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Antagonism of Myostatin Enhances Muscle Regeneration During Sarcopenia

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A reduction in muscle mass and strength is often observed with aging, and this phenomenon is known as sarcopenia. This age-related atrophy frequently correlates with insufficient levels of muscle regeneration resulting from impairment of satellite cell involvement and myogenesis brought about by the aged environment. Using *myostatin*-null mice, we recently showed that negative regulators of muscle mass such as myostatin play an active role in the regulation of myogenesis during aging. The present study specifically tests the therapeutic value of a myostatin antagonist in sarcopenia. We report here that a short-term blockade of myostatin, through stage-specific administration of a myostatin antagonist, significantly enhanced muscle regeneration in aged mice after injury and during sarcopenia. Antagonism of myostatin led to satellite cell activation, increased Pax7 and MyoD protein levels, and greater myoblast and macrophage cell migration, resulting in enhanced muscle regeneration after notexin injury in aged mice. In addition, the antagonist demonstrated a high degree of efficacy, as only minimal doses during the critical period of regeneration after injury were sufficient to restore the myogenic and inflammatory responses in the aged environment. Thus, we propose that the antagonism of myostatin has significant therapeutic potential in the alleviation of sarcopenia.

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INTRODUCTION

Mammalian skeletal muscle mass is dependent on the number of muscle fibers, their type, and size. All three aspects are believed to be influenced by a variety of conditions, including aging, which can lead to atrophy of individual muscle fibers, loss of fiber numbers, and switching between fiber types.^{1–5} A prominent component of muscle fiber size is the involvement of satellite cells, a population of myogenic precursor cells associated with muscle fibers. Satellite cells are located between the basal lamina and sarcolemma⁶ and exist predominantly in a mitotically quiescent state.⁷ These cells are believed to be largely responsible for muscle regeneration⁸ by giving rise to myoblasts, thereby enabling additional nuclei to fuse

to existing fibers or form new myofibers.^{9,10} For the progression of this process, satellite cells must enter the cell cycle from the quiescent state and undergo myogenesis. Clearly, the number of satellite cells as well as their proficiency to undergo this process would impact the degree to which muscle regeneration can occur. One factor that is thought to severely impact satellite cell behavior is aging. Satellite cells isolated from aged animals have demonstrated a significant lag when entering the cell cycle,¹¹ a decreased proliferation and differentiation potential,^{12,13} and an increased susceptibility to apoptosis.^{14,15} Additionally, various studies indicate that aged muscle is restricted in its ability to promote satellite cell activation.^{16–18} Using cross-age muscle transplantation, it has been demonstrated that the poor regeneration associated with old animals is a function of the aged environment.¹⁹ In addition, it has also been suggested that insufficient up-regulation of Notch signaling is directly responsible for the impaired activation propensity of the aged satellite cells.¹⁷ More recently, parabiotic pairing experiments have indicated that systemic factors from young mice restored the activation of Notch signaling and also promoted the proliferation and regenerative capacity of satellite cells in aged mice.²⁰ Alternatively, negative regulators, such as myostatin, may inhibit satellite cell activity in the aged environment.

Myostatin is a transforming growth factor- β superfamily member, and functions as a potent inhibitor of muscle growth.²¹ It is expressed both pre-natally in the developing myotome and post-natally in adult skeletal muscle,²¹ thereby suggesting that myostatin plays a continual role in myogenesis. Indeed, *myostatin*-null animals display significantly greater muscle mass resulting from muscle fiber hyperplasia and hypertrophy.²¹ Studies indicate that myostatin influences myogenesis in C2C12 myoblast cultures through the regulation of cell cycle progression and myogenic regulatory factors.^{22,23} Moreover, myostatin has been shown to inhibit satellite cell activation in mice,²⁴ and this would have significant consequences in the context of age-related muscle wasting and regeneration. Interestingly, increased myostatin has been correlated with muscle atrophy during unloading in mice,²⁵ muscle wasting in human immunodeficiency virus patients,²⁶ and cachexia induced by systemic administration of myostatin.²⁷ Although the exact role of myostatin during age-related muscle wasting remains unclear, its prolonged absence from the pre-natal stage, as seen in *myostatin*-null mice, has been demonstrated to result in an overall reduction in sarcopenia.^{28,29} This being the

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case, an antagonist of myostatin will have significant therapeutic potential in the alleviation of sarcopenia and impaired muscle regeneration that is observed in aged animals. In fact, we report here that a short term blockade of myostatin function through the administration of a truncated protein was able to significantly enhance muscle regeneration in aged mice. This regeneration occurred by a process of restoration of the myogenic and inflammatory responses in the aged mice, leading to increased satellite cell activity and enhanced macrophage and myoblast migration.

RESULTS

Production of the antagonist Mstn-ant1

Previously we have shown that a prolonged absence of myostatin reduced sarcopenia in *myostatin*-null mice.²⁸ We elected to test whether a specific molecule, a truncated version of myostatin, could antagonize myostatin and potentially offer a therapeutic option for treating sarcopenia. Myostatin protein is proteolytically processed at amino acid 266, giving rise to the biologically active mature myostatin²¹ which spans amino acids 266–375. This 110-amino-acid protein binds to activin type IIB receptor as a dimer to transduce signaling.³⁰ In order to antagonize myostatin, we produced a C-terminal truncation spanning amino acids 266 to 350, as a dominant negative (mimetic) protein. This truncation, named Mstn-ant1, along with its amino acid length, is illustrated in **Figure 1a**. The Mstn-ant1 protein was expressed in an *Escherichia coli* expression system and purified to homogeneity (**Figure 1b**).

Antagonism of myostatin enhances muscle regeneration following injury

Next we tested the utility of Mstn-ant1 in a regenerating model in mice. Typically after a notexin type injury, there is initially an increase in the weight of the injured muscle due to oedema, followed by a decrease caused by necrosis of the damaged muscle fibers. Muscle weight then begins to increase again as regeneration of the fibers occurs. The results indicated a trend in which the loss in muscle weight was less pronounced in the Mstn-ant1-treated muscles as compared with the saline-treated muscles at days 7 and 10 (**Figure 2a**). The observed trend could have been

due to a decreased muscle loss during the necrosis period or, conversely, due to advancement in new fiber formation resulting from the *in vivo* antagonism of myostatin by the Mstn-ant1 treatment. In support of this, an analysis of the tibialis anterior (TA) muscle sections indicated that nascent muscle fiber formation (regenerated areas) was taking place earlier, and there was an associated reduction in necrotic areas (unregenerated areas) in the muscles treated with Mstn-ant1 as compared to saline-treated muscles at days 7 and 10 (**Figure 2b–f**) ($P < 0.05$). Measurement of fiber areas can indicate the progression of regeneration occurring within a recovering muscle. Individual fiber areas were therefore measured to assess fiber regeneration 28 days after injury, and the results

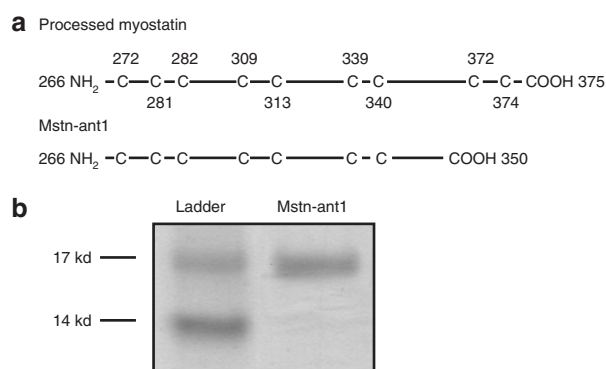


Figure 1 Structural representation of the antagonist Mstn-ant1. **(a)** Mstn-ant1 and processed myostatin are depicted, illustrating the length of the truncated protein (C = cysteine, numbers = amino acid number). **(b)** The Mstn-ant1 protein was separated on a sodium dodecyl sulfate gel to confirm its purity.

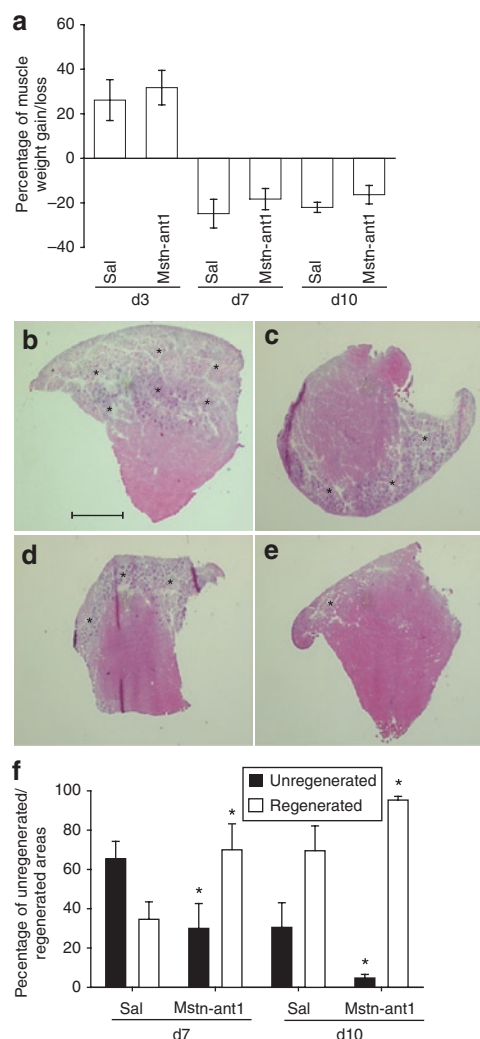


Figure 2 Antagonism of myostatin after notexin injury enhances muscle regeneration. **(a)** After notexin injury and treatment with Mstn-ant1, the percentage of muscle weight gain or loss from saline (Sal)- and Mstn-ant1-treated muscles was analyzed on days 3, 7, and 10 (d3, 7, and 10) ($n = 4$). Sections were cut from each tibialis anterior muscle and stained with hematoxylin and eosin (H & E) so as to visually determine the extent of muscle injury and regeneration on day 7 in **(b)** Sal-treated and **(c)** Mstn-ant1-treated muscles, and on day 10 in **(d)** Sal-treated and **(e)** Mstn-ant1-treated muscles ($n = 4$, * denotes unregenerated areas) (scale bar = 1 mm). **(f)** Using the H & E stained sections, the percentages of unregenerated and regenerated areas within each muscle were measured ($n = 4$). * $P < 0.05$ when comparing Sal-treated with Mstn-ant1-treated.

indicated that the regenerated fibers from the muscles of Mstn-ant1-treated mice were significantly larger than those from the saline-treated mice (**Figure 3c**) ($P < 0.05$). Also, because collagen deposit levels can be measured within the regenerated muscle³¹ following notexin injury, Van Gieson staining of the saline-treated and Mstn-ant1-treated muscles was carried out (**Figure 3a and b**). Reduced levels of collagen were found in the Mstn-ant1-treated muscles as compared to saline-treated muscles at days 10 and 28 ($P < 0.05$) (**Figure 3d**).

Expression of MyoD and Pax7 in regenerating muscle is altered by antagonist treatment

It has been established that both Pax7 and MyoD are potent markers of myogenesis. While Pax7 levels can signify the extent of the satellite cell pool as well as satellite cell self-renewal,^{32,33} MyoD can signify the level of myogenesis occurring within a muscle.³⁴ To investigate Pax7 and MyoD protein levels in the notexin injured muscles, Western blot analyses were performed. Analysis of the saline-treated and Mstn-ant1-treated muscles indicated that Pax7 protein levels were higher with Mstn-ant1 treatment at days 3, 7, 10, and 28 (**Figure 4a and b**) ($P < 0.05$). Similarly, MyoD levels were also higher with Mstn-ant1 treatment than with saline treatment at days 3, 7, and 10 (**Figure 4a and c**) ($P < 0.05$). Comparable levels of MyoD were seen between the treatment groups at day 28; however, a higher level of Pax7 was observed at the same time point with the administration of Mstn-ant1. As in the case of a notexin injury, though to a lesser extent, aged muscle also displays a level of ongoing myogenesis due to muscle maintenance and repair associated with the aging process. Therefore, Pax7 and MyoD were also analyzed in the muscles isolated from aged mice treated for 6 weeks with saline

or Mstn-ant1. Protein isolated from gastrocnemius muscle at the completion of the trial indicated significantly higher Pax7 and MyoD protein levels in the Mstn-ant1-treated mice compared to those in the saline-treated mice (**Figure 4d, f and h**) ($P < 0.05$). In addition, protein isolated from actively growing primary myoblasts also displayed increased levels of both Pax7 and MyoD (**Figure 4e, g and i**) after Mstn-ant1 treatment.

Satellite cell activation and proliferation, and muscle strength increase in response to Mstn-ant1 treatment

To investigate how Mstn-ant1 would affect satellite cell activation, isolated single fibers from 1- and 24-month-old mice were cultured with or without Mstn-ant1. Satellite cell activation was determined through immunocytochemistry using an antibody for proliferating cell nuclear antigen, a marker for DNA replication (**Figure 5a**). The fibers cultured with Mstn-ant1 consistently displayed a higher percentage of activated satellite cells per fiber than fibers cultured in media alone (**Figure 5c and d**) ($P < 0.05$).

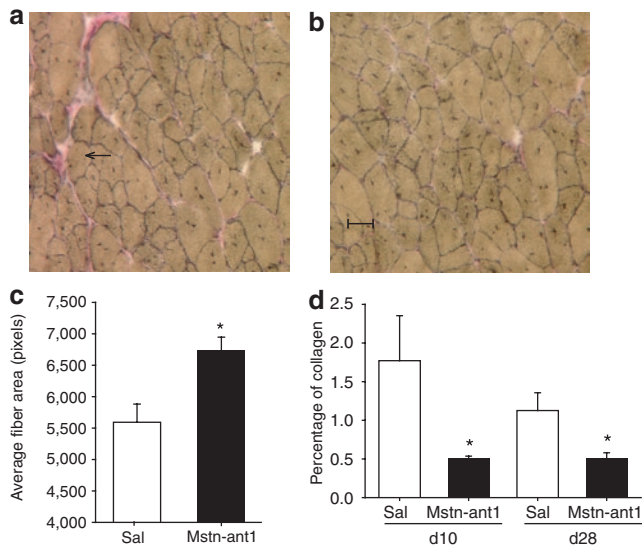


Figure 3 Increased fiber area and decreased collagen are observed in Mstn-ant1-treated muscles. Representative images of Van Gieson-stained (a) saline (Sal)-treated and (b) Mstn-ant1-treated muscle sections are shown (scale bar = 20 μ m). Collagen is identified by pink staining (indicated by arrow). (c) Individual regenerated fiber areas as well as (d) the percentage of collagen within each section was measured in the Sal- and Mstn-ant1-treated day 28 muscles using the Van Gieson-stained sections ($n = 4$). * $P < 0.05$ when comparing Sal-treated with Mstn-ant1-treated.

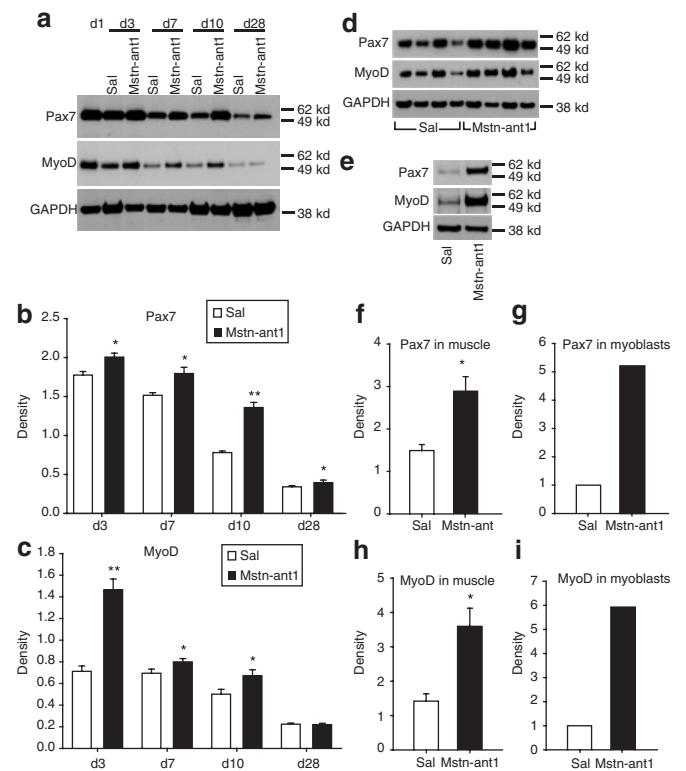


Figure 4 MyoD and Pax7 expressions in regenerating muscle are increased by Mstn-ant1 treatment. (a) Western blot analysis was performed on days 1, 3, 7, 10, and 28 on saline (Sal)- and Mstn-ant1-treated notexin injured muscles in order to determine Pax7 and MyoD protein levels. Each blot was analyzed by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels. Normalized data were used for generating the (b) Pax7 and (c) MyoD graphs depicted ($n = 4$). ** $P < 0.001$, * $P < 0.05$ when Sal-treated are compared with Mstn-ant1. Western blot analysis was also performed on (d) muscles and (e) primary myoblasts isolated from aged mice treated for 6 weeks with Sal or Mstn-ant1 to determine Pax7 and MyoD protein levels during sarcopenia-related muscle regeneration. Each blot was analyzed by densitometry and normalized to GAPDH protein. Normalized data were used for generating the (f and g) Pax7 and (h and i) MyoD graphs shown ($n = 4$). * $P < 0.05$ when comparing Sal-treated with Mstn-ant1-treated.

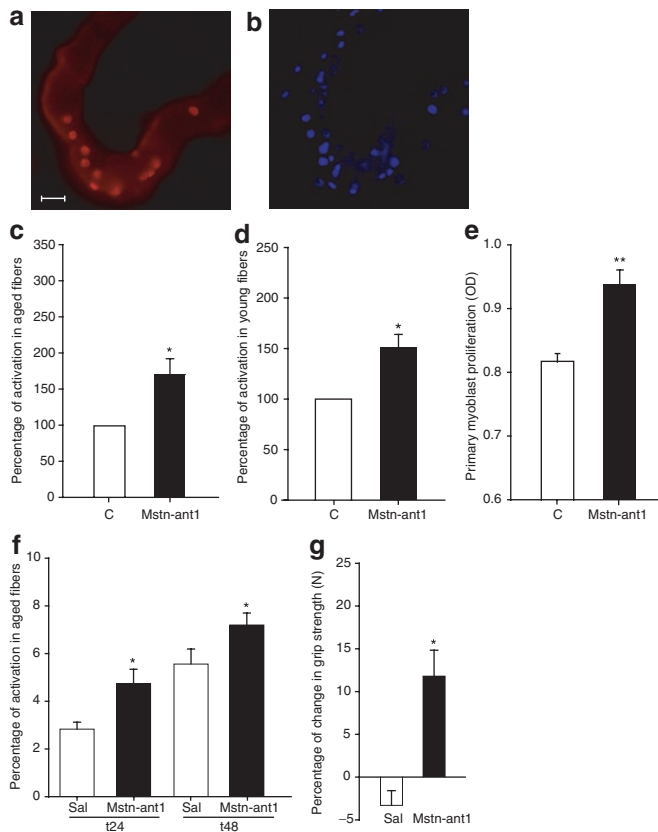


Figure 5 Satellite cell activation is increased by *in vitro* and *in vivo* myostatin antagonism. Activated satellite cells on isolated single fibers were visualized by immunostaining for (a) proliferating cell nuclear antigen and (b) total nuclei by 4',6-diamidino-2-phenylindole (scale bar = 20 μ m). The number of satellite cells per 100 myonuclei was determined in muscle fibers isolated from (c) 24-month-old and (d) 1-month-old mice after culturing for 48 and 24 hours, respectively, with or without (c = control) Mstn-ant1. Fibers were isolated from 12 animals and more than 1,000 nuclei per group were counted. * $P < 0.05$ when comparing Mstn-ant1 compared with control. (e) A proliferation assay was performed with primary myoblasts isolated from 1-month-old mice. The myoblasts were cultured with or without Mstn-ant1 and cell proliferation was determined using a methylene blue photometric endpoint assay. Proliferation after 96 hours is graphically shown. Assays were performed in triplicate using cells isolated from four animals. ** $P < 0.001$. (f) Satellite cell activation was also assessed in single fibers isolated from aged mice treated for 6 weeks with saline (Sal) or Mstn-ant1. Single fibers were cultured for 24 and 48 hours (t24, t48) before the percentage of activated satellite cells was determined as described above. Fibers were isolated from 10 animals in each treatment group and in excess of 1,000 nuclei per group were counted. * $P < 0.05$ when Sal is compared to Mstn-ant1 treated. (g) Grip strength was assessed in the aged mice treated for 6 weeks with Sal or Mstn-ant1. Grip tests were performed on each animal at the commencement and completion of the trial ($n = 10$ per treatment group) giving a measurement in Newtons (N). * $P < 0.05$ when comparing Sal-treated with Mstn-ant1*-treated.

Myostatin is also known to affect cell proliferation. Thus, to investigate the efficacy of the antagonist in increasing myoblast proliferation, primary myoblasts from 1-month-old mice were cultured with or without Mstn-ant1. The results indicated that culturing with Mstn-ant1 increased myoblast proliferation by 15% (Figure 5e) ($P < 0.001$).

In addition to culturing isolated fibers with Mstn-ant1, satellite cell activation was also investigated in the fibers isolated

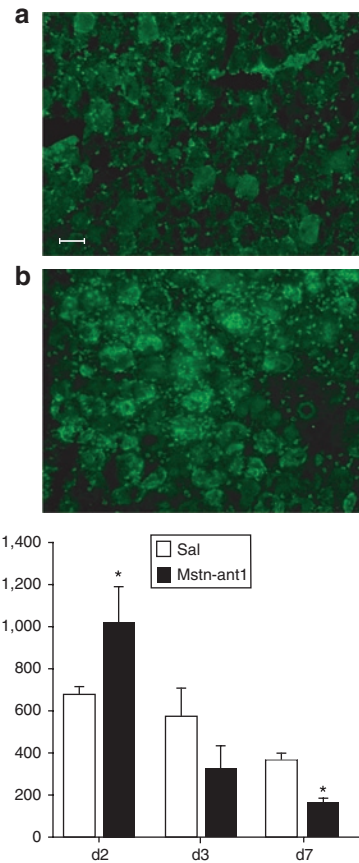


Figure 6 Antagonism of myostatin enhances macrophage infiltration. Representative images of Mac1-immunostained, day 2 (a) saline (Sal)-treated and (b) Mstn-ant1-treated muscle sections after notexin injury (scale bar = 50 μ m). (c) The number of macrophages in Sal-treated and Mstn-ant1-treated muscle sections was determined at days 2, 3, 7, and 10 ($n = 4$ per day and treatment group). * $P < 0.05$ when comparing Mstn-ant1 treatment with Sal treatment.

from aged mice injected with saline or Mstn-ant1 for 6 weeks. The fibers isolated from the Mstn-ant1-treated mice displayed a significantly higher percentage of activated satellite cells per fiber than those from the saline-treated mice (Figure 5f) ($P < 0.05$). Furthermore, to test whether the 6-week treatment could increase muscle strength, grip tests were performed at the commencement and completion of the treatment period. The results indicated that the administration of Mstn-ant1 significantly increased the grip strength of the aged mice by 12% ($P < 0.05$) (Figure 5g).

Antagonist treatment alters macrophage migration

Myostatin is believed to inhibit macrophage migration.³¹ Therefore notexin injured muscles treated with saline or Mstn-ant1 were examined for the presence of macrophages, and anti-Mac1 antibodies were used for determining the efficacy of the antagonist in enhancing macrophage migration. Indeed, on day 2 after injury, the muscles that had been treated with Mstn-ant1 showed a greater percentage of Mac1-positive infiltrated nuclei than did the saline-treated muscles (Figure 6a–c) ($P < 0.05$). By day 3, this percentage had dropped in the Mstn-ant1-treated muscles to below that of the saline-treated day 3 muscles, and continued to be lower at days 7 and 10.

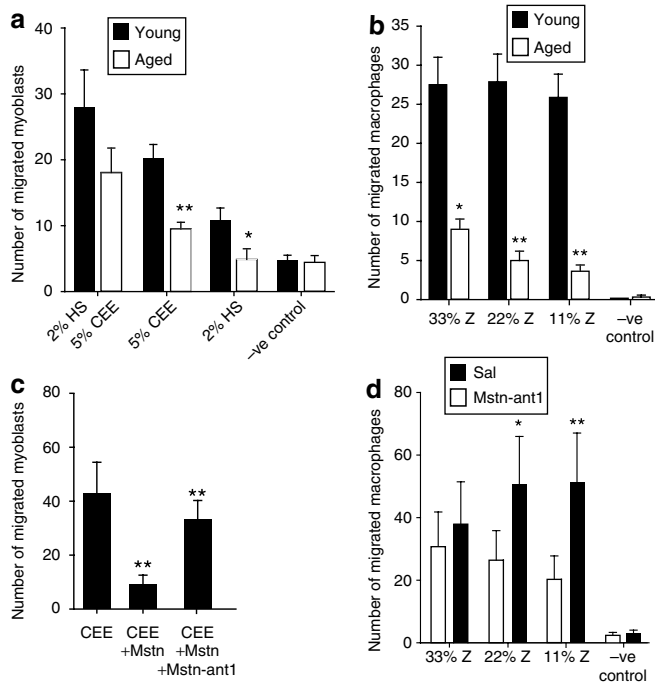


Figure 7 Aging decreases cell migration while Mstn-ant1 enhances migration. **(a)** Primary myoblasts isolated from 1- and 24-month-old mice were used in a migration assay with three concentrations of chemoattractant, 2% horse serum (HS) + 5% chicken embryo extract (CEE) (optimum concentration), 5% CEE, or 2% HS (suboptimal), while Dulbecco's modified eagle's medium (DMEM) alone was a negative control (-ve control). $**P < 0.001$, $*P < 0.05$ when 1-month old is compared with 24-month-old. **(b)** Peritoneal macrophages isolated from 1- and 24-month-old mice were used in a migration assay with three concentrations of the chemoattractant Zymosan A activated mouse serum (ZAMS) (Z), 33% (optimum concentration), 22 and 11% (suboptimal) and DMEM alone as a negative control. $**P < 0.001$, $*P < 0.05$ when 1-month old is compared with 24-month-old. **(c)** Primary myoblasts isolated from 24-month-old mice were used in a chemotaxis assay. CEE medium was a positive control, while CEE + myostatin (Mstn; 2.5 $\mu\text{g}/\text{ml}$) was used as a negative control. Mstn-ant1 was added to wells containing CEE medium + Mstn to rescue the chemo-inhibitory effect of Mstn. $**P < 0.001$ when CEE was compared with CEE + Mstn, and also when CEE + Mstn was compared with CEE + Mstn + Mstn-ant1. **(d)** Bone marrow-derived macrophages from the saline (Sal)-treated and Mstn-ant1-treated mice were used in assaying macrophage migration after treatment. Three concentrations of DMEM containing ZAMS were used, while DMEM was used as a negative control. $**P < 0.001$, $*P < 0.05$ when Mstn-ant1 treatment was compared with Sal treatment. For all migration assays, migrated cells were counted on four representative fields per membrane and the average number was plotted.

Myoblast and macrophage migration is altered with age and Mstn-ant1 treatment

Both myoblasts and macrophages are known to be influenced by chemotactic signals which affect their movement.^{35,36} In order to elucidate the effects of myostatin and aging on the migration of myoblasts and macrophages, a series of chemotaxis assays were performed. The results suggested that migration was significantly retarded in the myoblasts isolated from 24-month-old mice as compared to those from 1-month-old mice, in the presence of 5% chicken embryo extract or 2% horse serum (Figure 7a) ($P < 0.05$). Similarly, migration of macrophages isolated from 24-month-old mice was also lower compared to

those from 1-month-old mice, irrespective of the chemoattractant concentration (Figure 7b) ($P < 0.001$). The efficacy of the Mstn-ant1 in rescuing cell migration in response to myostatin treatment was then examined, using primary myoblasts isolated from 24-month-old mice. When Mstn-ant1 was added, it was possible to rescue cell migration by hindering the chemo-inhibitory effect of myostatin (Figure 7c) ($P < 0.05$). In order to test the efficacy of Mstn-ant1 in enhancing cell migration after prolonged *in vivo* treatment, bone marrow-derived macrophages were isolated from mice injected for 6 weeks with saline or Mstn-ant1. In fact the macrophages from Mstn-ant1-treated mice were able to migrate more efficiently than macrophages from saline-treated mice (Figure 7d) ($P < 0.05$).

DISCUSSION

Numerous studies indicate that aging considerably influences myogenesis because of the muscle environment or systemic factors,^{19,20} expression of myogenic regulatory factors,³⁷ and satellite cell behaviour.^{16,17} Recently, it has been shown that a prolonged absence of myostatin reduces sarcopenia in *myostatin*-null mice.^{28,29} However, as these mice lack functional myostatin from a pre-natal stage, it was necessary to carry out further studies to determine the effects of short-term inhibition of myostatin during old age. A previous study indicated that an increase in skeletal muscle mass and strength occurs following treatment with a myostatin inhibitory antibody.³⁸ In this study, we show that a short-term administration of a myostatin antagonist restores the regenerative and myogenic capacity of aged muscle.

In agreement with past studies that showed that the lack of myostatin significantly enhances muscle regeneration and reduces fibrosis,^{28,29,31} the results reported here suggest that a blockade of myostatin by administering Mstn-ant1 immediately after notexin injury can replicate this effect. Muscle weights, histology, greater regenerated areas at days 7 and 10, as well as significantly larger individual fiber areas at day 28, all indicated advancement of regeneration with Mstn-ant1 treatment (Figures 2 and 3c). In addition, Mstn-ant1-treated muscles displayed reduced levels of collagen (Figure 3d), thereby suggesting that myostatin is involved in fibrosis and collagen formation. This is supported by the fact that during fibroblast migration assays myostatin can behave as a fibroblast chemoattractant (data not shown). Collectively, these results indicate that a prolonged absence of myostatin, as observed in the *myostatin*-null mice, is not essential for obtaining enhanced muscle regeneration and reduced fibrosis. Rather, a short-term blockade of myostatin during the regeneration period is sufficient to enhance the regeneration process.

During muscle regeneration, MyoD is expressed earlier and at higher levels in *myostatin*-null muscle as compared with wild-type muscle.³¹ Similarly, Western blot analysis performed on the regenerating muscle from mice treated with Mstn-ant1 showed increased levels of MyoD during regeneration, thereby suggesting that the increase in myogenesis is the direct result of a blockade of myostatin by Mstn-ant1 (Figure 4c). In addition, Pax7, which is expressed in quiescent and proliferating cells,³³ was higher with Mstn-ant1 treatment than with saline treatment throughout the trial period, thereby suggesting a comparative increase in satellite cell number, activation and/or self renewal (Figure 4b). These

higher Pax7 and MyoD levels could be due to increased numbers of satellite cells and the subsequent myogenesis, and increased satellite cell self renewal. It is noteworthy that while similar levels of MyoD were observed in the Mstn-ant1- and saline-treated regenerated muscles at day 28, higher levels of Pax7 were seen in the Mstn-ant1-treated muscles than in the saline-treated ones. Since Pax7 is a marker for satellite cell self-renewal,^{32,39} the higher level of Pax7 indicates that Mstn-ant1 must have enhanced this self-renewal process. This is in accordance with an earlier finding that indicates that myostatin inhibits satellite cell self-renewal.²⁴ Similarly, an extended treatment with Mstn-ant1, administered to aged mice for 6 weeks, also resulted in increased MyoD and Pax7 protein levels in both gastrocnemius muscle and primary myoblasts (Figure 4f–i). Aged muscle can undergo wasting due to a loss of fibers and atrophy of existing fibers;¹ therefore a level of continued myogenesis is required for maintaining and repairing the muscle. Correspondingly, MyoD expression has been shown to be high in young animals, significantly decreasing in the adult, and later increasing again as the animal continues to age.⁴⁰ However, although the levels of MyoD and of other myogenic regulatory factors increase in the aged muscle, this does not restore the muscle's myogenic ability to the level seen in a young muscle.^{20,41,42} Because myostatin is known to reduce the expression of MyoD,²² it would be expected that myostatin would negatively regulate this age-related myogenesis. Indeed, by antagonizing myostatin, we were able to increase the level of myogenesis, as reflected by the increase in the levels of both MyoD and Pax7 protein. In support of these results, increased satellite cell activation was observed in 1- and 24-month-old single fibers when cultured in the presence of Mstn-ant1 (Figure 5c and d). Furthermore, when Mstn-ant1 was administered *in vivo* to aged mice, it was able to increase the percentage of activated satellite cells per fiber (Figure 5f). This would suggest that antagonism of myostatin through subcutaneous injections of Mstn-ant1 can provide a means to increase myogenesis, possibly by increasing satellite cell number, or by priming the satellite cells to continue along the myogenic pathway. If this is indeed the case, then potentially, antagonism of myostatin in aged animals could contribute to reversing the decline in satellite cells and/or their activation potential that has been reported to occur in the aged muscle environment.^{17,43} An increase in satellite cell activation and numbers would be expected to result in an augmentation of strength. As Figure 5g indicates, after a 6-week treatment with Mstn-ant1, grip strength showed an increase in the treated animals ($P < 0.05$).

As previously stated, a major component of the regeneration process is the inflammatory response. After injury, macrophages as well as myoblasts migrate to the site of injury in response to signaling from inflammatory cytokines and various growth factors. Corroborating the results of a study using *myostatin*-null mice,³¹ an accelerated migration and enhanced accretion of macrophages was observed with Mstn-ant1 treatment (day 2, Figure 6c), thereby suggesting that Mstn-ant1 rapidly and effectively antagonizes the inhibitory effect of myostatin on macrophage migration. Similar findings emerged from chemotaxis assays, wherein Mstn-ant1 effectively blocked the inhibitory effect of myostatin, thus rescuing the cell migration (Figure 7c). Aging was also shown to produce a significantly negative effect both on primary myoblasts

and on macrophage migration (Figure 7a and b). This could be on account of a reduction in the propensity of the 24-month-old myoblasts and macrophages to respond to their respective chemoattractants, or on account of a reduced expression of receptors. Interestingly, after 6 weeks of Mstn-ant1 treatment, the migratory ability of macrophages was restored (to an unknown extent) in aged mice (Figure 7d).

Collectively, the results presented here suggest that a short-term blockade of myostatin and its function through antagonist treatment can effectively enhance muscle regeneration in aged mice after injury and during age-related muscle wasting. The ramifications of myostatin antagonist treatment for human health are potentially extensive. The efficacy of an antagonist treatment has been demonstrated here, as only four doses of an antagonist during the critical period of regeneration after injury were sufficient to significantly improve muscle recovery. Therefore we propose that antagonism of myostatin is a viable option for treatment of deficient muscle regeneration and sarcopenia in humans, by restoring myogenic and inflammatory responses and decreasing fibrosis.

MATERIALS AND METHODS

Generation of Mstn-ant1. An *E. coli* expression system was used for producing the truncated myostatin protein. Amplified complementary DNA from the C-terminal region of myostatin was purified and subsequently inserted into the cloning vector pET 16-B (Novagen, Madison, WI), as previously described.⁴⁴ In order to generate Mstn-ant1, the complementary DNA was truncated at the amino acid 350, producing a truncated portion of the processed region. The protein was purified utilizing a nickel-nitriloacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) affinity column.⁴⁴ In order to test the purity of the antagonist, 3 μ g of it was separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA), stained with Coomassie blue, and then destained.

Animals. The wild-type mouse strain C57BL/10 was bred at the Ruakura Small Animal Colony. All animals were handled in accordance with the guidelines of the Ruakura animal ethics committee (AgResearch, Hamilton, New Zealand).

Administration of Mstn-ant1 to notexin injured mice and aged mice. On day 0, 1-year-old mice were anesthetized with 10% ketamine hydrochloride (100 mg/ml)/5% Rompun (20 mg/ml) at 0.1 ml/7 g body weight. A small incision was made over the left TA, and 10 μ l of notexin (10 μ g/ml; Venom Supplies, Australia) was injected into the muscle. On days 1, 3, 5, and 7, the antagonist treatment group received Mstn-ant1 at 6 μ g/g body weight subcutaneously, while the control group received the equivalent volume of saline. Mice were euthanized on days 1, 2, 3, 7, 10, and 28 (4–6 mice per group per day) for tissue collection. Additionally, mice aged 13–16 months were injected subcutaneously three times a week with Mstn-ant1 at 6 μ g/g body weight, or the equivalent volume of saline, for 6 weeks (10 per group). TA and gastrocnemius muscles were collected for single fiber isolations and muscle protein, respectively, while the remaining hindlimb muscle was collected for myoblast isolation. Bone marrow was also collected for macrophage cultures used in the chemotaxis assays.

Assessment of muscle regeneration and strength. TA muscles were dissected from each notexin injured mouse, weighed and frozen for protein isolation or tissue sectioning. Transverse sections (10 μ m) were cut from the midbelly region of each muscle. Muscle sections were stained with hematoxylin and eosin in order to visualize and measure unregenerated and regenerated areas. For visualizing areas of collagen deposited 10 and 28 days after injury, muscle sections were stained with Van Gieson

staining (stains collagen pink). The amount of collagen was then measured within each section as a percentage of the total section area. Muscle sections relating to days 2, 3, 7, and 10 were immunostained for Mac1, an antibody with specificity for infiltrating peripheral macrophages, as previously described.³¹ Double-blind measurements of muscle strength was measured in the aged mice injected with saline or with Mstn-ant1, for 6 weeks at the commencement of the trial and again at the completion, using a grip strength apparatus (MK-380S, Muromachi; Tokyo, Japan). The mice were pulled backwards by the tail, and the maximal force exerted by the mouse was digitally recorded. The mean of three grip tests for each animal was calculated and the mean maximal force of 10 animals per group was determined and expressed in Newtons as mean \pm SEM.

Single fiber isolation and analysis of satellite cell activation. TA muscles collected from the Mstn-ant1- or saline-injected mice were used for isolating single fibers following an adaptation of the protocol of Rosenblatt *et al.*⁴⁵ previously described.²⁸ Isolated fibers were then cultured for 24 or 48 hours at 37°C in 5% CO₂. In addition, single fibers were isolated from non-treated 1-month-old and 24-month-old mice. These fibers were cultured with or without Mstn-ant1 at 5 μ g/ml for 24, 48, or 72 hours at 37°C in 5% CO₂. After the required culturing time, fibers were immunostained for proliferating cell nuclear antigen and counterstained with 4',6-diamidino-2-phenylindole (refer to **Supplementary Data S1**). Proliferating cell nuclear antigen positive nuclei were counted as a percentage of total myonuclei in order to normalize the data and exclude any discrepancies in the lengths of the fibers analyzed.

Isolation of primary myoblasts and peritoneal- and bone marrow-derived macrophages. Satellite cells were isolated from the hindlimb muscles of the Mstn-ant1- or saline-injected mice according to published protocols.^{31,46,47} After 48 hours of culturing, the cells were collected for protein isolation. In addition, primary myoblasts were isolated from non-treated 1-month-old and 24-month-old mice for proliferation and chemotaxis assays. For a proliferation assay, the myoblasts were cultured with or without Mstn-ant1 at 10 μ g/ml for 96 hours. Cell proliferation was later assessed using a methylene blue photometric endpoint assay, as previously described.⁴⁸ Bone marrow-derived macrophages were obtained by plating bone marrow cells at 5×10^6 cells/plate in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum + 10% L929 conditioned medium (containing colony stimulating factor-1) for 5 days to induce macrophage differentiation.⁴⁹ Peritoneal macrophages were obtained by lavage of the peritoneal cavity of 1-month-old and 24-month-old non-treated mice with cold phosphate-buffered saline.

Myoblast and macrophage chemotaxis assays. Chemotaxis assays were performed in duplicate using cell culture inserts containing polyethylene terephthalate 0.8 μ m membranes (BD Biosciences, San Jose, CA), as previously described.³¹ For macrophage chemotaxis, DMEM + Zymosan A (Sigma, St. Louis, MO) activated mouse serum was used as a chemoattractant at 33, 22, and 11% (vol/vol). For myoblast chemotaxis, DMEM + 2% horse serum + 5% chicken embryo extract (optimal), DMEM + 2% horse serum (suboptimal) or DMEM + 5% chicken embryo extract (suboptimal) were used as chemoattractants. Rescue experiments were performed with either 2.5 or 5 μ g/ml of recombinant myostatin in the presence or absence of Mstn-ant1 at 5 \times the myostatin concentration. The cells were incubated for 4 and 7 hours for macrophages and myoblasts, respectively. Migrated cells were counted on four representative fields per membrane and the average number was plotted.

Protein analysis. TA muscles from notexin injured mice were homogenized in 1 ml protein lysis buffer [0.05 M Tris pH 7.5, 0.25 M NaCl, 5 mmol EDTA, Complete protease inhibitor tablet (Roche, Annheim, Germany) and 0.1% NP40]. Bradford's reagent (Bio-Rad Laboratories, Hercules, CA) was used for estimating total protein. Total protein (10 μ g) was separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to

nitrocellulose membrane (Bio-Rad) before immunostaining for MyoD or Pax7 (refer to **Supplementary Data S1**). Additionally, protein was collected from primary myoblasts²⁴ and gastrocnemius muscle isolated from the mice injected with saline or with Mstn-ant1 for 6 weeks. Total protein (15 μ g) was used for Western blot analyses of MyoD and Pax7.

Image and statistical analysis. Unregenerated/regenerated areas and collagen deposition were analyzed using an Olympus SZ-PT stereomicroscope (Olympus, Tokyo, Japan), a three charge-coupled device camera (Dage MTI, Michigan City, IN) and Scion Image software (Scion, MD). The obtained images were then measured using ImageJ software (National Institute of Health). Positive Mac1 staining was viewed under fluorescent illumination using an Olympus BX50 microscope, SPOT-RT 4.01 camera and software (Diagnostic Instruments Inc., Sterling Heights, MI), and images of each section were obtained for analysis. All data are presented as means and standard errors. Analysis of variance with Tukey test was used for determining the significance ($P < 0.05$) of data variations between groups.

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SUPPLEMENTARY MATERIAL

Data S1.

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