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# The need to more precisely define aspects of skeletal muscle regeneration☆

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ABSTRACT

A more precise definition of the term 'skeletal muscle regeneration' is required to reduce confusion and misconceptions. In this paper the term is used only for events that follow myofibre necrosis, to result in myogenesis and new muscle formation: other key events include early inflammation and revascularisation, and later fibrosis and re-innervation. The term 'muscle regeneration' is sometimes used casually for situations that do not involve myonecrosis; such as restoration of muscle mass by hypertrophy after atrophy, and other forms of damage to muscle tissue components. These situations are excluded from the definition in this paper which is focussed on mammalian muscles with the long-term aim of clinical translation to enhance new muscle formation after acute or chronic injury or during surgery to replace whole muscles. The paper briefly outlines the cellular events involved in myogenesis during development and post-natal muscle growth, discusses the role of satellite cells in mature normal muscles, and the likely incidence of myofibre necrosis/regeneration in healthy ageing mammals (even when subjected to exercise). The importance of the various components of regeneration is outlined to emphasise that problems in each of these aspects can influence overall new muscle formation; thus care is needed for correct interpretation of altered kinetics. Various markers used to identify regenerating myofibres are critically discussed and, since these can all occur in other conditions, caution is required for accurate interpretation of these cellular events. Finally, clinical situations are outlined where there is a need to enhance skeletal muscle regeneration: these include acute and chronic injuries or transplantation with bioengineering to form new muscles, therapeutic approaches to muscular dystrophies, and comment on proposed stem cell therapies to reduce age-related loss of muscle mass and function. This article is part of a directed issue entitled: Regenerative Medicine: the challenge of translation.



## 1. Introduction

One aim of this paper is to discuss semantics with respect to the rather casual use of the word 'regeneration' of skeletal muscle in the literature, as applied to various forms of damage and changes in muscle mass in mammals. Wide use of 'regeneration' for a range of very different cellular processes can lead to confusion and wrong assumptions. Classic muscle tissue regeneration involves myofibre necrosis (Fig. 1A). This results in a sequence of cellular events including inflammation and myogenesis to form new muscle to replace the damaged portion of the original tissue. Epigenetic regeneration is another process that occurs after limb amputation in some species (Godwin et al., 2013) and also in mammals during deer antler regeneration (Li et al., 2014). While epimorphic regeneration of skeletal muscle (that involves formation of a blastema) occurs in vertebrates such as amphibians and other species, it is not normally a feature of mature mammals (Brockes and Kumar, 2008) and thus will not be considered further here. A more detailed account of the cellular events involved in tissue and limb (epimorphic) regeneration and new muscle formation is provided elsewhere (Grounds, 2011).

In this paper, the term 'regeneration' is applied only to situations where myofibre necrosis has first occurred. Unfortunately, the same term 'muscle regeneration' is also used to describe 'repair' after damage to other components of muscle tissue, such as sarcomeric structure within myofibres (*e.g.* Z-band streaming), or interstitial extracellular matrix (ECM): this is very different to classic muscle regeneration.

Another situation where the term 'regeneration' is sometime misused relates to myofibres that undergo hypertrophy to return

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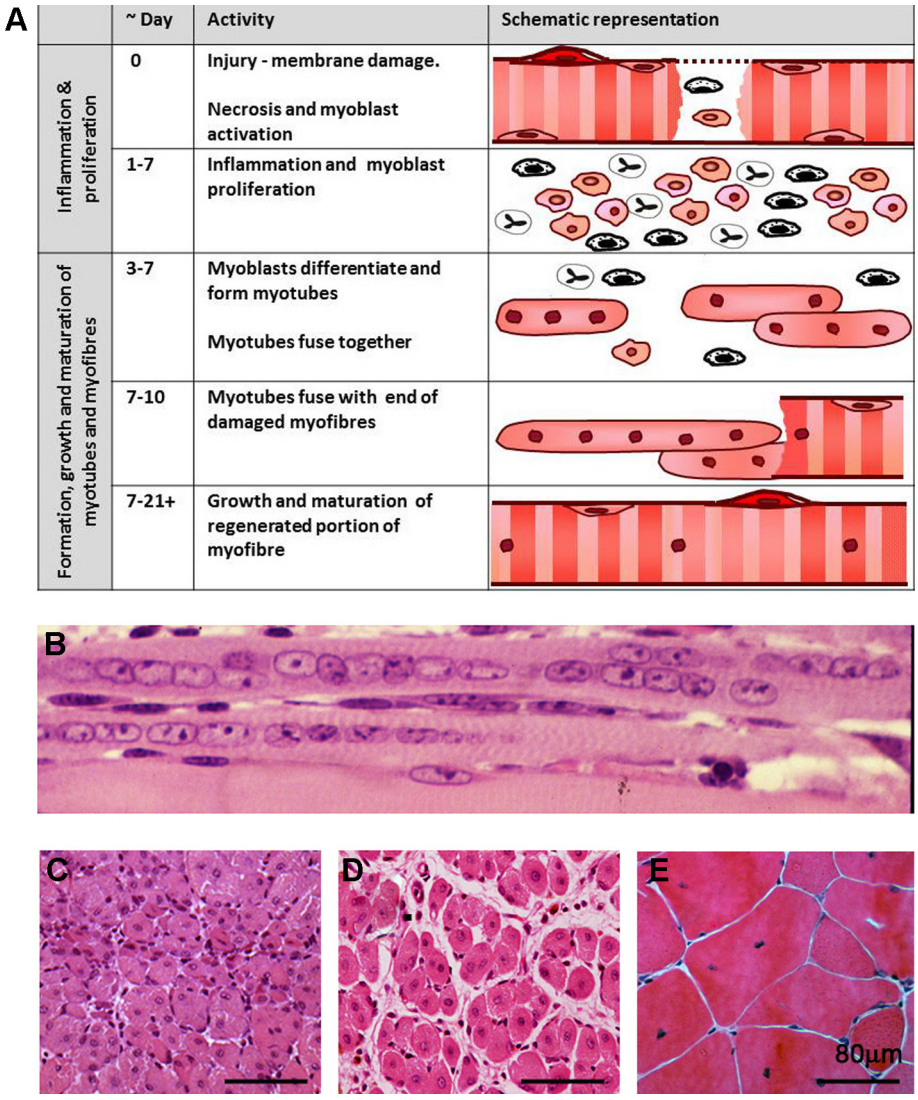


**Fig. 1.** The progression of cellular events in skeletal muscle during regeneration in response to myofibre necrosis, and examples of central myonuclei. (A) The time course of regenerative events after necrosis of a portion of a myofibre. Within 1 day of damage, necrosis is evident histologically as fragmented sarcoplasm and the presence of inflammatory cells. Coincidently there is myoblast activation and proliferation (by day one) and myoblast differentiation and fusion into myotubes occurs mainly between days 3 to 7. The myotubes fuse with more myoblasts and each other over the next few days, and by 10 days myotubes have fused with the ends of the damaged myofibres; inflammation decreases by this stage. Subsequently, myotubes and newly repaired segments of damaged myofibres undergo hypertrophy and mature to attain a stable adult size by about 3 weeks (adapted from Radley-Crabb et al., 2014). Regenerated myofibres in mice are identified by the presence of central myonuclei (*i.e.* myonuclei that are displaced and not in the classical sub-sarcolemmal peripheral position) that persist for many months. (B) Chains of central myonuclei are conspicuous in several newly formed myotubes/myofibres in a longitudinal section of adult mouse muscle regenerating after experimental injury: a portion of an undamaged myofibre with peripheral nuclei is shown at the bottom of the field [stained with Haematoxylin & Eosin (H&E)]. (C–E) Transverse sections through mature muscles stained with H&E showing myofbres with central or displaced myonuclei in three situations. (C) Dystrophic mdx muscle after endogenous myonecrosis showing recent regeneration with small newly-formed myotubes and larger older regenerated myofibres, all with central myonuclei. (D) Experimentally induced regeneration in young autograft of whole extensor digitorum longus muscle (from normal C57Bl/6J mouse aged 3 months) sampled at 10 days after transplantation, with many regenerated new myofibres with central myonuclei (from Shavlakadze et al., 2010b). (E) Myofibres of old quadriceps muscle (from sedentary male C57Bl/6J mouse aged 24 months), showing displaced myonuclei, even though there is no evidence of myonecrosis in these old muscles (Z. Soffe et al., manuscript accepted for publication).

to their original mass after some forms of muscle wasting (atrophy) that occurs in many clinical situations including disease, malnutrition, disuse, cachexia, denervation and ageing. Usually, the number of myonuclei remains unaltered throughout this process of decrease and increase in size, and only the myonuclear domain size changes: there is no involvement of satellite cells and this is essentially 'restoration' of muscle mass (discussed in Sections 3 and 4) Where pronounced hypertrophy occurs beyond normal size, numbers of myonuclei can increase at later stages due to fusion of satellite cells with the growing myofibre (in the absence of myonecrosis) to maintain the myonuclear domain (see Sections 3 and 4). Such exercise (or drug) induced hypertrophy is 'adaptation' and should also carefully be distinguished from the classical skeletal muscle regeneration that follows myonecrosis *in vivo*.

It is also pertinent to comment on myogenesis in tissue cultured cells (since these are so widely used experimentally) where myoblasts fuse to form young immature myotubes: this is not a situation of regeneration but represents only early events of myogenesis. These myotubes are not equivalent to mature innervated myofibres and this *in vitro* situation lacks the many key interacting events (e.g. inflammation, vascular interactions, blood-borne factors, ECM, innervation) involved in regenerating muscle and full function *in vivo*.

More precise use of 'muscle regeneration' is recommended to clarify discussions, especially in the context of potential clinical

therapeutic applications. Before engaging in this discussion, a brief introduction to the developmental formation and homeostasis of skeletal muscles is presented.

## 2. Setting the scene for skeletal muscle formation and maintenance

The focus of this discussion is on mammals such as mice and humans. However, there is remarkable similarity in the cellular events and molecular regulation of myogenesis during embryogenesis and skeletal muscle growth, homeostasis, atrophy and hypertrophy across many species including insects, fish and amphibians (Brockes and Kumar, 2008; Rai et al., 2013; Siegel et al., 2013), and for muscle regeneration in mammals and many vertebrates *e.g.* amphibians (Carlson, 1973; Carlson, 2003). There has been intense research into molecular regulatory mechanisms that control the formation of different groups of muscles during embryogenesis (Pownall et al., 2002; Siegel et al., 2013). The classic myogenic precursor cell (myoblast) in post-natal/adult vertebrate skeletal muscles is called the satellite cell. These are located on the surface of the myofibre beneath the basal lamina in vertebrates and are normally quiescent: they are considered as essential reserve precursor myogenic (stem) cells for myogenesis and skeletal muscle regeneration in response to necrotic damage of mature myofibres (Lepper et al., 2011; Relaix and Zammit, 2012). The fact that satellite cells are not present in mature muscles of insects, such as the fruit fly *Drosophila*, implies that satellite cells are not essential for normal homeostasis and function of skeletal muscles, in the absence of gross injury (discussed in Rai et al., 2013).

Mice and rats are valuable mammalian research models due to their ready availability, small size and many sophisticated tools available for modulation of gene expression, with cellular events in skeletal muscles and other tissues generally corresponding with the situation in humans. Once muscles are formed, the duration of the life span and the overall size of the animal will affect the kinetics of growth and tissue maintenance and also the overall loading of different muscles between species. Another factor to consider is the marked impact of different forms of locomotion on specific muscles: for example the bipedal posture of humans means far greater loading on leg compared with arm muscles, in contrast with the relatively shared load on all limb muscles in quadrupedal rodents. There are also important differences between muscle of mice and men with respect to myofibre type composition and activity patterns (discussed in Thornell, 2011)

The rapid postnatal growth of limb muscles in mice does not result from an increase in the number of myofibres, but initially involves fusion of myoblasts (derived from satellite cells) with the elongating myofibres (hyperplasia). After 3 weeks of age myofibre size increases mainly by hypertrophy alone (without addition of new myonuclei), growth slows after about 6 weeks and has almost ceased by 3 months (Grounds and Shavlakadze, 2011; White et al., 2010). Since the number of myonuclei at this later stage of hypertrophy appears fairly constant, the ratio of myonuclei to cytoplasm (myonuclear domain) changes with increasing myofibre size (discussed in Rai et al., 2013). Once bones stop growing and myofibre elongation ceases in the adult, homeostasis of the individual undamaged mature normal myofibres is usually then sustained with minimal (or no) contribution from satellite cells.

In mature myofibres of rodents and humans, a decrease (atrophy) or increase (hypertrophy) in size of the cross-sectional area (CSA) can alter the myonuclear domain size without any change in numbers of myonuclei (Brooks and Myburgh, 2014; Bruusgaard et al., 2012). Studies using mice that were genetically engineered to deplete most (>90%) satellite cells in adult muscles, confirm that hypertrophy of mature muscle can certainly occur initially in the absence of satellite cells (Jackson et al., 2012; McCarthy et al., 2011) reviewed in Fry et al. (2014b). In humans, significant myofibre hypertrophy can also occur without any change in numbers of myonuclei, as demonstrated in young males after resistance training where myofibre CSA was increased by 6% after 30 days and 17% after 90 days (Kadi et al., 2005). Where muscles undergo very marked hypertrophy beyond their normal size, this may involve fusion of satellite cells with the expanding myofibre: for example, increased numbers of myonuclei (~50%) are seen in very large myofibres (~9000 μm) of highly trained athletes using anabolic steroids (discussed in Kadi and Ponsot, 2010). Other studies in humans show that while 12 weeks of aerobic training increased the size (CSA) of both type 1 and type II human myofibres, only the type 1 myofibres showed increased numbers of satellite cells with accretion of myonuclei: emphasising important differences between myofibre-type responses to a specific exercise regime (Fry et al., 2014b). Apart from these situations of exercise or drug induced hypertrophy and adaptation, there may be little or no role for satellite cells in most healthy adult uninjured muscles, although this remains unclear. This controversial proposal is strongly supported by studies in ageing mice where satellite cells had been genetically depleted in adult muscles (at 4 months), yet muscles appeared normal throughout life with no acceleration or exacerbation of the age-related loss of muscle mass (sarcopenia) at 24 months of age (Fry et al., 2014a).

There are about 600 muscles in the human body, with many studies based on observations using less than a dozen mature muscles, mainly of the limbs. Great complexity exists across different anatomical muscles as elegantly demonstrated for the diaphragm (Stuelsatz et al., 2012). The tentative consensus is that for normal myofibres in homeostasis, the myonuclei do not turnover. However, some craniofacial muscles such as the extraocular and laryngeal muscles appear to maintain a population of activated/proliferating satellite cells in adults of various species, although their precise role and extent to which they fuse with mature myofibres is unclear (McLoon et al., 2004, 2007): these muscles seem the exception to the rule and will not be considered further in this paper. Such differences between diverse muscle types provide a cautionary note regarding generalisations across all muscles.

## 3. Incidence of myofibre necrosis and regeneration in normal ageing muscles and impact of exercise

The incidence of myofibre necrosis and hence of regeneration in mature muscles may normally be very low or non-existent for healthy individuals. While the opposite is sometimes assumed (i.e. that such myofibre damage is frequent), there is generally little or no evidence of regeneration reported in many muscles of ageing rodents and humans (Thornell, 2011), although the situation remains unclear (discussed in detail in Grounds, 2014). While physiological exercise is widely presumed to damage normal muscles this is not the case for mild exercise since voluntary running on wheels or treadmills for mice, and walking, running or swimming for humans, does not appear to result in any significant myonecrosis (Loenneke et al., 2014), even after an ultramarathon run of 160 km mainly downhill (Thornell, 2011). A wealth of studies in animals and human show that even mild exercise can activate satellite cells and cause proliferation (Aarimaa et al., 2004; Kadi and Ponsot, 2010; Macaluso and Myburgh, 2012), although it seems that these myogenic precursor cells often do not differentiate nor fuse with the myofibres. Thus the role and fate of this increased pool of satellite cells is intriguing (Macaluso and Myburgh, 2012). It is proposed that this high responsiveness of satellite cells to exercise serves to replenish the satellite cell pool (Kadi and Ponsot, 2010) and recent data suggest that satellite cells can modulate the ECM and limit fibrosis (Fry et al., 2014a). It remains unclear what this transient

activation of satellite cells signifies, or even whether many of these satellite cells/myoblasts may be lost from the muscles as occurs in other situations (see Section 5.5). In contrast, extreme exercise may result in myofibre necrosis: this is influenced by many factors, including the muscles involved and training status of the individual and the response can be very variable (Irintchev and Wernig, 1987). It is known that the soleus is susceptible to myonecrosis after reloading (after unloading) (Tidball and Wehling-Henricks, 2007) or a bout of unaccustomed mild exercise in mice, emphasising variation in the response of different muscles: however, the extent to which normal muscles of mice and especially of humans are injured by different forms of exercise remains unclear (Irintchev and Wernig, 1987; Thornell, 2011). This important issue of 'an inherent need for regeneration' requires further critical investigation especially for healthy human (and rodent) muscles that are not normally subjected to extreme exercise. This is especially relevant to the view that there is some inherent regular stimulus for muscle regeneration (and hence need for satellite cells and myogenesis) in normal ageing muscles, that forms the basis for the concept of 'failed regeneration' over time (see Section 6.3).

## 4. Classic identification of necrotic myofibres and key events during regeneration

The extent of initial myofibre death (necrosis) depends on the type of injury: some may affect only a portion of the long multinucleated myofibre, when this is known as focal or segmental necrosis, since formation of new cell membrane can 'seal off' the damaged segment (Papadimitriou et al., 1990). Necrotic myofibres are identified initially by fragmented sarcoplasm within about 24 h of the damage (evident histologically by light microscopy) and this is closely associated with infiltration of inflammatory cells from the vasculature. The ECM is especially important for the early steps in inflammation including immune cell migration and differentiation (Sorokin, 2010). Inflammatory cells include neutrophils, macrophages, mast cells and lymphocytes that secrete a wealth of enzymes and cytokines that help to initiate myogenesis and to remove the necrotic tissue within the myofibre to allow replacement by new muscle: the inflammatory response with interactions of many cytokine networks and especially the role of different classes of macrophages is essential for full muscle regeneration (Fig. 1A) (Forbes and Rosenthal, 2014; Kharraz et al., 2013; Smith et al., 2008; Tidball et al., 2014; Tidball and Wehling-Henricks, 2007). In response to such necrotic damage the quiescent satellite cells/myoblasts on the surface of myofibres become activated, proliferate (starting by 24 h) and move to the site of damage, at least in part by response to chemotaxis (Goetsch et al., 2013; Griffin et al., 2010; Robertson et al., 1993a; Torrente et al., 2003). Within a few days these myoblasts differentiate and then fuse to form small multinucleated cells called myotubes (from about 3.5 days) (Abmayr and Pavlath, 2012). Subsequently the myotubes fuse together and mature to replace the damaged myofibre and these myotubes also fuse with the ends of the sealed persisting segments of the parent myofibre (after about a week) to replace the damaged segment (Jarvinen et al., 2013; Robertson et al., 1993b). This whole process is completed within 2 weeks, followed by full maturation of the new muscle (Fig. 1) with a similar time course of events in rodents and humans (Jarvinen et al., 2013). Such classic necrosis/regeneration occurs in response to injury (physical, chemical, thermal or toxins) and after muscle transplantation, accidental trauma, or inherent pathologies such as in muscular dystrophies.

Care must also be taken to be specific when referring to 'muscle degeneration' as this can occur in many situations (*e.g.* autolysis, denervation) that need to be carefully distinguished from myofibre necrosis.

Problems with accurate interpretation of events after myofibre necrosis can arise where the focus is only on the process of myogenesis as a measure of muscle regeneration. Consideration should also be given to altered kinetics of other key events during regeneration, as briefly outlined below.

### 4.1. The amount of necrosis will influence the extent of muscle regeneration

Various drugs and interventions are tested experimentally for their ability to impair or enhance muscle regeneration, yet they may have reduced or increased the amount of initial damage. Thus the amount of initial myofibre necrosis in response to an intervention needs to be carefully assessed first, before evaluating the amount of new muscle formed. This is demonstrated by use of anti-inflammatory drugs that protect dystrophic muscle from myonecrosis induced by exercise (Hodgetts et al., 2006); consequently the amount of newly-formed muscles is reduced. This has sometimes been mis-interpreted as 'improved regeneration' and recovery from damage, when in reality it was due to reduced myonecrosis.

### 4.2. Inflammation must occur for complete new muscle formation

It is emphasised that inflammation is essential to allow full regeneration of new muscles to occur (see Section 1.4). If inflammation is prevented or delayed, then efficient removal of necrotic tissues will impair new muscle formation. This is an important point, since some studies interpret a delay in new muscle formation as impaired myogenesis, whereas the true explanation may instead be problems with inflammation; a very different interpretation that may have profound implications if extended to therapeutic interventions. This is illustrated for experimental whole muscle autografts that undergo myonecrosis, excellent new muscle is normally formed by 5 days in young adult mice: however this process is prevented by drugs that blockade the inflammatory cytokine TNF (Tumour necrosis factor) and significantly delay the influx of inflammatory cells, with consequent delayed initiation of myogenesis and overall regeneration (Grounds et al., 2005a). This is further demonstrated in autografts in very old mice (aged 29 months) where there is minimal inflammation initially and no myogenesis at 5 days (in striking contrast with young autografts), although there is subsequent 'catch-up' in old autografts with formation of excellent new muscle by 10 days in all grafts (Shavlakadze et al., 2010b). Similar observations of delayed kinetics of early regenerative events, but excellent long-term new muscle formation have been demonstrated in other models of injury/regeneration in old mice (Lee et al., 2013) and issues of accurate interpretation of some studies of regeneration in aged muscles are discussed in detail elsewhere (Grounds, 2014; Lee et al., 2013; Shavlakadze et al., 2010b).

### 4.3. Revascularisation may also be required in some models of injury

Some models of injury do not damage the vascular or nervous supply, such as myotoxins that kill only the myofibres (Lee et al., 2013) or minor focal injury. However, other models require revascularisation (*e.g.* contusion injury) and reinnervation of the damaged muscles—both of these apply to transplanted muscle grafts (Shavlakadze et al., 2010b) or whole muscles that are left *in situ* but the vascular and nerve supply is severed (Lee et al., 2013). In these situations, revascularisation is essential in order for the early events of regeneration to progress (Roberts and McGeachie, 1992). Thus a delay in angiogenesis and revascularisation needs to be clearly identified, since this can also lead to misinterpretation of events (as for the situation of delayed inflammation). Interventions

to enhance angiogenesis (Smythe et al., 2002), including exercise (Roberts and McGeachie, 1992), are potential strategies to accelerate new muscle formation in clinically transplanted muscle grafts or other surgical situations.

### 4.4. Reinnervation is essential for full maturation and function of regenerated myofibres

In striking contrast with the early need for inflammation and revascularisation, innervation is not required initially for regeneration, with myogenesis and new muscle formation unimpaired in denervated muscles after experimental damage *in vivo* (McGeachie and Grounds, 1989). In many models of muscle injury the innervation of myofibres probably remains intact. However, innervation is essential for full maturation of the newly formed myofibres *in vivo*: if reinnervation does not occur then function will be impaired. This was beautifully demonstrated for muscle grafts transplanted between young and old rats where excellent new muscle was formed initially but the long-term function of (young or old) grafts was impaired when implanted into old host rats and examined at 60 days: it was concluded that this was due to problems with reinnervation by the old hosts (Carlson et al., 2001).

### 4.5. Fibrosis impairs myogenesis and regeneration

The deposition of ECM molecules including collagens and fibronectin is increased by inflammation that follows necrosis. After a single bout of minor necrosis/inflammation this is not normally a problem. However, if the area of tissue damaged is very large and revascularisation is impaired, ischaemic conditions will favour fibroblast proliferation, fibrosis and scar tissue formation (Serrano and Munoz-Canoves, 2010). Fibrosis becomes a major problem in situations of chronic repeated damage/inflammation where the accumulating fibrotic tissue alters the ECM environment and cellular response. This occurs in myopathies like Duchenne muscular dystrophy in young boys (see also Section 6.2.2), where the progressive accumulation of fibrosis favours conversion of myoblasts into fibroblasts, regardless of their intrinsic myogenic capacity (Alexakis et al., 2007; Cisternas et al., 2013; Kharraz et al., 2014). The ECM composition and extent of fibrosis affects many aspects of muscle function, formation and the regenerative process including reinnervation (Cisternas et al., 2013; Grounds, 2008; Serrano and Munoz-Canoves, 2010). Fibrosis is also a major problem in pathological situations in many tissues, such as lungs, liver and kidney, with much interest in interventions to reduce or reverse this process (Cisternas et al., 2013). Age also alters the ECM and increases collagen deposition and fibrosis (Grounds, 2008). Accumulating molecular modifications to the cross-links of collagens (that turn over very slowly), with the dramatic acceleration of these changes in diabetes, leads to stiffness and impaired function in aged animals. Since the extrinsic ECM environment can impact on many aspects of muscle regeneration, the ECM composition will influence interpretation of altered cellular dynamics. Recently, it was reported that satellite cells contribute to modulation of the ECM in ageing muscles (without any essential role for satellite cells in maintenance of myofibre mass) (Fry et al., 2014a).

## 5. Potential confusion when identifying regenerated myofibres

As mentioned above, necrosis is identified by fragmented sarcoplasm and the presence of inflammatory cells within myofibres (Fig. 1). If this aspect is never observed, then changes of myofibres over time may instead be due to some other pathological cellular event. Where necrosis/inflammation has occurred, the early stages of myogenesis can be identified histologically in transverse or longitudinal sections of muscles stained with haematoxylin and eosin: initially myoblasts cuffing within the contour of the necrotic myofibres can be observed, followed by small myotubes with central myonuclei that enlarge and mature over time (Fig. 1).

### 5.1. Central myonuclei

Central myonuclei are widely considered a hallmark of a myofibre that has undergone regeneration (*e.g.* after experimental injury or endogenous necrosis of dystrophic muscles), and such displaced (*i.e.* non-peripheral) myonuclei after regeneration can persist for many months in mice. However such central myonuclei can also occur for different reasons and these need to be considered. For example, central myonuclei are also a feature of denervated myofibres (Lu et al., 1997). Therefore, when observing muscle biopsies or old normal muscles without any knowledge of the history of preceding cellular events, the presence of a myofibre with a central or misplaced myonucleus can be difficult to interpret: is it a regenerated or a denervated myofibre or due to some other pathology? It is noted that misplaced myonuclei (not secondary to muscle regeneration) are also a feature of centronucleated myopathies, which result from mutations in genes for myotubularin, amphiphysin 2 or dynamin 2 (Cowling et al., 2014; Romero, 2010).

### 5.2. Expression of embryonic or neonatal myosins

Expression of embryonic or neonatal myosins (often visualised by immunostaining) is another parameter used to identify newly formed *i.e.* regenerating myofibres, in mature muscles; however, this can also be subject to ambiguity. While new myotubes express embryonic myosin initially (recapitulating embryogenesis) this is replaced by mature myosin isoforms during maturation. Conversely, denervated myofibres are known to re-express embryonic myosins (Jakubiec-Puka et al., 1990; Schiaffino et al., 1988). The strong immunostaining for neonatal myosin in dysferlinopathy patient muscle biopsies (and presence of central myonuclei in dysferlin-deficient mouse muscles) that show little evidence of classic endogenous necrosis/regeneration (Chiu et al., 2009), may also be due to some form of myofibre degeneration. In ageing muscles of male rats, the increasing expression of embryonic and neonatal myosins (pronounced by 18 months of age), seems more likely due to denervation or some other pathological change, since no evidence of myonecrosis or inflammation was reported (Ibebunjo et al., 2013). Thus the precise reason for a myofibre expressing embryonic or neonatal myosin needs to be carefully considered.

### 5.3. Expression of myogenin

Newly formed muscles re-express many genes associated with myogenesis, such as embryonic myosins (discussed above in Section 5.2) and the myogenic regulatory transcription factors MyoD and myogenin. In addition, newly formed myotubes express genes associated with lack of innervation such as nicotinic Acetylcholine Receptor (nAChR) subunits γ and δ (that are down-regulation with innervation). It is well documented that genes such as nAChR-γ and-δ are also re-expressed in denervated muscles, but less widely recognised is the fact that this also occurs for myogenin and MyoD (Chai et al., 2011; Dedkov et al., 2003; Eftimie et al., 1991; Kostrominova et al., 2000; Moresi et al., 2010; Voytik et al., 1993). Elevated myogenin (mRNA and immunostaining) was also reported in denervated human biceps muscles (Chen et al., 2011) with highest mRNA levels after 7 months (~38 times more than normal) and sustained elevation even after 26 months. In very old muscles of mice (aged 24 to 29 months) where denervation is well described

(Chai et al., 2011), levels of mRNA for myogenin were strikingly elevated, as were other genes like nAChR-γ and-δ (Barns et al., 2014) and similar observations were made in muscles of very old rats (Dedkov et al., 2003; Ibebunjo et al., 2013). Thus in some situations such as ageing muscles it can be difficult to identify the precise reason for increased myogenin levels, since degeneration of NMJs is well-documented and the extent to which necrosis/regeneration may have occurred is sometimes unclear. In our study in old female C57Bl/6J quadriceps (Barns et al., 2014), there was no histological indication that necrosis or regeneration had occurred, since areas of fragmented sarcoplasm with inflammation and young myotubes were not observed at any age and myofibres with non-peripheral nuclei were not conspicuous before 29 months, and even at this old age their number was low (∼5% of the total myofibre number). However, flattened atrophic myofibres with peripheral myonuclei were observed in quadriceps from mice aged ≥24 m and these are considered a feature of denervated muscles.

### 5.4. Split or branched myofibres

Split or branched myofibres are also a feature of short-and long-term regenerated myofibres and persist, along with the presence of central myonuclei (discussed in Grounds, 2014; Head, 2010). While split myofibres arise after necrosis of mature myofibres during myogenesis (within or outside the damaged myofibre), some may result from incomplete lateral fusion during myogenesis. They can also be formed by cleavage of mature 'undamaged' myofibres (Irintchev and Wernig, 1987) especially when myofibres become very large due to a hypertrophic stimulus (Shavlakadze et al., 2010a), and such split myofibres appear to increase with age (Pichavant and Pavlath, 2014).

### 5.5. Activated and proliferating satellite cells

Activated and proliferating satellite cells are early features of *bona fide* muscle regeneration, but they can also occur in other situations without any subsequent fusion with myofibres: they may simply revert back to the quiescent state or indeed may disappear from the muscle tissue. Such ephemeral activation and proliferation of satellite cells (in the absence of myofibre necrosis) without apparent fusion with myofibres is widely reported after many forms of exercise (discussed in Section 3). These activated/proliferating myogenic cells can be identified by expression of myogenic markers such as Pax7 or MyoD, or DNA synthesis confirmed by immunostaining for PCNA (Proliferating Cell Nuclear Antigen) or more permanent labelling by markers incorporated into new strands of DNA, such a tritiated thymidine or bromodeoxyuridine. Transient activation of satellite cells (as seen after exercise) appears to be quite common, although it is not clear what the significance and consequences may be. Another classic example of satellite cell activation (in the absence of myonecrosis) is in response to experimental denervation, which has been described extensively. Experiments in the 1970s with transplanted whole muscle grafts in rats reported that when muscles had been denervated for several weeks prior to transplantation, more myoblasts were present and the necrotic muscles (resulting from ischaemia due to grafting) regenerated more rapidly (reviewed in McGeachie and Allbrook, 1978); this is because the prior denervation had already activated the satellite cell population. These early studies used tritiated thymidine to label cells synthesising DNA, and showed in tibialis anterior and tongue muscles that denervation (and to a lesser extent tenotomy) rapidly increased the proliferation of satellite cells and connective tissue cells within 7 days (McGeachie and Allbrook, 1978). A series of papers in denervated mouse muscles (McGeachie, 1985, 1989; Murray and Robbins, 1982a,b) showed a peak of satellite and connective tissue cell proliferation by 3–4 days after denervation and, when cells were labelled either in week 1, 2, 3 or 4, the satellite cell proliferation was sustained for at least one month after denervation and it seems likely that this might be ongoing (reviewed in McGeachie, 1989). These labelled cells did not survive; many were lost by 8 days and over 90% were gone from the muscle tissue by 6 weeks (Murray and Robbins, 1982b). Importantly, there was no fusion of the labelled satellite cells with the atrophic denervated myofibres even after 4 weeks (McGeachie, 1985). Such rapid satellite cells activation and proliferation after denervation is reported in many others muscles. Further evidence that this response is abortive and there is no fusion with the denervated myofibres was provided by a study in rats where the spinal cord was transected: satellite cells were activated and some proliferated and this was increased by exercise, but there was no fusion of satellite cells into the myofibres within the time studied (Dupont-Versteegden et al., 1999).

A similar situation is reported for cachexia in tumour-bearing mice and patients with pancreatic cancer, where (in the absence of any experimental injury) there was associated activation of many satellite cells (and other myogenic precursors) that did not differentiate and did not fuse with myofibres (He et al., 2013). Thus the presence of activated or proliferating satellite cells can be a transitory event in response to some disturbance of homeostasis and certainly is not always a prelude to formation of new muscle.

These examples emphasise that caution must be applied for each of these parameters that are not exclusively a feature of regenerating skeletal muscles (central myonuclei, embryonic myosin isoforms, elevated myogenin, split myofibres and activated/proliferating satellite cells), when assessing and interpreting the histomorphology (and gene expression) of muscle tissues.

## 6. Clinical situations which require enhanced skeletal muscle regeneration

Skeletal muscle necrosis and the need for new muscle formation may be an infrequent occurrence for relatively sedentary normal humans (as discussed in Section 3), which applies to a large proportion of people across the world. Yet without dispute there is clearly a major need for muscle regeneration in many situations after myonecrosis. Two clinical areas where strategies to enhance muscle regeneration are of key interest are briefly outlined: one relates to severe acute injury or transplantation (and includes bioengineering) and the other to chronic muscle degeneration, in diseases like muscular dystrophies. A third situation is also mentioned regarding a controversy related to proposed stem cell therapies for ageing muscles.

### 6.1. Acute and chronic injury and the need for regeneration and new muscle formation

Severe injuries that result in necrosis can occur after accidents, road trauma, war casualties, surgery, muscle transplantation and other surgical and orthopaedic situations, as well as damaging exercise in sports medicine with high incidence of contusion injury associated with many sports (Smith et al., 2008), and disease (Shin et al., 2014). Skeletal muscle has a proven capacity for regeneration to cope with many of these situations. The overall functional disability resulting from such muscle damage that starts with myonecrosis, will directly depend of the size and nature of the injury combined with the speed and capacity to optimise all of the components of regeneration outlined above: i.e. inflammation to remove the damaged tissue, revasularisation to reconnect the blood supply, myogenesis to form new muscle, and innervation to optimise function, plus restoration of the ECM components. This must be done while minimising fibrosis. The multitude of

interventions and drugs that are used or being investigated to modulate and hopefully enhance these many processes required for optimal inflammatory response (Smith et al., 2008), new muscle formation *in situ* with functional regeneration and minimal fibrosis, are beyond the scope of this paper (Grounds, 2011).

#### 6.1.1. Tissue bioengineering to form new muscles

Where large areas of muscle need to be replaced, muscle grafting was once the only option. Clearly the classic events of regeneration (inflammation, revascularisation, myogenesis and innervation) are targets for interventions to accelerate the speed and efficacy of new muscle formation in grafts after transplantation: however, the source of autologous muscles for such grafting (to avoid immune rejection) is a problem. To address this, novel strategies are being intensively explored to replace severely damaged muscles that have failed to regenerate, or muscles that have been lost by prolonged denervation, aggressive tumour ablation or other circumstances. Tissue bioengineering aims to develop either bioscaffolds that can be implanted to enhance new muscle formation *in vivo*, or to construct complex muscle structures *ex vivo* for subsequent implantation to replace the missing muscles (Wang et al., 2014). These approaches have many technical challenges and focus on topics related to suitable biomaterials and ECM for functional bioscaffolds, various sources of myogenic stem cells (ideally autologous) to populate the bioscaffolds, along with the design of vascular networks and factors to enhance innervation (Juhas and Bursac, 2013; Klumpp et al., 2010; Li et al., 2011). There is much creativity in this field of regenerative medicine although, at this stage, bioengineering of skeletal muscles is still mainly experimental rather than a clinical reality.

### 6.2. Therapies for muscular dystrophies that target aspects of muscle regeneration

It is appropriate to mention the lethal childhood disease Duchenne muscular dystrophy (DMD) where repeated cycles of myofibre necrosis over many years, (probably exacerbated in growing muscles), results in increasing fibrosis and failed regeneration with progressive replacement of many skeletal muscles by fibrous and fatty connective tissue. While other forms of muscle diseases also result in replacement of myofibres with fibrous fatty connective tissue, this discussion will focus on DMD. Many interventions are being developed using dystrophic mdx mouse and dog models and trialled for DMD (reviewed in De Luca, 2012; Foster et al., 2012; Malik et al., 2012). This paper makes no attempt to review the field but instead will draw attention to three major therapeutic approaches that involve aspects of regeneration related to: (1) myoblast or stem cell therapy, (2) prevention of myofibre necrosis and (3) reduction of fibrosis.

#### 6.2.1. Myoblast or stem cell therapy

Myoblast or stem cell therapy is highly attractive as a gene replacement strategy since, theoretically, it can introduce normal donor myonuclei (carrying the normal dystrophin gene) into the multinucleated myofibres of DMD boys where host myonuclei have gene defects for the protein dystrophin. This approach takes full advantage of the normal events of regeneration and new muscle formation where many myoblasts fuse together and with the damaged myofibres so that myonuclei (dystrophic host and normal donor) share a common syncytium, with the normal (donor) dystrophin protein then distributed within this single long muscle cell. Such myoblast cell therapy requires that muscles are damaged and regenerating, as occurs repeatedly in DMD. This approach has been intensively investigated for over 30 years and remains attractive although, unfortunately, many problems remain to be resolved before gene replacement using this cell-based strategy becomes a clinical reality (reviewed in Grounds and Davies, 2007; Negroni et al., 2011; Skuk et al., 2014).

#### 6.2.2. Prevention of myofibre necrosis

Prevention of myofibre necrosis is the main aim of many interventions for DMD. The dystrophin protein is located beneath the sarcolemma and forms a link from the cytoskeletal contractile proteins of the sarcomeres (where force is generated), across the sarcolemma (through the transmembrane dystrophin–dystroglycan complex) to laminins and other ECM proteins (where force is manifested mainly *via* the collagens) (Grounds et al., 2005b). Defects in the dystrophin protein result in susceptibility of the sarcolemma to mechanical loading and a high incidence of myofibre necrosis. This endogenous myonecrosis occurs repeatedly and seems to be exacerbated by exercise and growth (Grounds and Shavlakadze, 2011). While regeneration and new muscle formation is normally effective in most dystrophic mdx limb muscles, over time it fails in the DMD boys and muscles are replaced by fibro-fatty connective tissue. It has been widely proposed that the failure to form new muscle over time in DMD is due to exhaustion of myogenic stem cells: yet it seems more likely that the problem may instead be the changing ECM environment of the myogenic cells that suppresses the intrinsic myogenic potential. Every cycle of necrosis and inflammation deposits fibrous connective tissue that progressively alters the ECM composition (as discussed in Section 4.5). Where this occurs over years in DMD boys (compared with only weeks in the acute phase of the disease in the dystrophic mice) the accumulating fibrosis results in an adverse environment that no longer favours myogenesis

The focus on the vulnerable dystrophin-deficient sarcolemma has provided much insight into how myofibre necrosis is initiated, with inflammation, calcium dysregulation and oxidative stress being the main pathways involved. Thus drugs that target many components of these pathways have been intensively investigated, with many promising therapeutic possibilities emerging (reviewed De Luca, 2012; Malik et al., 2012; Radley et al., 2007). Wider recognition of different metabolic demands of dystrophic muscles with targeted nutritional interventions to meet the high energy needs (Radley-Crabb et al., 2014) is another approach to help protect dystrophic myofibres from necrosis: such tailored dietary interventions are attractive to use in combination with other therapies.

While it is also proposed that therapeutic interventions to enhance regeneration are required for DMD, this seems to focus on the wrong aspect. Quite simply, it seems far more potent to reduce the need for regeneration by preventing myonecrosis, in DMD or mdx muscles. Although there is excellent regeneration of limb muscles in adult mdx (C57Bl/10Scsn) mice, it is not uncommon to read that a particular intervention has 'improved muscle regeneration'. What exactly does this mean in this context of already excellent new muscle formation in this species? Instead, evidence of less regeneration, that reflects less initial damage, is desirable. More precise specification of what has occurred is required to help ascertain the mode of action of the candidate therapeutic agent. Prevention of myofibre necrosis will spare dystrophic muscles from the energy consuming cellular processes involved in regeneration, new muscle formation and re-growth, and will also prevent the accumulation of fibrosis that has such adverse consequences.

#### 6.2.3. Reduction of fibrosis

The accumulation of fibrous and fatty connective tissue in DMD and mdx muscles results from the repeated cycles of necrosis and inflammation (Kharraz et al., 2014) and fibrosis in mdx mice can be increased by a range of experimental strategies (Pessina et al., 2014). Transforming growth factor beta (TGF-β) is widely recognised as a major pro-inflammatory and pro-fibrotic cytokine (Ceco and McNally, 2013) and other factors within dystrophic

muscles can exacerbate fibrosis. Recently it has been emphasised that genetic background and modifier genes can alter disease severity, as shown for the DBA/2 mouse strain that has increased fibrosis due to mutations in the gene that codes for latent TGF-β -binding protein 4 (LTBP) (Ceco and McNally, 2013; Heydemann et al., 2009). Increased severity of dystropathologies on the DBA/2 background was first demonstrated for the mouse model of human Limb Girdle Muscular Dystrophy 2C that is due to sarcoglycan deficiency (Heydemann et al., 2009). Subsequently, mdx mice were bred with DBA/2 mice, with resultant increased fibrosis, poor quality of regenerated muscles and pronounced dystropathology that more closely resembled the human DMD condition (Fukuda et al., 2010): the extent to which this can be accounted for primarily by increased fibrosis is unclear.

Anti-fibrotic drugs show benefits with reduced fibrosis in dystrophic muscles of mdx mice (Riquelme et al., 2014; Swiderski et al., 2014; Turgeman et al., 2008) with some translation to clinical trials in DMD boys (reviewed in Kharraz et al., 2014). It seems likely that combinations of drug and nutritional interventions *e.g.* to reduce both myonecrosis and fibrosis, will provide additive benefits in the quest to optimise therapies to protect the dystrophic myofibres and reduce the severity of dystropatholgy in DMD boys.

### *6.3. Is impaired regeneration due to lack of myogenic stem cell capacity a cause of age-related muscle wasting (sarcopenia)?*

This topic is mentioned because myogenic stem cell therapy has been promoted as a potential intervention to enhance regeneration and reduce the progressive age-related loss of skeletal muscle mass and function, known as sarcopenia. This therapy is based on the assumptions that (a) there is some turnover of myonuclei and satellite cells, with ongoing necrotic damage and hence a need for regular regeneration using myogenic stem cells in normal aging muscles; and (b) that these processes exhaust the satellite cells with impaired myogenic stem cell capacity *in vivo* and inability to form new muscles over time in very old muscles. Yet as discussed above (Sections 1.2 and 1.3) it seems that satellite cells may not play a role during homeostasis of normal mature muscle (in the absence of injury), plus there is little evidence of necrosis/regeneration in most healthy old humans or rodents in response to moderate exercise (discussed in Section 1.3). Indeed Thornell (2011) who has worked extensively with ageing human muscles, states that 'in my view, the satellite cells are not a key factor in the decline of muscle mass in humans'. This view is further endorsed by studies in mice genetically engineered to conditionally deplete satellite cells in adult mice, where sarcopenia was unaffected in most muscles aged 24 months (Fry et al., 2014a).

The concept of an 'exhaustion of the pool of satellite/stem cells' required for muscle regeneration is widely proposed for various situations in diseased and normal muscle. Yet studies using many repeated bouts of experimental muscle damage (with intramuscular injections of bupivacaine) for 6 months in normal rats (Sadeh et al., 1985) and 50 bouts of additional damage in mdx mice (Luz et al., 2002), show that new myofibres continue to be formed, confirming that the pool of satellite cells (intrinsic capacity) has not been exhausted even by such excessive demands. However, the repeated damage progressively has adverse effects on the quality of the regenerating muscles reflecting the impact of altered extrinsic factors such as increasing fibrosis. It is important to distinguish between possible loss of intrinsic myogenic capacity versus a sustained myogenic capacity that cannot be manifested due to adverse extrinsic factors such as altered ECM composition, especially fibrosis. The fact that very old rodent and human muscles show an excellent capacity to form new muscles after experimental myonecrosis, indicates a persisting myogenic capacity even of old satellite cells *in vivo* (Alsharidah et al., 2013; Barberi et al., 2013; Carlson et al., 2001; Collins et al., 2007; George et al., 2010; Lee et al., 2013; Shavlakadze et al., 2010b; Zhang et al., 2014). These and other factors question the premise for promoting stem cell therapy for sarcopenia. It seems appropriate to more critically evaluate the actual need, and also the likely clinical reality, of such a proposed invasive intervention for muscles of elderly people (discussed in Grounds, 2014).

Instead of some fundamental problem with myogenesis in old muscles, it seems more likely that sarcopenia results from age-related deterioration of the intact myofibres *per se*, related to altered metabolism, denervation of myofibres and changes in the local environment. These complex changes over time (starting after about 15 months in mice) are strongly supported by transcriptome and proteomic analyses of ageing mouse and rat muscles that demonstrate progressive alterations related to denervation, metabolism and the ECM (Barns et al., 2014; Ibebunjo et al., 2013). It clearly is of central importance to determine the reasons for sarcopenia, in order to monitor progression of this muscle loss and to develop the most appropriate therapeutic interventions to maintain the health of ageing muscles.

## 7. Conclusions

Classic muscle regeneration is the response to myonecrosis. The term 'muscle regeneration' should be used precisely. It should not be applied to other situations of minor muscle tissue damage, nor to muscle atrophy followed by a subsequent increase in muscle mass (hypertrophy) that should be considered as 'restoration of muscle mass' and does not usually involve satellite cells and their fusion with myofibres. When cellular changes that are widely considered as 'markers of regeneration' are observed in muscles (e.g. central myonuclei, expression of embryonic/neonatal myosins and/or myogenin, split myofibres, or activated/proliferating myogenic cells), care must be taken in interpretation since these markers can also occur in other situations that do not involve myonecrosis and regeneration (e.g. pathologies, denervation, normal ageing and various forms of exercise). There are many clinical situations where acute or chronic necrotic damage occurs that requires interventions to enhance muscle formation *in situ* or to replace entire muscles that have been irreversibly damaged. This translational field is ripe for creative solutions that rely on a good understanding of the overall sequential cellular steps required for effective new muscle formation and function (including inflammation, revascularisation, ECM remodelling, myogenesis and innervation): each of these steps is a potential target for clinical therapies to enhance skeletal muscle regeneration.

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