

Therapies for sarcopenia and regeneration of old skeletal muscles

More a case of old tissue architecture than old stem cells

Miranda D Grounds

School of Anatomy, Physiology and Human Biology; University of Western Australia; Crawley, Australia

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Age related loss of skeletal muscle mass and function (sarcopenia) reduces independence and the quality of life for individuals, and leads to falls and fractures with escalating health costs for the rapidly aging human population. Thus there is much interest in developing interventions to reduce sarcopenia. One area that has attracted recent attention is the proposed use of myogenic stem cells to improve regeneration of old muscles. This mini-review challenges the fundamental need for myogenic stem cell therapy for sarcopenia. It presents evidence that demonstrates the excellent capacity of myogenic stem cells from very old rodent and human muscles to form new muscles after experimental myofiber necrosis. The many factors required for successful muscle regeneration are considered with a strong focus on integration of components of old muscle bioarchitecture. The fundamental role of satellite cells in homeostasis of normal aging muscles and the incidence of endogenous regeneration in old muscles is questioned. These issues, combined with problems for clinical myogenic stem cell therapies for severe muscle diseases, raise fundamental concerns about the justification for myogenic stem cell therapy for sarcopenia.

Background

Sarcopenia, the age-related loss of skeletal muscle mass and function, is now recognized as a major clinical problem for older people and research in the area is expanding exponentially.¹ The aim of this mini-review is not to discuss the many dimensions of sarcopenia and potential therapies, but instead to focus on one specific intervention related to muscle regeneration. There is a controversy regarding the efficacy of new skeletal muscle formation in very old animals in response to injury. There is good evidence that regeneration of many tissues can be slower (although not necessarily impaired) in old compared with young animals due to a range of factors that include age-related changes in the

speed and efficacy of the inflammatory response (an essential pre-requisite for subsequent new tissue formation), alterations in vascular architecture and blood supply, changes in extracellular matrix composition and architecture with increasing fibrosis in old age, and less effective re-innervation of the damaged old tissue. These major components of the complex integrated architecture of muscle tissue are essential for healthy muscle function and for regeneration after major damage. What has recently captured attention is the extent to which the intrinsic capacity of stem cells might, or might not, be compromised with age. Accordingly, myogenic stem cells and the proposal for stem cell therapies to treat sarcopenia, based on the notion of “failed regeneration due to impaired myogenesis of old muscles,” are the central focus of this review.

Support for Excellent In Vivo Capacity of Myogenic Precursor Cells from Old Muscles

The controversy pertaining to regeneration of old muscles, concerns the capacity of skeletal muscle precursor cells (widely referred to as myogenic stem cells, satellite cells or myoblasts) to form new muscle in old animals in response to experimental damage. This was addressed by a comprehensive recent study of skeletal muscle regeneration induced by different types of injury in young adult (3 mo), old (22 mo), and geriatric (28 mo) normal mice.² The 3 injury models were a myotoxin (notexin from snake venom) which leaves the blood vessels and nerves intact; freezing that damages local muscle, nerve and blood vessels; and denervation and devascularization which dissociates the nerves and blood vessels from the whole muscle (this is similar to transplantation of whole intact muscles). It is well documented in young rodents that all of these experimental models cause necrosis (death) of the myofibers and stimulate regeneration with replacement of the damaged tissue by new muscles. Studies were done in female C57Bl/6J mice (the important influence of gender was discussed) and tissues were sampled at several times from 7 to 30 d after injury to allow for the full gamut of regenerative events

Correspondence to: Miranda D Grounds;
Email: miranda.grounds@uwa.edu.au
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to occur. Histological analyses revealed successful architectural regeneration with excellent new muscle formation following notexin injury with negligible fibrosis and fully restored function, regardless of age. This confirms that there is no detectable problem with the *in vivo* intrinsic myogenic capacity of myogenic precursor cells to form new muscle even in very old mice aged 28 mo. However, this study did emphasize that problems with the vascular and neural supply can influence effective overall regeneration (this was an issue for all ages) and thus the impact of different models of injury needs to be considered. These findings directly refute the idea that the myogenic capacity of muscle satellite cells in aged muscle is decreased and emphasize that other aspects of regeneration are a more relevant focus for therapies. Clearly there is much clinical interest to improve the regeneration of old human muscles in response to accidental or surgical trauma.

This study supports earlier *in vivo* studies in rodents that show excellent myogenic capacity with good muscle formation in very old muscles after injury. Over 25 y ago, Sadeh (1988)³ performed a comprehensive time-course analysis of regeneration in young, mature, and old rats (aged 3, 12, and 24 mo, gender not specified) after muscle injury resulting from intramuscular injection of the toxin bupivacaine; tissues were sampled for histological analysis at 2, 3, 4, 5, 7, 14, 21, 28, and 56 d.³ Even in old rats, myotubes were formed by day 7 indicating no problem with inherent myogenic capacity. What was striking was that the process of inflammation and consequent regeneration was progressively delayed with aging and subsequent maturation of the new myofibers was impaired in the old rats. This most likely reflects problems with re-innervation and fibrosis that is well documented to prevent functional restoration of damaged old myofibers⁴: indeed, inherent problems with neuromuscular activity are now increasingly recognized as a major issue in age-related loss of muscle mass and function in rodents and humans.⁵⁻¹¹

A series of studies performed since the 1980s used a model of whole muscle transplantation to study muscle regeneration, with cross-transplantation of extensor digitorum longus (EDL) muscles between young and old rodents (heterochronic grafts) being an excellent model to distinguish between the influence of factors intrinsic within the muscles, compared with systemic factors. Long-term studies in rats assessed the functional properties of muscles at 60 d, and unequivocally showed that young or old muscles in old hosts had impaired function (probably due to issues with re-innervation in old hosts), whereas muscles from old rats aged 24 or 32 mo showed excellent new muscle formation and functional properties in young hosts or after injury with Marcaine (reviewed in⁶), indicating no intrinsic problem with myogenesis of the old muscles. The question of whether the kinetics of early regenerative events and myogenesis were altered in old muscles was addressed by a time course study of regeneration in 74 grafts (at days 2, 3, 4, 5, 6, and 7) using the cross-transplantation model between young and old mice aged up to 21 mo.¹² While a slight age-associated delay in inflammation and neovascularisation was seen in old hosts (and a marked age-related delay in neovascularisation induced by old muscle was demonstrated using another assay), this did not significantly affect the

formation of myotubes at 7 d in any grafts. Subsequent cross-transplantation studies using much older mice (female C57Bl/6J) aged 27–29 mo¹³ showed a marked delay in inflammation (and hence no initiation of myogenesis) at 5 d after transplantation for old autografts, a time when new myotubes were conspicuous in young autografts and there were no adverse effects of the old host on young autografts. Thus age-related delays and alterations in the inflammatory response need to be considered.¹⁴ However, by 10 d after transplantation, excellent new muscle was present in all grafts regardless of age.¹³ Thus this transient delay in the onset of myogenesis did not significantly compromise the excellent overall *in vivo* myogenic capacity of even very old muscles in rodents. There is certainly evidence for subsequent disturbed progression and resolution of long-term muscle maturation after regeneration in some very old muscles but, rather than an inherent problem with the myogenic precursor cells, this seem more likely to be due to factors like impaired re-innervation and the adverse impact of the environment of denervated old muscles (discussed in refs. 2 and 13).

Two variants on this approach of muscle transplantation also support excellent *in vivo* myogenic capacity of old muscles in vertebrates. In mice, single myofibers isolated from muscles of old mice (aged about 24 mo) had fewer Pax7+ satellite cells compared with young myofibers and yet, when examined at 4 wk after engraftment into irradiated muscles of young *mdx*-nude mice, they showed excellent new muscle formation *in vivo* from donor myoblasts equivalent to that seen with grafts from young myofibers:¹⁵ it was concluded that a minor subset of stem-like satellite cells survives the effects of aging. Additional experiments in this study showed that isolated myofibers derived from young and old muscles, after *in vivo* engraftment and subsequent notexin injury, contained similar numbers of newly-regenerated donor myofibers. Despite this similarity between the myogenic capacity of young and old myogenic stem cells *in vivo*, there were marked differences in tissue culture, emphasizing that *in vitro* results are not necessarily representative of what happens *in vivo*.¹⁵ Another model transplanted xenografts of small strips of human muscle into immunocompromised mice: when this was done for autopsied muscle removed from an octogenarian cadaver (at 2 d post-mortem), excellent new muscle was formed, unequivocally demonstrating robust *in vivo* capacity of myogenic cells even from very old humans.¹⁶

From the clinical perspective, it is especially relevant that tissue culture studies of human muscles show no apparent differences between the behavior of muscle precursor cells isolated from vastus lateralis muscles of young (20–25 y) compared with elderly (67–82 y) people,¹⁷ in their capacity for proliferation, differentiation, and senescence. Another study using human satellite cell cultures derived from old active or sedentary subjects (aged 68–80 y) similarly concluded that the satellite cells “do not show any difference in their proliferative capacity, nor do they differ from those derived from young donors” (aged 15–24 y)¹⁸ and stated that while there were some problems with satellite cell proliferation “this limit is not reached during normal aging”¹⁸: both of these human studies support the above *in vivo* studies in rodents. In addition, no adverse effect on myogenesis

was observed for young satellite cells grown in serum taken from elderly people (aged 69–84 y).^{18,19} Such human results are very significant when considering feasible clinical interventions. These combined studies do not support proposed stem cell therapy to treat sarcopenia, since the intrinsic myogenic (stem) cells seem quite adequate and excellent new muscle can be formed in vivo even in geriatric muscles.

The Opposite View of Age-Impaired Myogenic Stem Cells

In contrast, a series of complex studies using parabiosis to conjoin the blood supply of young (2–3 mo) and old (19–26 mo) C57Bl/6J mice, followed by freeze injury, in conjunction with extensive tissue culture studies,^{20,21} concluded that myogenic stem cell activity is significantly impaired in old muscles. These in vivo studies only examined the tissues up to 5 d after damage in vivo and did not take into account the delayed time-course of inflammation and other essential early regenerative events in old tissues (discussed in ²). A transient delay in early events required for regeneration of old muscles is not the same as ‘impaired’. The concept of impaired myogenic stem cells per se is further promoted by three recent papers in the journal *Nature*: Cosgrove et al. (2014)²² conclude that there is a “cell-autonomous functional decline in skeletal muscle stem cells” that supports “localized autologous muscle stem cell therapy for the elderly”; Sousa-Victor et al. (2014)²³ similarly state that their findings “provide the basis for stem-cell rejuvenation in sarcopenic muscles”; and Bernet et al. (2014)²⁴ using tissue culture studies of isolated satellite cells and combinations of cultured satellite cells on isolated myofibers as heterochronic grafts, also propose that “age-associated deregulation of a satellite cell homeostatic network” presents new therapeutic opportunities for sarcopenia. These are sophisticated studies with many implications that also rely on extensive tissue culture studies of purified populations of myogenic precursor cells.

It is well recognized that “data from tissue cultures do not provide a reliable guide to in vivo behaviour” since “tissue culture, lacks both the architecture and, usually, the metabolic fidelity of the normal tissue in vivo.”¹⁶ One specific example is that the behavior of satellite cells is heavily influenced by adjacent cells such as fibroblasts with crucial dynamic interactions between these cells types in vivo,²⁵ yet fibroblasts are absent from most in vitro experiments using satellite cells. Fibroblasts are very important for production of many extracellular matrix (ECM) molecules and it is well documented that the ECM composition is critical for skeletal muscle function, regeneration, and myogenesis in vivo.²⁶ Many questions remain concerning how accurately tissue culture studies represent what may happen in vivo. The promotion of stem cell therapies to alleviate human sarcopenia hinges on the notion that myogenic stem cell function is impaired in old muscles: yet this is clearly strongly disputed. Such stem cell therapy requires much more critical consideration and should also take into account three other issues briefly outlined below.

Balanced Discussion of the Literature

One concern is that many proponents of such stem cell therapy, e.g.^{22,23} rarely cite (let alone critically discuss) literature that supports the contrasting view that there is nothing significantly wrong with the intrinsic quality of the myogenic stem cells from old muscles in vivo. Such omission does not provide a strong foundation for progression of rigorous science and meaningful discussion of realistic clinical applications.

Is Myogenesis Required for Homeostasis of Mature Uninjured Muscles?

A second major concern relates to the assumption that there is an inherent need for regular muscle regeneration (that involves myofiber necrosis and subsequent myogenesis) in adult and old muscles and that satellite cells play some ongoing role in the homeostasis of normal uninjured adult and aging muscles. Yet, there is remarkably little evidence to support this wide-spread view. Instead, the opposite seems to be the case for normal sedentary people, even those who partake in regular mild exercise, and it may be that years and even decades pass by without any myofiber necrosis: myogenesis is not a feature of most mature muscles of normal aged rodents and humans (reviewed in ref. 27). As indicated below, studies in very old mice generally provide evidence against any significant endogenous necrosis/regeneration in normal old muscles. It is widely reported that numbers of satellite cells decrease with age and this was supported by very detailed studies that show highly variable numbers of satellite cells between individual myofibers, with a decrease in numbers (but no change in myogenic potential) from about 12 mo of age up to 33 mo in aging male C57Bl/6J mice^{28,29}; however, this may be of little in vivo consequence during normal muscle homeostasis (in the absence of major or repeated damage). The numbers of satellite cells can be influenced by the muscle examined (often limb muscles) and the label used to identify satellite cells (e.g Pax7), and a study in rat diaphragm muscles of male rats aged up to 24 mo concluded that the satellite cell number ‘may not be affected by aging at least in a muscle functioning constantly’.³⁰ While the incidence of severe damage that results in necrosis of myofibers may be a relatively rare events in many situations for those with sedate lives, clearly satellite cells remain absolutely essential for myogenesis to repair necrotic damage, if and when it occurs. This question regarding the actual incidence (if any) of satellite cell activation and fusion with normal aging myofibers, is of central importance as a foundation for the proposed need for myogenic stem cell transplantation, but also in the context of therapies that propose “rejuvenation” of the niche for myogenic stem cells. A critical appraisal of the evidence for any ongoing contribution of satellite cells to normal myofibers in homeostasis and especially for the incidence of myofiber necrosis/regeneration needs to be provided and carefully evaluated for muscles of both rodents and humans.

Most muscle damage, e.g that may result from abnormal exercise overload, is more likely to disrupt the interstitial connective

tissue structure (with 'tearing' here protecting the myofiber from actual damage), or may result in 'disruption of the sarcomeric structure' e.g., seen as Z-line streaming that then reduces force production (and hence protects the myofiber from sarcolemmal damage and necrosis) rather than myofiber necrosis/regeneration per se.³¹ Indeed a review of the response of humans to low-intensity exercise suggests that minimal to no muscle damage is occurring with this type of exercise.³² Even eccentric exercise may not result in myonecrosis: "it has been shown that even a single eccentric stretch in rabbits may be sufficient to result in temporary reduced biomechanical capacity and to stimulate the dormant satellite cells to divide. Such an injury is, however, very mild since the offspring of the activated satellite cells did not seem to mature further into myoblasts expressing muscle specific proteins nor fuse with the parent myofiber."³³ Thus more precise language needs to be used to accurately describe the exact nature of any muscle "damage." These other aspects of muscle damage can also be associated with inflammation and sometimes (transitory) activation of satellite cells (also seen in denervated muscles), although the myoblasts will not normally fuse with the undamaged sarcolemma of a mature or atrophic myofiber. Thus inflammation and satellite cell activation (unrelated to new muscle formation) can occur in many other situations apart from myofiber necrosis.

A pronounced feature of myofiber necrosis is sarcolemma disruption that leads to fragmentation of the sarcoplasm that is subsequently invaded by inflammatory cells and becomes replaced by newly formed myotubes/muscle.³³ Once such necrosis has occurred the regenerated myofibers contain myonuclei that are not in the classical sub-sarcolemmal peripheral position, referred to as internal or centrally located myonuclei, and these can persist for many months in rodents. While central myonuclei are widely used to identify regenerated myofibers, caution is required since central myonuclei also result from myofiber denervation, as shown within 2 mo after experimentally denervation in rats.³⁴ The incidence of such central myonuclei appears to be rare in many normal adult muscles even in very old mice. Shefer et al. (2006)²⁹ reported that myofibers with central nuclei were not conspicuous in soleus and EDL muscles of old male C57Bl/6J mice aged up to 33 mo, and it was stated that: 'Regardless of mouse age, the majority of myofibers did not demonstrate centrally localized nuclei or segments of myonuclei chains as typically seen in myofibers from regenerating muscle';²⁹ a very low number of central myonuclei was also reported at all ages in EDL muscles up to 28 mo of age in female C57Bl/6J mice.² In quadriceps muscles, central myonuclei were also rare in female C57Bl mice before 29 mo of age³⁵ indicating little or no regeneration in aging laboratory mice; and seemed most likely due to denervation. However, another study of quadriceps in 24 mo old C57Bl/6J mice reported that many 'atrophic' myofibers with central myonuclei were evident³⁶; while the gender was not stated, many pathological features are more pronounced in old male than female rodent muscles (unpublished data; Soffe, Shavlakadze, Grounds). In contrast with the studies in mice, in aging male rats some central myonuclei (and split myofibers) were present in soleus by 21–25 mo and these features were further pronounced in very old animals (27 mo)

where they also affected EDL, indicating a gradual involvement of different types of muscles with advancing age; however, it was concluded that this probably resulted from denervation rather than regeneration.¹⁰

In addition to central myonuclei, the architecture of regenerated myofibers is commonly altered with a range of malformations from simple splitting or forking to more complex branching of the myofibers (reviewed in^{37,38}). The precise mode of formation of split myofibers has been controversial, since they occur in a wide range of conditions: they arise after necrosis of mature myofibers during myogenesis (within or outside the damaged myofiber) and some may result from incomplete lateral fusion during myogenesis, but they can also be formed by cleavage of mature 'undamaged' myofibers (discussed³⁹). These branched myofibers, along with central myonuclei, were evident in normal adult mouse EDL muscles after a single bout of necrosis/regeneration induced experimentally by transplantation of whole muscles⁴⁰ or by notexin injury of EDL muscles in male C57Bl/6J mice.⁴¹ They are notably very pronounced in many dystrophic muscles of mdx mice that are endogenously subjected to multiple cycles of necrosis/regeneration.³⁸ However, neither of these features, central myonuclei nor branched myofibers, were conspicuous in normal EDL muscles from male C57Bl/10Scsn mice aged up to 28 mo;³⁸ again supporting the conclusion that endogenous regeneration either does not occur or is rare in normal (uninjured) aging muscles of laboratory mice. This was also supported by a recent report of an increase in split myofibers (with one branch) in old 20–21 mo C57Bl mice (gender unspecified) that was more pronounced (~12%) in EDL compared with gastrocnemius (~6%) muscles: this study emphasized that these did not contain central myonuclei and concluded that they resulted from splitting of undamaged myofibers.⁴² Caged mice are relatively sedentary and their normal activity seems insufficient to cause significant myofiber damage. However, it appears that the initial response to unaccustomed mild voluntary wheel exercise can be necrosis of some muscles with subsequent regeneration: it is important to note that this is not repeated with subsequent bouts of the same exercise over many months, presumably due to some adaptation after the first acute bout of myonecrosis.³⁹ Striking differences were observed between mouse strains (6 were examined) and especially between muscles, with the soleus being the muscle most susceptible to such initial exercise-induced myonecrosis.³⁹ Overall, the extent to which endogenous necrosis and regeneration may occur in the many different muscles in the body during normal daily living and the precise situation for various species, especially normal aging human muscles, remains unclear.

Studies of old normal human muscles provide relatively little evidence for myofiber regeneration although many changes in myofibers are noted often associated with denervation.⁴³ In contrast with experimental animals where all muscles are readily sampled, it is important to note that many human analyses are limited to only a very small biopsy sample of the vastus lateralis muscle (long segments of individual myofibers can also be isolated from this biopsy). Detailed studies of aging human myofibers by Cristae et al. (2010)⁴⁴ observed many morphological changes but relatively few central myonuclei in a comparison of

myofibers isolated from biopsies of vastus lateralis muscles from young (aged about 21–32 y) and old (aged 65–96 y) men and women and stated: “In muscle fibres expressing the type I MyHC isoform, internal nuclei were observed in a small number of muscle fibres from old men (1 of 30) and women (2 of 51) and none were observed in the young men and women. No internal nuclei were observed in the 85 fibres expressing the IIa MyHC isoform, irrespective of age and gender. Internal nuclei were observed in three out of four type IIx fibres in old individuals^{7,44} Studies by Andersen (2013)⁴⁵ of old myofibres from 12 frail elderly men and women aged 85–97 years described altered size and morphology of myonuclei and myofibres with complex changes in myosin isoforms,⁴⁵ and Frontera et al. (2012)⁴⁶ from a study of isolated myofibres also concluded that age-related changes within myofibres *per se*, e.g. related to myosin isoforms and myofibrillar protein structure and function, contribute to the loss of myofibre quality (reviewed in⁴⁶). A wealth of other human studies exist and a wider review is required to ascertain the likely incidence of necrosis/regeneration in normal adult and ageing human muscles.

Overall, these available data from rodents and humans emphasize the need for a more rigorous justification of the fundamental assumption of a “constant” need for myonuclear replacement (due to regeneration) and replenishment of satellite cells in normal aging skeletal muscles.

Major Challenges of Myogenic Stem Cell Therapy and Unrealistic Claims

Finally, myogenic stem cell therapies have classically been intensively investigated using cell transplantation in the well-justified situation of correction of the gene defect in the lethal muscle diseases such as Duchenne Muscular Dystrophy. Since the 1990s, dozens of studies have been conducted in animal models using a diversity of creative approaches with some resultant clinical trials, yet the results for clinical translation have been dismal with major problems related to supply of suitable donor myogenic stem cells (ideally need autologous donor myogenic cells that can be rapidly expanded *ex vivo*), massive rapid death of transplanted donor cells, and minimal cell dispersal that is required to supply all muscles throughout the body (reviewed in^{47–50}). Such cell therapy is highly challenging, expensive, invasive, and not to be treated lightly. It also requires that muscles are damaged and regenerating (as occurs in muscular dystrophy) in order for the donor stem/myogenic cells to participate in new

muscle formation: yet such damage is not a feature of healthy normal old muscle. The proponents of such myogenic stem cell transplantation therapy for sarcopenia should take into account the clinical reality of such a proposed invasive intervention for muscles of elderly people.

Conclusion

One year after publication of the comprehensive *in vivo* paper by Lee et al. (2013)² it is disappointing that proponents of stem cell therapy for sarcopenia continue to avoid discussing data that conflict with their conclusions. As it stands, myogenic stem cells therapy has not yet worked realistically for clinical treatment of severe muscle diseases and thus multiple injections of donor cells into elderly humans without any prospect of positive outcome is difficult to justify at this time. More fundamentally, the lack of evidence to substantiate the proposal that satellite cells and regeneration are required routinely for homeostasis of normal adult and aging muscles, challenges the notion of “failed regeneration” as a central cause for sarcopenia. Even where myofiber necrosis does occur (induced experimentally in most studies), there is strong evidence that myogenic cells of very old muscles of mice and humans have excellent myogenic capacity and can form good new muscle *in vivo*. As an alternative explanation for sarcopenia, there is increasing evidence for age-related alterations and deterioration of the myofibers and the integrated architectural components of the connective tissue and nerve supply of skeletal muscles. These complex changes are strongly supported by detailed time-course transcriptome and proteomic analyses of aging rat and mouse muscles that demonstrate progressive alterations, especially related to denervation, the extracellular matrix and metabolism^{35,51}; these all have adverse consequences for maintenance of old muscle function and mass. Thus molecular changes within myofibers and their environment seem more promising targets for therapies to reduce sarcopenia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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