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SHORT COMMUNICATION

AAV-based shRNA silencing of NF-κB ameliorates muscle pathologies in *mdx* mice

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Chronic inflammation, promoted by an upregulated NF-kappa B (NF-κB) pathway, has a key role in Duchenne muscular dystrophy (DMD) patients' pathogenesis. Blocking the NF-κB pathway has been shown to be a viable approach to diminish chronic inflammation and necrosis in the dystrophin-defective *mdx* mouse, a murine DMD model. In this study, we used the recombinant adeno-associated virus serotype 9 (AAV9) carrying an short hairpin RNA (shRNA) specifically targeting the messenger RNA of NF-κB/p65 (p65-shRNA), the major subunit of NF-κB associated with chronic inflammation in *mdx* mice. We examined whether i.m. AAV9-mediated delivery of p65-shRNA could decrease NF-κB activation, allowing for amelioration of muscle pathologies in 1- and 4-month-old *mdx* mice. At 1 month after treatment, NF-κB/p65 levels were significantly decreased by AAV gene transfer of p65-shRNA in the two ages of treatment groups, with necrosis significantly decreased compared with controls. Quantitative analysis revealed that central nucleation (CN) of the myofibers of p65-shRNA-treated 1-month-old *mdx* muscles was reduced from 67 to 34%, but the level of CN was not significantly decreased in treated 4-month-old *mdx* mice. Moreover, delivery of the p65-shRNA enhanced the capacity of myofiber regeneration in old *mdx* mice treated at 4 months of age when the dystrophic myofibers were most exhausted; however, such p65 silencing diminished the myofiber regeneration in young *mdx* mice treated at 1 month of age. Taken together, these findings demonstrate that the AAV-mediated delivery of p65-shRNA has the capacity to ameliorate muscle pathologies in *mdx* mice by selectively reducing NF-κB/p65 activity.

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Keywords: AAV vector; ShRNA; NF-kappa B; DMD

INTRODUCTION

Duchenne muscular dystrophy (DMD), caused by recessive mutations in the dystrophin gene, remains an untreatable genetic degenerative muscle disease.[1,2] In DMD patients, loss of sarcolemmal dystrophin and the dystrophin-associated glycoprotein complex promotes muscle fiber damage during muscle contraction,[3–7] resulting in the recruitment of T cells, macrophages and mast cells to the damaged muscle, leading to progressive myofiber necrosis.[2] Muscle regeneration is the initial response to damage, but muscle progenitor cells become exhausted by continuous cycles of necrosis and regeneration over time. This gives way to accumulated fibrosis and fatty deposits that exacerbate the characteristic wasting process of DMD.[8]

Chronic inflammation has a key role in the progressive muscle wasting and degeneration observed in DMD patients.[9] Previous studies demonstrate that in muscular dystrophies, chronic inflammation is promoted by an upregulated NF-κB signaling pathway.[10,11] Elevated levels of NF-κB activation have been detected in more than one type of muscular dystrophy, including DMD[12] and limb girdle muscular dystrophy.[13] Chronic activation of the classic NF-κB signaling pathway is required for DMD pathology by acting on both immune cells and damaged skeletal muscles to promote inflammation and to inhibit myogenic differentiation of muscle precursors in *mdx* mice.[12] Of note, the NF-κB subunit p65 is the key subunit associated with the chronic inflammation of DMD. The p65 subunit has been implicated in the regulation of DMD muscle pathology using transgenic mouse model carrying only one allele of p65 in the *mdx* background. Such *mdx*-heterozygous p65 crosses result in an improved dystrophic pathology characterized by enhanced muscle regeneration.[9]

AAV vectors have the advantageous biological property of being capable of infecting a wide range of host cell types including muscle, which makes AAV a particularly good vector for muscle-directed gene therapy,[14–16] especially for muscular dystrophies.[17] In AAV vector-based mini-dystrophin gene replacement treatment of dystrophin-deficient (dys−/−) (*mdx*) or dystrophin/utrophin double knock-out (dys−/−:utr−/−) mice[17–19] and canine models,[20] as well as in human clinical trials for DMD,[21] the AAV vector delivery system thus far represents one of the most promising approaches to aid in the treatment of DMD. However, the efficiency of gene therapy in large animal model studies and in human clinical trials for DMD has been limited by the immune response, which is tightly associated with chronic inflammation. It is likely that a combination of gene replacement and anti-inflammatory therapies[22] is required in order to reduce muscle necrosis and degeneration.[23] We recently demonstrated that the AAV-mediated dominant-negative forms of IκB kinase, such as IKKα (IKKα-dn) and IKKβ (IKKβ-dn), diminish necrosis in both young and old *mdx* mice through an i.m. injection.[24] Interestingly, we observed improvement of muscle regeneration only in the older treated *mdx* mice. Although the transgenic mouse model with an ablation of the p65 was revealed to promote muscle regeneration in *mdx* mice by attenuating chronic inflammation, almost no work has been done with small interference RNA technology via viral gene transfer. In this study,

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we developed an AAV vector carrying short hairpin RNA (shRNA) specifically targeting p65 to block the classic NF-κB pathway in *mdx* muscles. Local gene transfer of the p65-shRNA to muscle resulted in the improvement of dystrophic muscle pathologies, especially a decrease in the pathological regeneration observed in young *mdx* mice.

RESULTS

Efficient gene transfer improves morphology in young and old mdx mice

In previous studies utilizing an adeno-associated virus serotype 9 (AAV9) vector, high gene transfer efficiencies have been observed in cardiac and skeletal muscles following systemic delivery.[25] More importantly, the AAV9-mediated delivery of the mini-dystrophin gene in the small and large DMD models did not stimulate a host immune issue.[20,25] We constructed an AAV vector expressing either a p65-shRNA or a control shRNA (ct-shRNA) from an U6 promoter and the ZsGreen reporter from the human cytomegalovirus (CMV) promoter. Initially, to determine the efficacy of AAV9 vector-mediated shRNA gene transfer, the extent of ZsGreen reporter gene expression in treated gastrocneminus (GAS) muscles was examined following injection of 3.5×10^{11} viral particles of AAV9_U6_p65-shRNA_CMV_ZsGreen. As shown in Figure 1, the gastrocneminus (GAS) muscles of *mdx* mice treated at the ages of 1 and 4 months showed efficient expression of AAV9 vectors carrying either the p65-shRNA or the ct-shRNA at 1 month after treatment (in green). Hematoxylin and eosin staining revealed fewer mononuclear cells (infiltrating immune cells) in the GAS muscles of the p65-shRNA-treated skeletal muscles compared with the ct-shRNA-treated or phosphate-buffered saline (PBS)-treated *mdx* mice. Moreover, the morphology of the p65-shRNA-treated *mdx* muscles was significantly improved when compared with the muscles of age-matched wild-type C57/BL mice.

Reduction of NF-κB/p65 levels by the AAV-p65-shRNA

To demonstrate the effectiveness of the p65-shRNA, the macrophage RAW cell line was transfected with an AAV plasmid DNA carrying p65-shRNA and ZsGreen reporter gene. The extent of inhibition of NF-κB was evaluated following lipopolysaccharide stimulation by measuring the level of phosphorylated NF-κB/p65 (P-p65) using an antibody specific to P-p65. Western blot analysis revealed that the p65-shRNA-transduced mouse macrophages had a lower level of P-p65 compared with the ct-shRNA-transduced and PBS-treated macrophages (Figure 2a). We next examined whether the levels of p65 messenger (mRNA) in p65-shRNA-treated *mdx* muscle were decreased, resulting in the reduction of p65 protein levels. Real-time quantitative PCR (qPCR) data analyses, by using one primer-pair that binds the region of p65 gene, demonstrated that the level of p65 mRNA in the p65-shRNA-treated muscles was decreased by 50% compared with the ct-shRNA-treated muscles at 1 month after treatment ($^*P < 0.05$) (Figure 2b). These results verify the decreased levels of p65 mRNA in the *mdx* muscles treated with AAV-p65-shRNA at 1month of age, but not in the ct-shRNA-treated muscles. Immunohistochemical analysis of muscle sections was also performed by immunofluorescence staining of P-p65 at 1 month after treatment. Compared with the ct-shRNA-treated or PBS-treated *mdx* mice, the immunofluorescence staining showed a decreased level of P-p65 in *mdx* GAS muscles treated by AAV vector carrying p65-shRNA (Figure 3a, in red). Next, quantitative analyses were carried out to identify the number of P-p65-positive myofibers and immune cells per visual frame. The statistical analysis of the data, using ten randomly chosen visual frames, showed that the P-p65-positive cells in AAV-p65-shRNA-treated groups were significantly less than the age-matched control groups. As shown in Figure 3, 1-month-old *mdx* mice treated with AAV-p65-shRNA had a significant decrease in P-p65-positive myofibers and immune cells (19.0 ± 2.1) compared with age-matched ct-shRNA-treated (53.2 ± 9.2) ($^*P < 0.05$) and PBS-treated (51.6 ± 6.5) ($^{\&}P < 0.05$) *mdx* mice. Similar results were obtained in 4-month-old treated *mdx* mice; the AAV-mediated p65-shRNA led to a decrease in the level of P-p65 in muscles (28.2 ± 6.9) when compared with ct-shRNA-treated and PBS-treated (66.0 ± 14.8) ($^{\&}P < 0.05$) *mdx* mice (58.6 ± 6.2) ($^*P < 0.05$) of the same age. No significant difference in immunofluorescence staining was found between ct-shRNA- and PBS treated *mdx* mice. In C57/BL6 wild-type mice, only a few P-p65-positive myofibers were revealed when compared with age-matched *mdx* mice treated by p65-shRNA, ct-shRNA or PBS. The findings demonstrate that the AAV vector-mediated p65-shRNA treatment reduces the activity of classic NF-κB pathway in the dystrophic muscles by decreasing p65 mRNA.

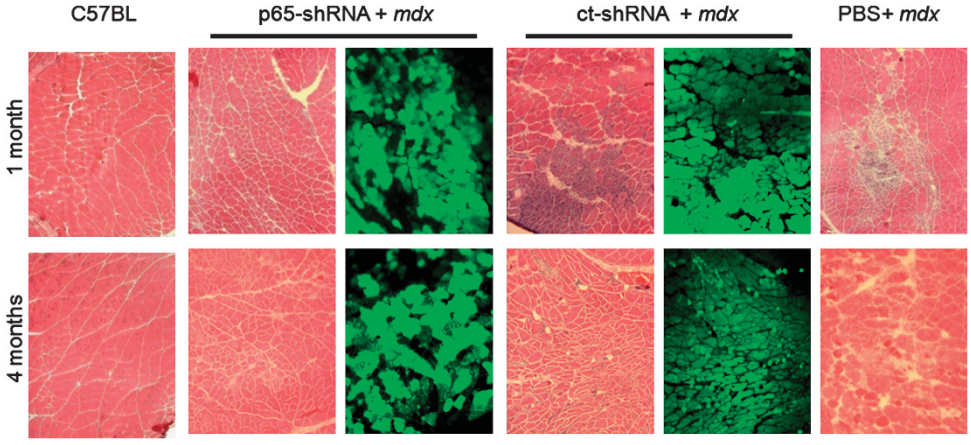
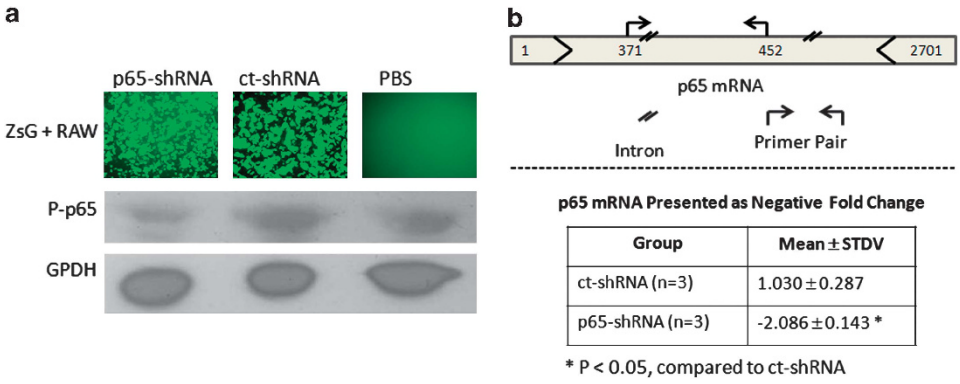


Figure 1. High efficiency gene delivery in *mdx* mice GAS muscles. After 1 month of treatment, GAS muscles from AAV vector-treated and PBS-treated *mdx* mice at 1 month and 4 months of age were harvested and cut into 10 μm thick cryosections for histological analysis. The ZsGreen-positive myofibers showed efficient expression of AAV9 vectors carrying p65-shRNA or ct-shRNA in both treated young and old *mdx* mice. Hematoxylin and eosin staining revealed fewer mononuclear cells (infiltrating immune cells) in the GAS muscles of p65-shRNA-treated muscles compared with ct-shRNA- and PBS-treated *mdx* mice. p65-shRNA-treated muscles also appeared to have been normal when compared with C57BL wild-type muscles in morphological analysis. All pictures are shown at 100 × magnification.



p65 mRNA Presented as Negative Fold Change

Group	Mean ± STDV
ct-shRNA (n=3)	1.030 ± 0.287
p65-shRNA (n=3)	-2.086 ± 0.143 *

* P < 0.05, compared to ct-shRNA

Figure 2. Reduction of p65 by AAV-p65-shRNA in non-muscle and muscle cells. (**a**) AAV plasmid DNA carrying either p65-shRNA or ct-shRNA showed high efficiency in transfected RAW cells, mouse macrophages, as monitored by the expression of the ZsGreen reporter. Western blot verified the reduction of P-p65 in AAV-p65-shRNA-treated mouse macrophages following lipopolysaccharide stimulation compared with AAV-ct-shRNA-treated macrophages. (**b**) qPCR analysis demonstrated reduction of p65 expression in AAV-p65-shRNA-treated young *mdx* muscle tissues at 1 month after treatment, showing 50% decrease compared with the control group (*$P<0.05$). All pictures are shown at 100 × magnification.

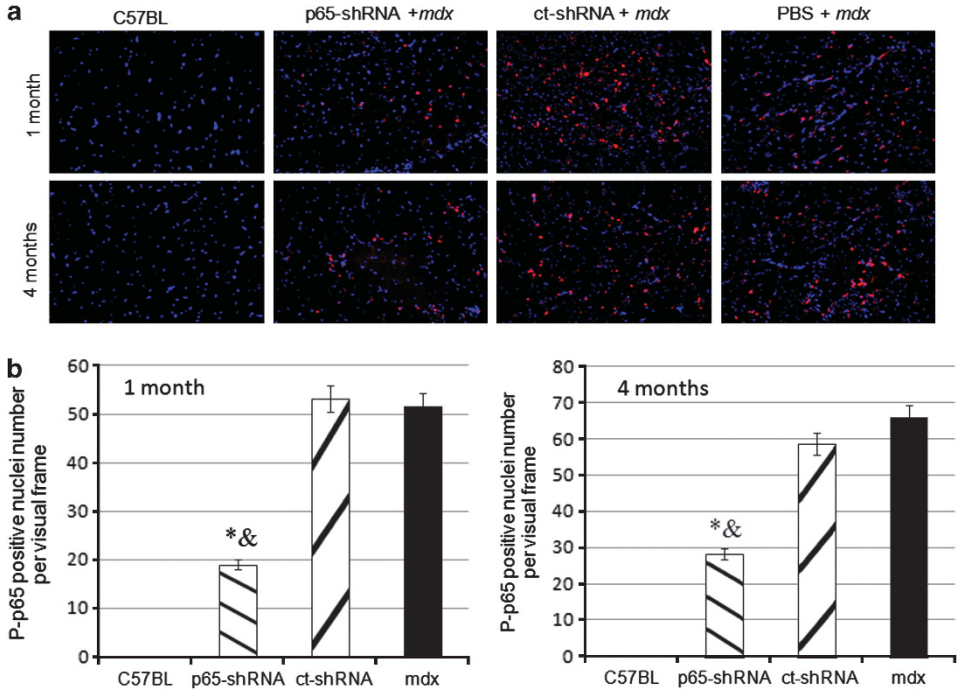


Figure 3. Reduction of p65-positive cells in muscle treated by AAV-p65-shRNA. (**a**) The phosphorylated NF-kB/65 unit immunostaining was performed to assess the NF-kB levels in 1-month-old treated *mdx* mice and 4-month-old treated *mdx* mice, and represented lower numbers of p65-positive cells (in red color) in p65-shRNA-treated muscles compared with ct-shRNA-treated and PBS-treated muscles. The normal muscle (C57BL) did not show significantly positive p65 cells. (**b**) The quantitative analysis of nuclei, positive for phosphorylation, showed significant decreases in p65 shRNA-treated *mdx* mice at the ages of 1 month and 4 months compared with age-matched ct-shRNA-treated or PBS-treated *mdx* mice. (*,&$P<0.05$). All pictures are shown at 200 × magnification (*p65-shRNA vs ct-shRNA; &p65-shRNA vs *mdx*).

Amelioration of necrosis in young and old mdx mice

Necrotic fibers are a feature of the dystrophic muscle pathology in DMD, responsible for progressive wasting of the affected fibers.[26] We investigated whether the resultant decrease in the p65 levels in dystrophic muscle could diminish the muscle necrosis, which is associated with chronic inflammation. At 1 month after treatment, the muscle sections were incubated with fluorescently labelled mouse IgG to determine the level of muscle fiber necrosis. The results in Figure 4 show a significant decrease in myofiber necrosis in *mdx* mice treated by p65-shRNA at the ages of 1 and 4 months when compared with age-matched control muscles from ct-shRNA- and PBS-treated *mdx* mice. No significant necrosis was revealed in C57BL muscles. Muscles from C57BL- and p65-shRNA-treated *mdx* mice had lower levels of inflammatory cell infiltration and necrosis, as determined by hematoxylin and eosin staining, compared with ct-shRNA- and PBS-treated *mdx* muscles.

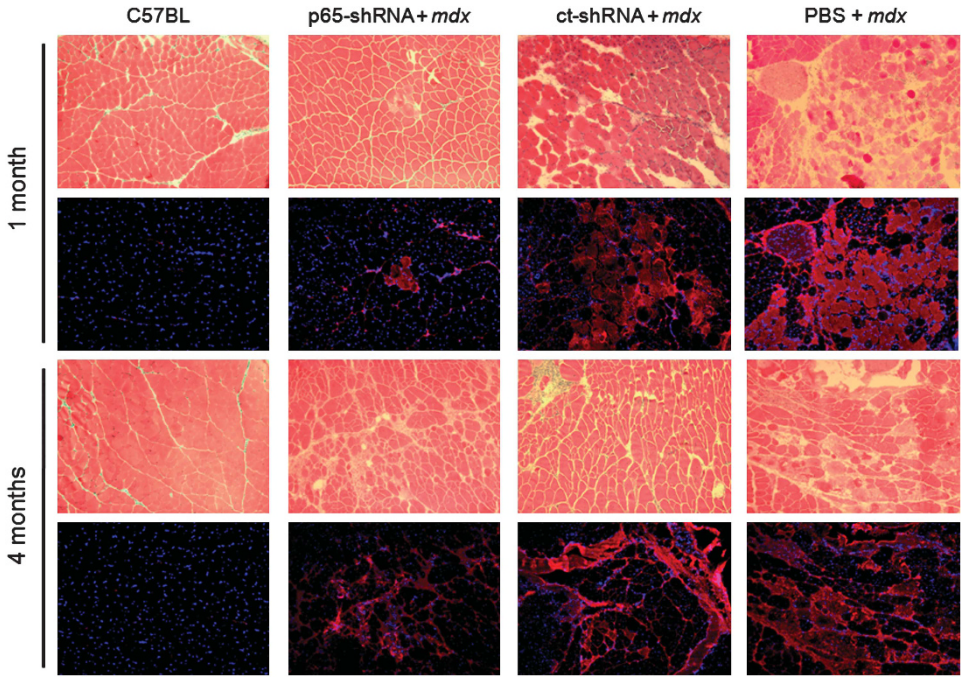


Figure 4. p65-shRNA treatment reduced myofiber necrosis in young and old *mdx* muscles. The muscle cryosections were stained by mouse IgG fluorescently labeled antibody to show necrotic myofibers. There were marked reductions of mouse IgG-positive myofibers (in red color) in *mdx* muscles treated by p65-shRNA vectors at 1 month of age and 4 months of age, not in ct-shRNA-treated or PBS-treated muscles. All pictures are shown at 100 × magnification.

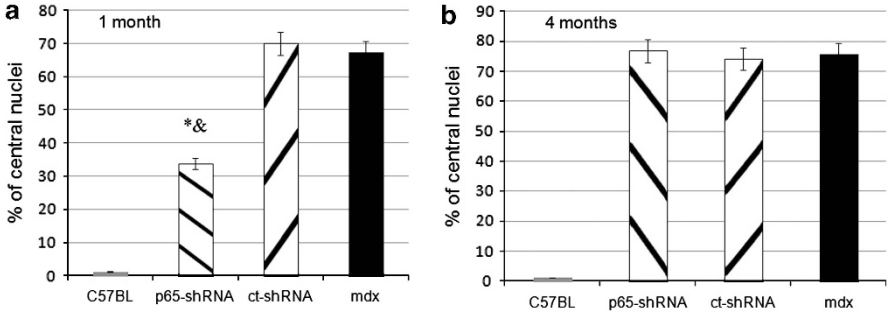


Figure 5. p65-shRNA treatment reduced CN of myofibers in young *mdx* mice, not in old *mdx* mice. (**a**) Decreased significantly in p65-shRNA vector-treated young *mdx* mice compared with the age-matched ct-shRNA vector-treated *mdx* mice ($^{*}P<0.05$) and the PBS-treated *mdx* controls ($^{\&}P<0.05$). (**b**) The rate of CN was not significantly different between the p65-shRNA vector-treated muscles of old *mdx* mice and age-matched ct-shRNA vector-treated or PBS-treated *mdx* mice. All numbers (see Results) were collected from the ZsGreen-postive myofibers (in green) that were infected by AAV vector containing either p65-shRNA or ct-shRNA. In control groups such as PBS-treated *mdx* and C57BL normal muscles, the numbers represented total myofibers by randomly counting the nuclei.

Amelioration of central nucleation (CN) in treated young mdx mice, not in old mdx mice

The evidence shows that *mdx* mice begin a cycle of myofiber degeneration and regeneration usually around 3 weeks of age.[27] This phenomenon can be detected by the observation of CN, a characteristic pathological sign of dystrophic muscles.[17] To investigate whether blocking the classic NF-κB pathway using AAV-based p65 gene silencing can be a potential approach for ameliorating such dystrophic pathology, quantitative analyses of CN from 1600 to 6500 myofibers were performed by hematoxylin and eosin staining of PBS-treated control (C57BL and *mdx*) muscles and ZsGreen-positive myofibers in *mdx* muscles treated by AAV vectors carrying p65-shRNA or ct-shRNA. The percentage of CN was calculated by dividing the number of total central nuclei by all counted numbers of myofibers in each group (four mice per group). As observed in Figure 5, ZsGreen-positive myofibers showed a significant reduction of CN (34%, 632/1872) in the *mdx* muscles treated with AAV-p65-shRNA at 1 month of age compared with the age-matched ct-shRNA-treated *mdx* mice (70%, 1915/2724) ($^{*}P<0.001$) or PBS-treated *mdx* mice (67%, 3583/5310) ($^{\&}P<0.001$). However, there was no significant difference between the p65-shRNA-treated (76%, 5028/6582), ct-shRNA-treated (74%, 1196/1616) and PBS-treated muscle (75%, 2273/3009) of 4-month-old *mdx* mice. These results indicate that the p65-shRNA treatment ameliorates the dystrophic pathology in young, treated *mdx* muscle, leading to the decrease in CN. They also suggest that beginning this treatment early could have more therapeutic effects or benefits than later stage treatment of DMD. In both age groups, the percentage of CN in C57BL/6 mice were <1%, consistent with our previous studies.[17]

Decrease in muscle regeneration in young mdx mice, but increase in the capacity of myofiber regeneration in old mdx mice

As embryonic isoform of MyHC (eMyHC) is present only in newly regenerated myofibers, it is a valuable marker for muscle regeneration. Therefore, we next performed eMyHC immunofluorescence staining to test whether antiinflammation by the p65-shRNA gene silencing has a role in dystrophic myofiber regeneration. As shown in Figure 6, we found that treatment by AAV-p65-shRNA at 4 months of age could increase eMyHC-positive myofibers when compared with the ct-shRNA-treated and PBS-treated mice of the same age. In contrast, a significant decrease in eMyHC-positive myfibers was observed in the dystrophic muscles treated by AAV-p65-shRNA at 1 month of age when compared with the age-matched controls. However, eMyHC-positive myofibers were not observed in the age-matched C57BL muscles. Quantitative analyses by counting 1500–5000 cells per group showed that the percentage of eMyHC-positive myofibers in AAV-p65-shRNA-treated *mdx* mice (16.2%, $n=2589$) at 4 months of age was significantly higher than the age-matched ct-shRNA-treated *mdx* mice (7.9%, $n=2248$) ($^{*}P<0.001$) or the PBS-treated control (9.1%, $n=2768$) ($^{\&}P<0.001$). In contrast, the percentage of eMyHC-positive myofibers was decreased in the p65-shRNA vector-treated *mdx* GAS muscles at 1 month of age (11%, $n=2891$), compared with the ct-shRNA vector-treated *mdx* mice (43%, $n=5241$) ($^{*}P<0.001$) or PBS control (46%, $n=1503$) ($^{\&}P<0.001$). These results suggest that the p65-shRNA treatment improves muscle regeneration in old *mdx* mice and improves the inflamed environment in dystrophic muscle at a young age, decreasing muscle regeneration.

Improvement of myofiber membrane integrity in mdx mice

Myofiber membrane integrity damage takes place during dystrophic muscle degeneration and regeneration. Evans blue dye (EBD) leaks into myofibers with disrupted membranes and can be detected visually using red fluorescent light under a fluorescent microscope.[17] However, it cannot leak into healthy myofibers, which have an intact membrane. To assess whether the AAV-p65-shRNA treatment protected the integrity of myofiber membranes, an integrity test was performed by tail vein injection of the EBD. As shown in Figure 7, less EBD permeated into the myofibers of both the young and old AAV-p65-shRNA treatment groups compared with the *mdx* control treated with PBS buffer at 1 month of age. In contrast, the ZsGreen-negative myofibers revealed a large amount of EBD uptake, indicating broken membranes. Although some myofibers that had low levels of ZsGreen expression still had EBD leakage in the cytoplasm, the results demonstrate that myofiber membrane integrity was functionally improved by the p65-shRNA silencing treatment in both young and old *mdx* mice.

DISCUSSION

The progressive muscle wasting and degeneration of DMD patients are associated with chronic inflammation, which occurs because of an upregulation of classical NF-κB signaling in immune cells and damaged muscle fibers. Dystrophic muscle has a higher immune alertness, owing to chronic inflammation and to the presence of a higher concentration of immune effector cells, presenting a high risk for AAV-based gene therapy, unless immunosuppression is used.[28] More recently, Mendell *et al*.[29] reported that dystrophin-specific T-cells were observed in AAV-mini-dystrophin-treated DMD patients before vector administration, associated with the severity of muscle inflammation. This suggests that using dystrophin replacement alone to treat the primary defect may not be successful in rescuing progressive muscle degeneration and wasting. As long as chronic inflammation

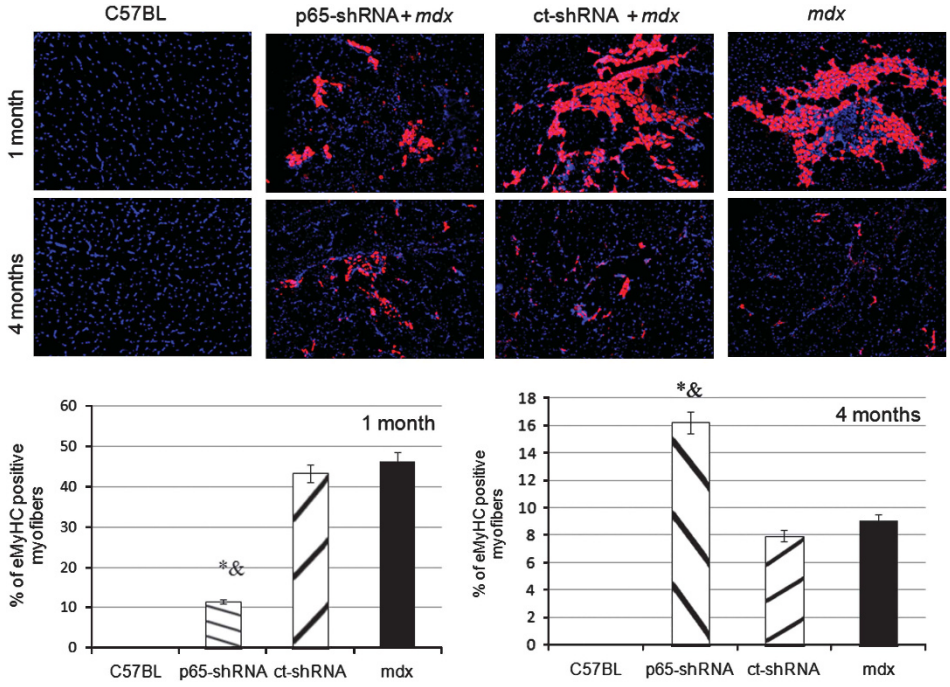


Figure 6. AAV-p65-shRNA treatment increased the myofiber regeneration in treated old *mdx* muscles, but not in treated young *mdx* muscles. eMyHC staining shows the new regenerative myofibers in the muscles. Interestingly, the percentage of eMyHC-positive myofibers (in red color) in p65-shRNA vector-treated *mdx* mice at 4 months of age was significantly higher than the age-matched ct-shRNA vector-treated ($^{*}P<0.001$) or PBS-treated *mdx* mice ($^{\&}P<0.001$). However, there was a significant decrease in the percentage of eMyHC-positive myofibers in the p65-shRNA vector-treated *mdx* GAS muscles at 1 month of age compared with the ct-shRNA vector-treated ($^{*}P<0.001$) or PBS-treated *mdx* mice ($^{\&}P<0.001$). All pictures are shown at 100× magnification.

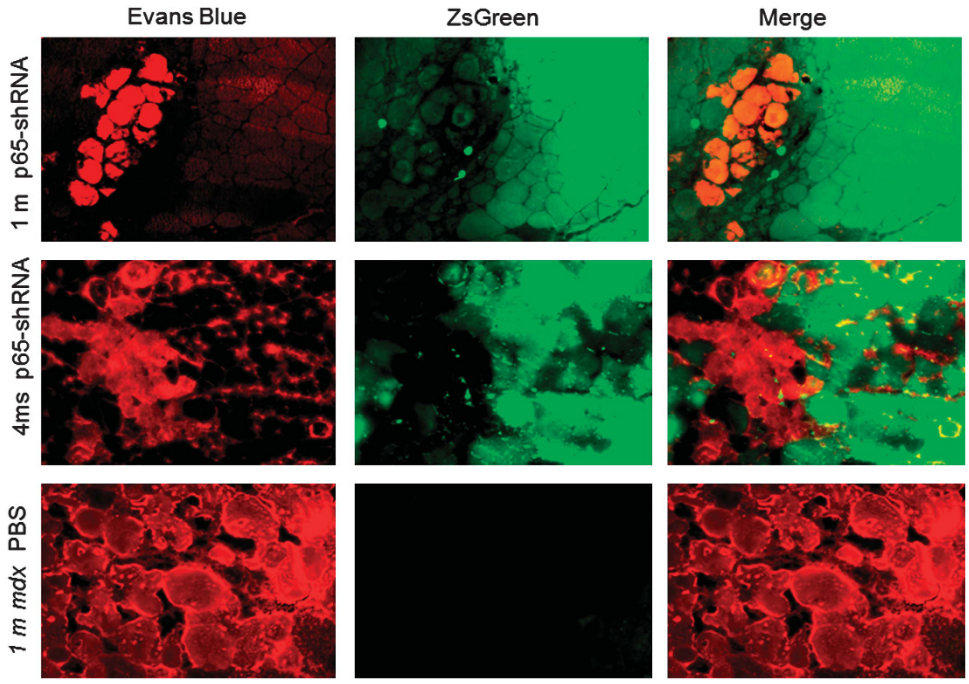


Figure 7. AAV-p65-shRNA improved membrane integrity of myofibers in both treated young and old *mdx* mice. EBD leakage into the myofibers indicates damage to the myofiber membrane (in red). There was severe EBD leakage in PBS-treated *mdx* mice, shown by the red color throughout muscle cell cytoplasm, but it was markedly reduced in both treated young and old *mdx* mice with protection from the AAV-based p65-shRNA treatment. As seen in the first and second row, the myofibers with EBD leakage existed in both 1-month- and 4-month-old treatment groups exactly where ZsGreen expression was negative. All pictures are shown at $\times$ 200 magnification.

is present, an AAV vector-induced immune response may still interfere with a successful outcome. The investigation of the potential for immunity in inflamed muscles should be considered when designing and monitoring experimental therapies for DMD.

Chronic inflammation and ongoing myofiber necrosis exist throughout the lives of *mdx* mice. NF-κB is a ubiquitously expressed transcription factor in mammals that has a key role in muscular dystrophy by promoting the inflammatory response.[10,11] Numerous studies have demonstrated the chronic inflammation in DMD dystrophic muscles, which has been evidenced to be regulated by the classical pathway of NF-κB activation[12] and results in secondary pathologies such as muscle necrosis and wasting. Recently, blocking IKKβ has been considered as a potential approach for anti-chronic inflammation in dystrophic muscles by targeting the upstream activator of the classical NF-κB pathway. The peptide inhibitor of IKKβ,[9] for example, reduced inflammation and necrosis while enhancing regeneration. However, these approaches require repeated treatments and have the potential for systemic side effects. In our recently published study,[24] we clearly demonstrate the therapeutic benefits of inhibiting NF-κB activation by AAV gene transfer of the dominant negative forms of IKKá (IKKα-dn) and IKKα (IKKβ-dn) in dystrophic muscle to promote regeneration, particularly in older *mdx* mice, and to block necrosis.

However, in the classical activation pathway, the downstream NF-κB signaling occurs primarily by way of a p50/p65 heterodimer,[30] especially through P-p65 entering the nucleus, where it binds to its cognate DNA site and interacts with the basal transcription factors and coactivators to promote gene expression.[31] On the basis of enhanced muscle regeneration of the *mdx* mice that were bred with mice carrying a heterozygous deletion of the p65 subunit,[9] the p65 subunit in the NF-κB signaling pathway is a potential therapeutic target for treating this disease. Of particular importance in this study is the fact that specific shRNA that targets the mouse NF-κB/p65 subunit[32] was packaged into a self-complementary AAV vector, and is controlled by a human U6 promoter that provides high levels of gene transfer and also ensures p65 gene silencing on both immune cells and dystrophic muscle fibers, thus diminishing the inflammation in dystrophic muscles. On the basis of high efficiency of AAV9 vectors that we observed in murine[25] and large DMD[20] models, the use of an AAV9 vector in this study ensures a high efficiency of shRNA delivery in *mdx* muscle by i.m. injection. We did not observe the ZsGreen marker gene expression in the liver following i.m. injection in our experiment that might be associated with the inactivation of the CMV promoter in mouse hepatocytes at 1 month after treatment (data not shown). The potentially systemic AAV9 vector dissemination in treated adult *mdx* mice was not tested in this study. Although *mdx* mice have less inflammation in dystrophic muscle compared with large animal models, such as golden retriever muscular dystrophy model, the success of using an AAV9 vector carrying the p65-shRNA in *mdx* mice for antiinflammation provides a feasible strategy for the treatment in a large DMD animal model.

The shRNA targeting NF-κB/p65 was hypothesized to degrade NF-κB/p65 mRNA, resulting in a block of the NF-κB signaling pathway.[9,32] Hence, we attempted to silence the p65 gene to improve the pathologies observed in *mdx* mice. Our findings indicate that AAV-based p65-shRNA has the capacity to decrease immune cell infiltration, to inhibit necrosis and to protect the myofiber membranes. More importantly, the treatment could diminish the muscle inflammation at the peak of pathological degeneration in dystrophic muscle, resulting in decreased regenerative myofibers in 1-month-old *mdx* mice. On the other hand, the treatment could enhance the numbers of regenerative myofibers in the treated old *mdx* mice after muscle progenitor cells were exhausted with continuous cycles of muscle degeneration and regeneration. As revealed in the young treated *mdx* mice at 1 month of age, decreased necrosis and CN followed the AAV-p65-shRNA treatment. Significantly, reduced necrosis was also found in *mdx* muscle at 4 months of age treated with AAV-p65-shRNA; however, no remarkable reduction in CN was revealed in

age-matched *mdx* muscle treated with AAV-p65-shRNA. Our results indicate that the anti-inflammatory effect is more efficient when we treat *mdx* mice at an earlier age. Hence, the rationale behind the AAV-p65-shRNA treatment is to therapeutically intervene before pathology is manifested to prevent, rather than reverse, disease. The findings also suggest that AAV-p65-shRNA treatment at a young age can delay or decrease the progressive muscle degeneration and regeneration. Moreover, treatment in old *mdx* mice can enhance therapeutic regeneration of myofibers and decrease degeneration. We speculate that an exhaustion of satellite cells during ongoing pathological degeneration and regeneration cycles results in the loss of the capacity to regenerate in response to injury signals in DMD. The relationship between NF-κB signaling and muscle stem cell differentiation has remained unknown up to now; our results demonstrate that the AAV-p65-shRNA treatment in dystrophic muscle reduces the adverse effects of NF-κB signaling, resulting in a therapeutic effect that is consistent with the results from the NF-κB/p65 knock-out *mdx* mice (*mdx*: p65 +/−).[9] Furthermore, our findings using the EBD injection membrane integrity test indicate that the AAV-mediated p65-shRNA can protect the muscle cell membrane from losing integrity, suggesting that the anti-inflammatory effect by decreasing the activity of NF-κB in muscle can be an additional strategy for restoring muscle function. However, as observed in ZsGreen-positive myofibers in Figure 7, the effect of protecting the membrane is dependent on the efficiency of delivering p65-shRNA.

Although the loss of dystrophin causes the primary clinical pathology in DMD, secondary processes involving persistent inflammation and impaired regeneration exacerbate disease progression. Necrosis presents from the age of 1 month as a consequence of the pathological degeneration and regeneration in *mdx* muscles;[33] hence, we treated *mdx* mice at the ages of 1 and 4 months to test the therapeutic effects in *mdx* muscle at different stages of inflammation. The successful anti-inflammatory effect of AAV-mediated p65-shRNA in adult *mdx* muscle provides a potential therapeutic approach for DMD. Moreover, anti-inflammatory approaches combined with gene replacement can also minimize the administration dose of viral vectors for gene replacement, and also diminish the potential immune response in the host through an improvement in the inflamed microenvironment. Furthermore, the carrying capacity of an AAV vector allows the p65-shRNA silencing cassette (<350 bp) and mini-dystrophin (Δ3849) expressing cassette package (<4.5 kb)[17,25] into a single-stranded AAV vector, which provides a possibility to combine gene replacement and anti-inflammatory therapy in a one-step treatment. In the future, a synergistic therapeutic effect could be achieved by combining dystrophin gene replacement with p65-shRNA-based anti-inflammation in a single AAV vector for DMD treatment, especially by muscle-specific expression of mini-dystrophin and gene silencing of NF-κB/p65 in damaged muscle cells and immune cells.

MATERIALS AND METHODS

Construction of the NF-κB shRNA and AAV vector production

Based on the sequence of siRNA (5′-TGTGT CCATT GTCTC ACTC-3′), which targets the mouse NF-κB/p65 subunit mRNA,[32] we designed a shRNA. The forward oligo is: 5′-GATC-19 bp sense-CTCGAG-19bp antisense-TTTTTTT-G-3′; the reverse oligo is: 5′-AATTC-aaaaaaa-19 bp sense-CTCGAG-19bp antisense-G-3′. As shown in Figure 8, these two complementary oligos formed a double-stranded fragment with *BamH* 1/*EcoR* 1 cohesive ends, and they were then cloned into the same restriction enzyme sites of a self-complementary AAV vector under the control of the human U6 promoter, followed by a ZsGreen expression cassette that serves as a marker to monitor the shRNA delivery efficiency. We also designed a control AAV vector, which contains a scramble shRNA that is not associated with any sequence in the cDNA of mouse NF-κB/p65, and a ZsGreen gene as above. An AAV9 vector carrying either p65-shRNA or ct-shRNA was made according to a three-plasmid co-transfection method and purified through CsCl density gradient ultracentrifugation, as per the published protocols.[34] The vector titers were checked by the DNA dot blot method and were approximately 7×10^{12}–1×10^{13} viral particles per ml.[17]

Mice and vector administration

All experiments involving animals were approved by the University of Pittsburgh Animal Care and Use Committee. The 1- and 4-month-old *mdx* mice (four mice for each group) were i.m. injected with 50 μl (3.5×10^{11} viral particles) of AAV9_U6_p65-shRNA_CMV_ZsGreen vectors into the right GAS muscle, and the left GAS muscles were treated by the same volume of PBS, serving as controls. In the ct-shRNA group, four mice at 1 month and 4 months of age were also treated by 50 μl (5×10^{11} viral particles) of AAV9_U6_ct-shRNA_CMV_ZsGreen into the right GAS muscle; the left GAS muscles were treated by the same volume of PBS buffer. Moreover, the age-matched C57BL/10 wild-type mice, four mice for each age group, also served as controls treated with the same volume of PBS buffer in their left GAS muscles. All of the mice were killed 1 month after AAV vector or PBS injection for histological analysis. The GAS muscle samples were collected, embedded into Cryo-gel (Instrumedics, St Louis, MO, USA) on cork, freshly submerged in liquid nitrogen-cooled 2-Methylbutane (Sigma, St Louis, MO, USA) for 1 min, and then stored at −80 °C or directly cut into 10 μm thick cryosections.

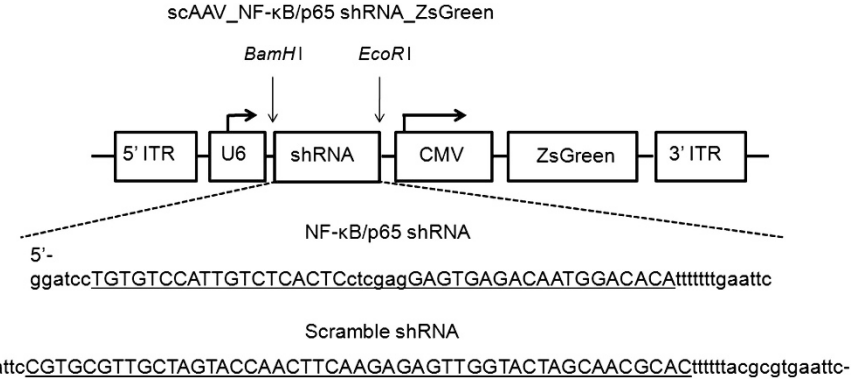


Figure 8. Construct of AAV vector containing both ZsGreen expression and mouse NF-κB/p65 shRNA silencing cassettes. A self-complementary AAV vector carrying the human U6 promoter controlled the shRNA cassette, followed by a ZsGreen reporter gene that is regulated by the universal (CMV) promoter to monitor the efficiency of the AAV vector. Both NF-κB/p65 shRNA and a scramble shRNA were located between *BamH* 1 and *EcoR* 1 restriction enzyme following the U6 promoter.

Muscle histology analysis

To test the levels of phospho-NF-κB/p65 (P-p65), the phospho-NF-κB/65 (Ser536) (93H1) rabbit mAb (Cell Signaling, Danvers, MA, USA) that detects NF-κB/p65, only phosphorylated at Ser536 was first applied at 1:50 dilution in 2% horse serum for 3 h at room temperature. The second antibody, Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), presenting as red color, was then applied at 1:500 dilution for 15 min at room temperature. To determine muscle fiber necrosis, muscle cryosections were immunofluorescently stained using biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA, USA), diluted in the ratio of 1:300 in 2% horse serum for 1 h at room temperature. The StreptavidinCy3 (Sigma) was used as a secondary antibody, presenting as red color, and was applied at a 1:500 dilution in PBS for 15 min. The muscle sections were stained using the VECTOR M.O.M Immunodetection Kit (Vector Laboratories) for eMyHC to evaluate the muscle myogenic regeneration, according to the manufacturer's protocol. The monoclonal antibody against eMyHC (F1.652, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA) was used at 1:50 dilution, for 2.5 h at room temperature. The antibody Streptavidin Alexa Fluor 594 conjugate (Invitrogen), presenting as red color, was used at 1:500 dilution in 2% horse serum at room temperature for 15 min. All muscle cell nuclei in the immunofluorescent staining were counterstained with 0.01% 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma), diluted 1:700 in PBS for 10 min. Photographs were taken with a (Leica Microsystems Inc., Wetzlar, Germany) inverted fluorescent microscope and were analyzed by using Norhtern Eclipse software (EMPIX imaging Inc., Cheektowaga, NT, USA). Hematoxylin and eosin staining was performed to show myofiber morphology and to calculate the percentage of central nuclei myofibers by analyzing images with Image J software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

To determine the effect of the p65-shRNA on the cultured immune cells, mouse macrophage RAW cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in six-well plates to achieve 80% confluence. Transfections with AAV plasmid DNAs were done with a Lipofectamine Kit (Invitrogen) according to a standard protocol,[24] and all transfection experiments were performed in triplicate. The medium was changed twice with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (1% Pen/Strep) at 6 and 48 h after transfection. The activation of NF-κB signaling pathway was activated by stimulation with lipopolysaccharide (Sigma) for 15 min, and cells were harvested for western blot assay that was carried out, according to a previously published method,[18] to determine the levels of P-p65 in mouse macrophages following the lipopolysaccharide stimulation. Cells were lysed in 20 μl of radioimmunoprecipitation assay buffer.[18] After adding loading buffer, all samples were boiled for 5 min and then were chilled on ice for 10 min. The samples were electrophoretically separated on 8% SDS-polyacrylamide gel, transferred to an Immobilon-P Transfer Membrane (Millipore, Tullagreen, Carrigtohill, Ireland), and analyzed by western blotting with phospho-NF-κB/65 (Ser536) (93H1) rabbit mAb (Cell Signaling) as the primary antibody and a goat anti-rabbit IgG (H + L) as the secondary antibody (Thermo Fisher Scientific, Peroxidase Conjugated, 1:5000, Rockford, IL USA). The P-p65 protein bands (65 kDa) were visualized with SuperSignal West Pico Chemiluminescence Substrate (Thermo Fisher Scientific) and exposed to *X* ray film.

qPCR analysis of p65 mRNA in treated tissues

In order to determine whether the treatment of the AAV-p65-shRNA decreased the levels of p65 mRNA in treated GAS muscle, we utilized reverse transcription-qPCR, a powerful and sensitive gene analysis technique. At 1 month after treatment, 50 mg of frozen muscle tissues (three samples per group) that were treated by either AAV-p65-shRNA or AAV-ct-shRNA at 1 month of age was homogenized in Trizol (Invitrogen), and total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocols. The reverse transcription of RNA to cDNA was performed with Superscript III reverse transcriptase (Invitrogen), according to manufacturer's protocols. Real-time-PCR was carried out using Taq Polymerase (Promega, Madison, WI, USA) on BioRad iQ5 Thermocycler (BioRad, Hercules, CA, USA) according to manufacturer's protocols. One primer pair specific to p65 (NCBI Reference Sequence NM_009045.4) includes forward primer (5′-TTCCCTCAGAGCCAGCCC AGG-3′) and reversed primer (5′-TAGCGGAATCGCATGCCCCG-3′). Beta actin (NCBI reference sequence NM_007393.3) was used as the reference gene, by using forward primer (5′-CCACACCCGCCACCAGTTCG-3′) and reversed primer (5′-TACAGCCCGGGGAGCATCGT-3′) for qPCR analysis. Each sample for qPCR was performed in triplicate, and the results analyzed using the comparative (ΔΔCt) method.

In vivo myofiber plasma membrane integrity

The treated *mdx* mice were given an i.v. injection of the EBD (Sigma), diluted at 10 mg dye per ml PBS buffer at a dose of 0.1 mg g^{-1} of body weight 1 month after treatment.[35] After the dye injection, mice were allowed continuous swimming in 37 °C water for 20 min. The mice were then killed, and GAS muscles were collected and cryosectioned as described above. The EBD-positive myofibers were observed directly under the fluorescent microscope.

Statistical analysis

Differences between different groups were analyzed by Student's *t*-test. A *P*-value <0.05 was considered statistically significant. Error bars in all figures indicate the standard error. In the result section we analyzed data by using Mean ± s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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