Changes in Myonuclear Number During Postnatal Growth – Implications for AAV Gene Therapy for Muscular Dystrophy

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Abstract. Adult skeletal muscle is a relatively stable tissue, as the multinucleated muscle fibres contain post-mitotic myonuclei. During early postnatal life, muscle growth occurs by the addition of skeletal muscle stem cells (satellite cells) or their progeny to growing muscle fibres. In Duchenne muscular dystrophy, which we shall use as an example of muscular dystrophies, the muscle fibres lack dystrophin and undergo necrosis. Satellite-cell mediated regeneration occurs, to repair and replace the necrotic muscle fibres, but as the regenerated muscle fibres still lack dystrophin, they undergo further cycles of degeneration and regeneration.

AAV gene therapy is a promising approach for treating Duchenne muscular dystrophy. But for a single dose of, for example, AAV coding for microdystrophin, to be effective, the treated myonuclei must persist, produce sufficient dystrophin and a sufficient number of nuclei must be targeted. This latter point is crucial as AAV vector remains episomal and does not replicate in dividing cells. Here, we describe and compare the growth of skeletal muscle in rodents and in humans and discuss the evidence that myofibre necrosis and regeneration leads to the loss of viral genomes within skeletal muscle. In addition, muscle growth is expected to lead to the dilution of the transduced nuclei especially in case of very early intervention, but it is not clear if growth could result in insufficient dystrophin to prevent muscle fibre breakdown. This should be the focus of future studies.

Keywords: Skeletal muscle growth, satellite cells, myonuclei, adeno-associated virus, Duchenne muscular dystrophy, gene therapy

SATELLITE CELLS AND SKELETAL MUSCLE TURNOVER

Skeletal muscle forms in development by the fusion of muscle precursor cells [1], giving rise to primary and secondary myofibres (reviewed [2]). Nuclei are initially added to the centre of the developing myofibres, but predominantly to the polar tendon insertion areas of the myofibres during later fetal life [3]. After birth, satellite cells (skeletal muscle stem cells) located under the basal lamina of myofibres, contribute to skeletal muscle growth, by adding myonuclei to the expanding myofibre [4]. Postnatal muscle growth in the mouse occurs by an increase in size (hypertrophy) rather than by hyperplasia (an increase in myofibre number) [5].

During adulthood, skeletal muscle has relatively low turnover [6], unless it is injured. An interesting study, using C\textsuperscript{14} levels to retrospectively birth date cells, estimated that nuclei from intercostal muscles of two human individuals had an average age of 15.1...
years, indicating that skeletal muscle in humans has a very slow turnover indeed [7].

Satellite cells respond to injury by becoming activated, proliferating and then differentiating to either repair damaged segments of myofibres, or fusing together to make new myofibres (reviewed [8]). But despite having this reservoir of muscle stem cells, skeletal muscle mass is lost with age [9] and in some neuromuscular conditions (reviewed [2]). More is known about skeletal muscle myonuclear number in the mouse than in other species, as it is more available for experimentation. There is also information on satellite cell number over the lifespan in the mouse, but fewer data are available for the human.

MECHANISM OF MUSCLE GROWTH

Studies in the rat [10, 11] and mouse have shown that muscle growth occurs by the addition of satellite cells to existing myofibres [12] and an increase in the myonuclear domain, leading to an increase in myofibre cross-sectional area and length and the addition of sarcomeres [13, 14]; reviewed [15]. In the mouse, the greatest increase in myofibre length occurs in the first 3 weeks of life. Studies by different labs, using different hindlimb muscles (extensor digitorum longus, tibialis anterior, gastrocnemius) from different strains of mouse [5, 13, 16], arrived at similar findings, indicating a 4–6x increase in myofibre length between birth and 21 days of age, with length increasing to a far lesser extent up to 6–12 months of age [16]. Although most growth in the mouse occurs over the first weeks of life, some growth continues after puberty [17].

SATELLITE CELL AND MYONUCLEAR NUMBERS IN GROWING MICE

It is very difficult to compare studies on satellite cell numbers, as different muscles, markers of satellite cells and denominators have been used (reviewed [2]). Nevertheless, early work using electron microscopy estimated that at birth, 32% of nuclei in mouse gastrocnemius muscles were in satellite cells, whereas in adults, only 6% of nuclei were in satellite cells [16]. These figures correlated with other work on mice [4, 18]. The length of the gastrocnemius myofibres and the total nuclei/myofibre (myonuclei and satellite cell nuclei) increased between birth and 60 days of age, but at a slower rate in the older mice [16]. More recent work in the mouse showed that satellite-cell derived myonuclei increase in number until 3–4 weeks of age, with a doubling of myonuclear number in extensor digitorum longus myofibres between 2 and 3 weeks of age [5, 19]. Interestingly, there is evidence that satellite cells continue to contribute to non-injured myofibres of adult mice. As this experiment was performed by labelling satellite cells with GFP at 6 months of age and then quantifying GFP + myofibres at 12 and 20 months of age, it is not possible to quantify the number of satellite cells that were added to each myofibre [20].

DIFFERENCES BETWEEN MDX AND C57BL/10 MUSCLE GROWTH

We will focus on Duchenne muscular dystrophy (DMD), as far more is known about muscle growth and regeneration in the mdx mouse model of DMD than in mouse models of other neuromuscular diseases. A comparison of mdx and control C57Bl/10 mice [19] revealed that the increase in myonuclear number/myofibre finished at 3 weeks of age in both strains, but the myonuclear domain continued to expand thereafter. Before the onset of florid pathology, mdx muscle growth lagged behind control muscle growth, due to a combination of fewer myonuclei and a smaller myonuclear domain. The myonuclear domain (μm³ x 10³) was significantly greater in wild type than in mdx at 2 weeks (approximately 12 vs 10) and 28 weeks (approximately 27 vs 14) of age, but similar [18–20] at 6 weeks of age. Extensor digitorum longus myofibre length during the first year of life (from 2–52 weeks of age) was similar in mdx and C57B/10, but volume and diameter were greater in mdx than in C57Bl/10 after 12 weeks of age. After 6 weeks of age, mdx myofibres (many of which would have undergone degeneration and regeneration) had more myonuclei than controls [21].

SATELLITE CELL AND MYONUCLEAR NUMBERS IN GROWING HUMANS

In children (sex not specified) there was a very small increase satellite cell number/fibre (determined from staining transverse sections) from 0–18 years of age [22]. Another study showed that satellite cell density (satellite cells/mm²) declined steeply in the first months of life [23]. Myonuclear content and fibre cross-sectional area increased significantly (30–40x) from birth to 15–18 years of age in humans [22, 23]. Data on the increase in myofibre length and
volume during growth in humans are sparse, as, unlike in the mouse [5, 13, 16], it is very difficult to isolate entire myofibres in order to easily obtain these measurements. Quantification of myonuclei is also challenging, due to difficulties in identifying the nuclei present within myofibres in skeletal muscle [24]. It is also very difficult to compare numbers of myonuclei found in different studies, as they have been quantified in different ways: length of isolated myofibre, [25] fibre in transverse sections [26], sarcomere [27]. It would be useful to agree on ways to quantify both satellite cells and myonuclei in order to be able to meaningfully compare different studies. Nonetheless, myonuclear number has been quantified in some human studies, but only in adults. Using single fibre segments from the vastus lateralis muscle from young healthy volunteers (both male and female), the number of myonuclei/sarcomere was approximately 0.3, and was not affected by training, detraining, or retraining [27]. In transverse sections of vastus lateralis muscles of adult males, the number of myonuclei/fibre in subjects undergoing training given creatine, protein, carbohydrate or no supplementation was quantified. In this study, myonuclear number was approximately 2/myofibre [26].

**HUMAN COMPARED TO RODENT SKELETAL MUSCLE GROWTH**

Relating the age of a mouse to a human is quite difficult, but it has been suggested that a 9 day old mouse is equivalent to a 1 year old child [28]. In the human, skeletal muscle mass increases throughout adolescence [29] to a far greater extent than in rats [30]. Hansson et al. [25] showed that the number of myonuclei in myofibres scaled sublinearly to cell volume, but close to linear with cell surface and this was similar in growing mouse muscles and in adult mouse and human muscles. This is despite differences in the size of human and mouse myofibres. Interestingly, and illustrating the challenge for gene therapy for skeletal muscle, it has been suggested that a single myofibre from the adult human sartorius muscle may contain more than 40,000 nuclei [25].

**RELEVANCE OF MYONUCLEAR TURNOVER TO GENE THERAPY FOR MUSCULAR DYSTROPHY**

It is becoming increasingly important to know how long-lasting myonuclei are, in the context of gene therapy for neuromuscular conditions. AAV-mediated gene therapy is a promising approach for several neuromuscular diseases, including DMD [31–36]). AAV cloning capacity is an issue for DMD, as the DMD gene is very large, but this has been overcome by using micro/mini-dystrophin constructs, leading to a smaller, but largely functional, dystrophin protein in muscle and heart fibres (reviewed [32]). The use of a tri-AAV vector, in which the entire full-length human dystrophin expression cassette was split into three fragments and each piece was packaged into an AAV vector, is another promising approach. When the three AAV vectors were co-injected into dystrophin-deficient mouse muscle, the complete expression cassette was reconstituted and a few human dystrophin+ fibres were seen [37]. However, for this strategy to be successful, the three vectors need to be transfected in the same nucleus; moreover, the toxicity related to the need to administer very high doses of each of the three AAVs raises concerns regarding the feasibility of this approach in the human.

But a major limitation to AAV-mediated gene therapy is that AAV induces an immune response, which makes re-administration a challenge [38, 39]. AAV enters nuclei where it usually remains episomal (reviewed [40]); integration into the host genome is minimal. There is very limited transduction of satellite cells after intra-muscular injection of AAV into mouse muscle [41, 42] and non-integrated AAV would be lost as the satellite cells divide (e.g. during muscle growth or regeneration). The extent of integration of AAV into satellite cells is not currently known. The use of promoters that drive dystrophin expression only within differentiated myofibres would help in satellite cell-mediated gene therapy approaches [43].

An alternative vector is lentivirus (LV), which becomes incorporated into the host DNA and is consequently long-lasting and persists in daughter cells. Lentivirus coding for mini-dystrophin injected intra-muscularly in the mdx mouse model of DMD gave long-lasting dystrophin expression and some satellite cell transduction [44, 45]. A second advantage of a lentivirus is that its cloning capacity is larger than that of AAV and it can accommodate the entire dystrophin cDNA [46]. But, in contrast to AAV, it is challenging to produce sufficient lentivirus titre to deliver to muscles bodywide and, unlike AAV [47], lentivirus has limited tropism to skeletal muscle [48]. And delivery to satellite cells, which are present at very small numbers compared to myonuclei [16], would also be a
challenge. But if lentiviral transduction of significant numbers of satellite cells were to be achieved, they would serve to deliver myonuclei, that can produce dystrophin, to growing or regenerating myofibres over a lifetime. Nevertheless, myonuclei may still be lost, for example as a consequence of minor injury or ageing, and unless sufficient lentivirally-transduced myonuclei are present, and/or genetically-modified satellite cells can compensate adequately for the lost myonuclei, this would lead to a reduction of dystrophin within the myofibre.

Crispr/Cas9 gene editing is another approach that has been applied to DMD animal models [49–54], but again, unless sufficient satellite cells are targeted (and this is very difficult to quantify, as it is technically impossible to extract all satellite cells from a muscle for analysis), loss of genetically-modified myonuclei will remain an issue. In order to investigate all, rather than just a sample, of the satellite cells within a muscle, a novel AAV9-mediated gene editing approach was used. An entire muscle (with all its satellite cells) from a donor Ai14 mouse treated systemically with AAV9-cre was grafted into an immunodeficient host mouse. A grafted muscle degenerates almost entirely and regenerates from its resident satellite cells. Within the regenerated, grafted muscle, there were both satellite cells and muscle fibres expressing the marker gene, evidence that at least some satellite cells within the donor muscle had been targeted and that they were functional, as they contributed to muscle regeneration within the host mouse [55].

Although quite modest levels of dystrophin seem to be required for functional benefit [56–62], loss of dystrophin to below these levels, due to loss of transduced or genetically-corrected myonuclei over time, would reduce the efficacy of the treatment. Also, to be fully-protective, dystrophin has to be spread all along the myofibre [63]. But myonuclei have domains [64–67] and dystrophin spreads only a few hundred microns from the nucleus that made it [68]. This means that myonuclei producing dystrophin must be quite closely spaced along the entire myofibre.

Although treating a patient early, before the onset of symptoms, would be preferable, in order to prevent as much muscle loss as possible, treatment of a very young, rapidly-growing child would lead to dilution of the AAV, possibly leading to insufficient dystrophin along the myofibre to prevent myofibre necrosis. This is assuming that the increase in myofibre volume from birth to adolescence that occurs in the mouse [5] is similar in the human. So is there an optimal time to deliver AAV-mediated gene therapy, after the child’s growth spurt is finished? For a LV-mediated gene therapy, as transduced satellite cells are incorporated rapidly into growing myofibres (in the mouse, at least), it would be essential that the “stem” satellite cells, that do not contribute to muscle growth, but do contribute to muscle regeneration [69] are also effectively transduced, for long-term patient benefit.

**WHAT IS THE EVIDENCE FOR THE LOSS OF AAV FROM MYOFIBRES?**

When either AAV U7ex23, designed to skip the mutated dystrophin exon and restore dystrophin protein production, or scrambled AAV, were injected intra-muscularly into the mdx mouse model of DMD and into non dystrophic controls, the viral genome number/nucleus was similar in mdx and control mice that had been given high doses of AAV U7ex23, that restored dystrophin expression in the mdx muscles. But the viral genome number/nucleus in mdx muscles that had been given the scrambled AAV three weeks previously was significantly less than in wild type muscles that had been injected with scrambled AAV. And when lower doses of AAV U7ex23 were given, leading to less dystrophin expression in treated mdx muscles, the viral genome number/nucleus was significantly less in mdx than control mice, 3 weeks later. The age of the mice was not stated, so whether the muscles were still growing or not at the time of AAV delivery is not known. Also, the use of a scrambled control is controversial, as it may have unexpected (possibly deleterious) consequences. Nevertheless, it seems that the loss of myofibres, as a consequence of them expressing insufficient dystrophin, led to the lower viral genome number/nucleus [70]. This was confirmed in wild type mice injected intramuscularly with AAV U7ex23 and then injected with cardiotoxin, to induce myonecrosis and regeneration - the viral genome number/nucleus was significantly less in muscles that had been treated with cardiotoxin than in non-cardiotoxin-treated muscles [70].

When the same AAV U7ex23 was delivered intravascularly to 3 week old mdx/utrophin knock-out mice (which would be expected to have finished satellite cell-mediated myofibre growth, but whose muscles (unlike mdx) would already be undergoing necrosis and regeneration [71], the viral genome...
number/nucleus was reduced in tibialis anterior and diaphragm between 3 months and one year after treatment, but to a lesser extent in the heart. This is likely to be because, although mdx heart does undergo necrosis [72], unlike skeletal muscle, it is capable of little, if any, stem cell-mediated regeneration. Any loss of AAV during skeletal muscle growth was however not investigated and possible differences in the biodistribution of the AAV in the two different mouse models were not taken into consideration.

In an alpha sarcogycan deficient mouse model of limb girdle muscular dystrophy type 2D, injected intramuscularly with either therapeutic or control AAV at one day of age, there was no loss of viral genomes in muscles treated with therapeutic AAV, between 4 and 12 months after treatment (though it would have been interesting to have examined earlier timepoints, to investigate the effect of muscle growth), but there was a significant loss in muscles treated with a control AAV. This adds to the evidence that it is myofibre loss that is the main cause of the reduction in viral genome and if the myofibre death is prevented (by an effective therapeutic construct, ideally delivered before the onset of muscle pathology), viral genomes are retained to a greater extent [73, 74].

Studies in canine models of DMD have provided evidence of persistence of AAV-delivered protein within skeletal muscle, although most of these studies have only looked fairly short-term, following intra-muscular delivery (up to 30 weeks, using immunosuppression [75], up to 8 weeks, with [76] or without immunosuppression [77]. There was evidence of restored dystrophin for up to 4 months after intravenous delivery of AAV-9 coding for microdystrophin into 2 month old immunosuppressed DMD dogs [78]. And promisingly, locoregional or systemic transfusion of AAV expressing canine microdystrophin into the GRMD dog model of DMD (at 3–4 months of age) gave functional dystrophin restoration and, following its intravenous delivery (with no immunosuppression) dystrophin expression persisted for up to 14 months and clinical improvement remained evident at 2 years of age [79].

It is possible that dilution of the viral genome/myofiber nuclei during muscle growth may not be as detrimental as its loss during myofibre degeneration, but this remains to be investigated. The co-occurrence of myofibre necrosis and regeneration and muscle growth [80] may compound any reduction in the number of viral genomes/nuclei. As the onset of muscle pathology in the dystrophin-deficient utrophin deficient mouse is earlier than in the mdx mouse, at 6 days of age [71], when growth is still very active, this model may be better than the mdx mouse to investigate the combination of growth and myofibre necrosis/regeneration on myonuclear loss.

Nevertheless, evidence for retention of AAV within non-dystrophic human skeletal muscle for up to 10 years [81] gives reason for optimism for treatment of neuromuscular diseases.

CONCLUSIONS

The ideal gene therapy for muscular dystrophies would be a one-shot approach, delivered as early as possible to prevent or reduce the pathological consequences of the genetic defect. The majority of myonuclei and satellite cells should be treated with an integrating virus, such as lentivirus, expressing full-length dystrophin. But currently there are challenges in producing lentivirus at high enough titre to all the muscles of a patient and the main hurdle to delivering lentivirus systemically is that this virus is prone to inactivation by human serum, contributing to inefficient muscle targeting [82]. AAV-mini or micro dystrophin have given encouraging results in pre-clinical models of DMD, but it is yet to be seen if this promise will be upheld in clinical trials. A greater knowledge of the dynamics of the loss of myonuclei and their replacement from satellite cells in children with DMD would help to pinpoint the optimal time for AAV delivery.

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