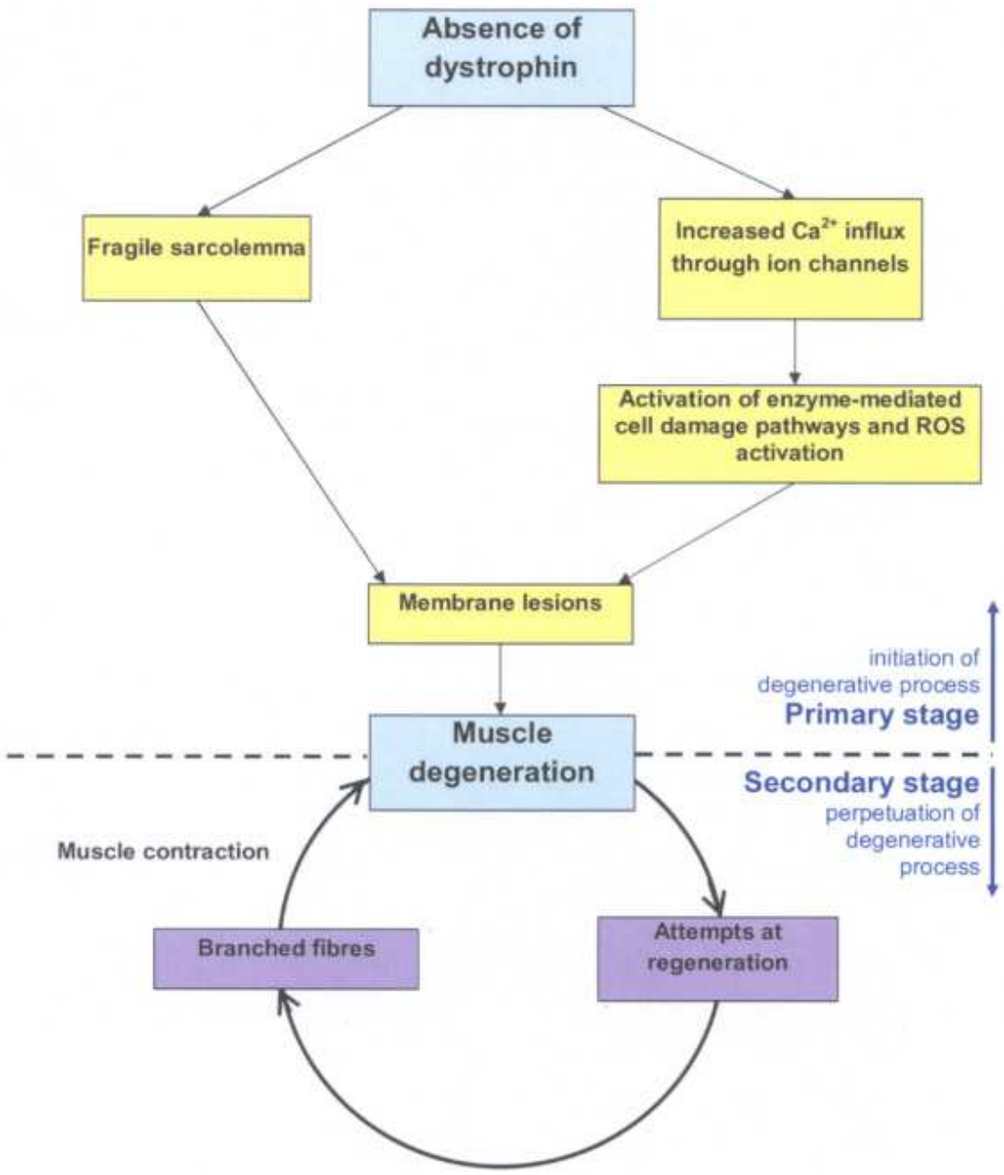

Fig. 13. **Proposed pathogensis of dystrophinopathy in *mdx* mice.** The primary effects of dystrophin's absence could be either a mechanical weakening of the sarcolemma (*left*) or an influx of Ca^{2+} through malfunctioning ion channels (*right*). Whatever the mechanism, muscle degeneration ensues. The secondary stage is a cycle in which attempted regeneration produces branched fibers, which are structurally compromised and easily damaged by contractile activity, resulting in further degeneration. In this scheme, research into dystrophin's function would be best directed at the primary stage, before fiber branching contributes to any observed susceptibility to contraction-induced damage in dystrophin-deficient muscle.

11. Conclusion and clinical implications of the two stage model

The two stage dystrophic process explains why there is a loss of muscle function over time, i.e. why the muscles are not simply destroyed on first use. Clinically this is potentially a very important point because it means if the initial pathological increase in calcium is prevented the disease process will be halted before it enters the 2nd stage degeneration/ regeneration cycle and the mechanically compromised branched fibers will not form. Research suggests the unbranched dystrophin-negative fibers are relatively normal in regard to their contractile properties (Williams *et al.,* 1993; Lynch *et al.,* 2001; Chan *et al.,* 2007). Thus if the initial calcium influx is prevented the dystrophin-negative muscle fibers will serve for day to day activities possibly for a normal life span.

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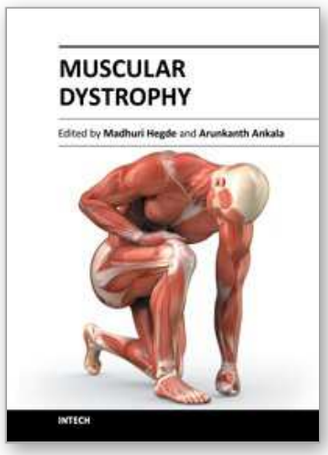

Muscular Dystrophy

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With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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