Cell Transplantation, Vol. 17, pp. 337–350, 2008
Printed in the USA.

0963-6897/08 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Inhibiting Myostatin With Follistatin Improves the Success of Myoblast Transplantation in Dystrophic Mice

Basma F. Benabdallah,* Manaf Bouchentouf,* Joel Rousseau,* Pascal Bigey,† Annick Michaud,* Pierre Chapdelaine,‡ Daniel Scherman,† and Jacques P. Tremblay*

*Génétique humaine, Centre de Recherche du CHUL, Québec, Canada
†Inserm, U640, CNRS, UMR8151, René Descartes Paris 5 University, Faculté des Sciences Pharmaceutiques et Biologiques, Chemical and Genetic Pharmacology Laboratory, Ecole Nationale Suprieure de Chimie de Paris, Paris, France
‡Ontogénie et reproduction, Centre de Recherche du CHUL, Québec, Canada

Duchenne muscular dystrophy is a recessive disease due to a mutation in the dystrophin gene. Myoblast transplantation permits to introduce the dystrophin gene in dystrophic muscle fibers. However, the success of this approach is reduced by the short duration of the regeneration following the transplantation, which reduces the number of hybrid fibers. Our aim was to verify whether the success of the myoblast transplantation is enhanced by blocking the myostatin signal with an antagonist, follistatin. Three different approaches were studied to overexpress follistatin in the muscles of *mdx* mice transplanted with myoblasts. First, transgenic follistatin/*mdx* mice were generated; second, a follistatin plasmid was electroporated in *mdx* muscles, and finally, follistatin was induced in *mdx* mice muscles by a treatment with a histone deacetylase inhibitor. The three approaches improved the success of the myoblast transplantation. Moreover, fiber hypertrophy was also observed in all muscles, demonstrating that myostatin inhibition by follistatin is a good method to improve myoblast transplantation and muscle function. Myostatin inhibition by follistatin in combination with myoblast transplantation is thus a promising novel therapeutic approach for the treatment of muscle wasting in diseases such as Duchenne muscular dystrophy.

Key words: Duchenne muscular dystrophy; Myoblast transplantation; Myostatin; Follistatin

INTRODUCTION

Duchenne muscular dystrophy (DMD), a severe X-linked genetic disease (5), is due to a dystrophin gene mutation, which causes an absence of dystrophin expression and is characterized by a progressive skeletal muscle atrophy (7). The absence (in DMD) or dysfunction (in Becker muscular dystrophy) of this protein leads to the myofiber membrane instability (3). Myoblast transplantation in dystrophic muscle results in the long-term restoration of this protein after the fusion of the transplanted myoblasts with each other or with damaged host muscle fibers (21). However, the success of this approach is reduced by the limited regeneration period following muscle damage produced by the intramuscular injections of myoblasts (26). This limitant factor can be bypassed by repetitive cell injections very close to each other as demonstrated previously (25).

Myostatin, a member of the transforming and growth factor-β family expressed predominantly in skeletal muscle tissue (19), is a powerful inhibitor of muscle growth (14). Indeed, mutations in the myostatin gene or inactivation of the myostatin protein in mice, cattle, and even in a human subject result in important increases in skeletal muscle mass (11,15,24). Actually, myostatin negatively regulates skeletal muscle growth by decreasing the myoblast proliferation rate and by delaying its terminal differentiation. Myostatin is also implicated in the muscle regeneration process (12,23), by blocking the satellite cell activation (17) and macrophage migration and thus the inflammatory response that occurs after muscle damage (18).

Follistatin is a secreted glycoprotein encoded by a single gene that is subject to alternative splicing. This mechanism generates two different forms of the protein: the short (FS288) and the long form (FS344). The short form of follistatin lacks the acidic tail in the C-terminal, which permits the protein to bind to the extracellular

Received June 11, 2007; final acceptance July 16, 2007.
Address correspondence to Jacques P. Tremblay, Ph.D., Unité de recherche en Génétique humaine, Centre de recherche de l'Université Laval, 2705, boulevard Laurier, RC-9300, Québec (Prov. Québec), Canada G1V 4G2. Tel: (418) 654-2186; Fax: (418) 654-2207; E-mail: Jacques-P.Tremblay@crchul.ulaval.ca

matrix and thus to be less diluted in the circulation (10). Follistatin is known to antagonize the function of myostatin (1). Indeed, experimental overexpression of follistatin in mice under a skeletal muscle-specific promoter results in muscle enlargement (15). This inhibition occurs after the formation of a latent complex between mature myostatin and follistatin, making myostatin unable to attach its specific receptor (6).

In addition, cell proliferation and differentiation are strongly influenced by protein acetylation. In skeletal muscle cells, it has been demonstrated that acetylation favors the differentiation process (8). Indeed, the inhibition of the histone deacetylase in myoblasts (C2C12 cell line and human muscle cells) results in the formation of larger myotubes with increased numbers of nuclei (8). Moreover, it has been recently reported that this enhanced fusion of myoblasts exposed to histone deacetylase inhibitor is mediated by a follistatin overexpression counteracting the myostatin activity on those myoblasts (9).

Our purpose was first to verify whether muscle function and myoblast transplantation success were improved in *mdx* mice overexpressing follistatin. We also blocked the myostatin signal in *mdx* mice with follistatin either directly by electroporating a plasmid coding for follistatin or indirectly by treating the mice with a histone deacetylase inhibitor (trichostatin A), and studied the consequences of this inhibition on myoblast transplantation and on muscle histological features.

MATERIALS AND METHODS

Animals

All the experiments were approved by the animal care committee of the CHUL (Centre Hospitalier de l'Université Laval). The *mdx* (dystrophic mouse model on a C57BL10J genetic background) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Transgenic mice overexpressing the human follistatin short form (on C57BL6J genetic background) were graciously given by Dr. Se-Jin Lee (Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD).

Transgenic Dystrophic Mouse Generation

Nondystrophic male mice overexpressing the human follistatin short form protein (hFst) were crossed with *mdx* female mice. All males in the F_1 generation were dystrophic because the dystrophin gene is carried on the X chromosome. Moreover, all those males were transgenic for the follistatin gene because the human follistatin short-form gene was located on the Y chromosome in the nondystrophic males crossed with the *mdx* females.

Follistatin Protein Quantification

A PCR for the human follistatin gene was performed on the cDNA [reverse transcribed from RNA prepared from tibialis anterior (TA) muscles of *mdx* and *mdx*/hFst). The forward primer was 5′-GCCTGCTTCCTCT GAGCAAT-3′ and the reverse primer was 5′-AGAGG GAACACCGGCCTCTC-3′. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also amplified as an internal control using 5′- GGAAGGGCT CATGACCACA-3′ as a forward primer and 5′-CCGTT CAGCTCTGGGATGAC-3′ as a reverse primer.

A Western blot on proteins prepared from TA of *mdx* or *mdx*/hFst mice and β-Gal or hFst electroporated mice was also done using a mouse anti-human follistatin antibody (R & D Systems Inc., MN, USA) and an anti-mouse horseradish peroxidase-coupled antibody (DAKO A/S, Denmark).

Rota Rod and Force Grip Assay

The Rota Rod (Columbus Instruments, OH, USA) started at stop position and accelerated at the rate of 18 revolutions per minute. Each mouse was allowed to remain on the rod until it felt off. Two trials were performed for each group of mice, and the best length of time that each mouse remained on the rod was recorded as endurance time for that animal. Mice were acclimated with the apparatus during six sessions (two sessions per week), and the represented results were recorded at the seventh session.

The automated grip strength meter (Columbus Instruments) was positioned horizontally and mice held by the base of the tail allowed to grasp the grid and were then pulled backward in the horizontal plane. The force applied to the bar at the moment the grasp was released was recorded as the peak tension [kg converted in N: force (in N) = mass (in kg) × 9.8 (N/kg)] by an electronic strain gauge. The test was repeated five consecutive times within the same session and the highest value from the five trials was recorded as the grip strength for that animal. Mice were acclimated with the apparatus during three sessions (three sessions per week), and the represented results were recorded at the fourth session. All mice were allowed to acclimate with the apparatus during 5 min before each trial. A total of four mice in each group were used in each test.

Cross-Section Area Measurements

A hematoxylin and eosin staining and an immunohistochemistry detection of mouse caveolin-3 (BD Biosciences, ON, Canada) were done on TA muscle sections from *mdx* and *mdx*/hFst mice. Each muscle section (two sections were analyzed for each group of mice) was photographed with a Nikon 4 MP camera. The total or single fiber cross-section area of each muscle was determined using Scion image software. For the single fiber cross-section area, three different zones of each section were photographed and the cross-section area of each

fiber on each muscle section was recorded; all fibers were then classified into different classes of fiber cross-section area. The percentage of fibers within each class was calculated.

Myoblast Preparation

Primary muscle cultures were prepared using a modified preplating technique from newborn (2–3 days old) C57BL/10J mice (22). Cell suspension, obtained after the enzymatic digestion of the muscle tissue, was allowed to attach for 48 h. Only adherent cells were used for the transplantations. Desmin labeling was performed on a sample of the culture to establish the percentage of myoblasts (about 70%). Cell viability was also verified using trypan blue staining.

Plasmid Electrotransfer

Six 3-month-old *mdx* mice were used for this experiment. Three mice were used for each plasmid. Forty micrograms of each plasmid (pCMV-β-Gal or pCMV-hFst) was slowly injected in a total volume of 40 μl of 0.9% sterile NaCl in TA muscles of *mdx* mice. After the intramuscular injection, electric pulses were applied using two stainless steel plate electrodes placed on either side of the hind limb (27).

Trichostatin A Treatment

Eight 2-month-old *mdx* mice were used for this experiment. Four mice were treated daily with IP trichostatin A (TSA) (Sigma Aldrich, MO, USA), and four were used as a controls and were injected daily with Hank's buffered salt solution (HBSS) (20).

Myoblast Long-Term Transplantation

Myoblasts were transplanted in TA muscles following the protocol described in Benabdallah et al. (2). Mice were immunosuppressed with daily injection of tacrolimus (FK506 at 2.5 mg/kg). In summary, 2 million myoblasts resuspended in HBSS were transplanted in each TA muscle of four *mdx* mice (4 months old) and four *mdx*/hFst mice (4 months old); all mice were immunosuppressed from the day of the transplantation and sacrificed 3 weeks after the transplantation. Myoblasts (500,000) resuspended in HBSS containing cardiotoxin (100 μg/ml) were transplanted 5 days after the electrotransfer in each electroporated (pCMV-β-Gal or pCMV-hFst) TA muscle of six *mdx* mice (3 months old). All mice were immunosuppressed from the day of the electrotransfer until their sacrifice 4 weeks later. Two million myoblasts, resuspended in HBSS containing cardiotoxin, were transplanted in each irradiated (12 Gy) TA muscle of eight mice (3 months old) administered or not with TSA. The myoblast transplantation was done 10 days after the beginning of the TSA injection. All mice were immunosuppressed from the day of the transplantation and sacrificed after 6 weeks. Dystrophin-positive fibers were revealed by immunohistochemistry as previously described (2).

Statistical Analysis

Differences among groups were statistically analyzed with an analysis of variance (ANOVA) test using the stat-view software (Brainpower, Cala-Basas, CA). A value of $p < 0.05$ was considered significant.

RESULTS

Overexpression of Human Follistatin in TA Muscles of Transgenic Dystrophic Mice

PCR and Western blot studies (data not shown) showed that the human follistatin transcript and protein were clearly overexpressed in TA muscle of transgenic dystrophic mice compared with those of nontransgenic dystrophic mice.

Improvement of Whole Body and TA Skeletal Muscle Weight in Transgenic Dystrophic Mice

Transgenic dystrophic males showed increased muscling; in fact, whole body and TA muscle weights were increased by 125% and 186%, respectively, in transgenic dystrophic males (*mdx*/hFst) relative to the same age control dystrophic animals (Fig. 1a, b).

Increasing of Cross-Section Area and Single Fiber Cross-Section Area in TA Muscle of Transgenic Dystrophic Mice

The results in Figure 1c show that the total cross-section area of whole TA muscles of transgenic dystrophic mice was increased by 1.7-fold compared to those of control mice. Moreover, single fiber cross-section areas were also bigger in *mdx*/hFst mice compared to those of *mdx* mice; this suggests that the increases of both muscle weight and whole muscle cross-section area are due in a large part to fiber hypertrophy. Indeed, the frequency histogram in Figure 1e clearly shows different size distributions in muscles from *mdx* and *mdx*/hFst mice, with a shift toward larger fibers observed in *mdx*/hFst mice. Indeed, below 900 units^2, there is a higher percentage of *mdx* than of *mdx*/hFst fibers, while above 900 units^2 there are more *mdx*/hFst fibers than *mdx* fibers. In addition, the mean fiber cross-section area is significantly higher in *mdx*/hFst TA muscle sections than in *mdx* TA muscle sections (Fig. 1f). These results are in accordance with those obtained in the nondystrophic transgenic mice overexpressing the human follistatin protein under a muscle-specific promoter (15).

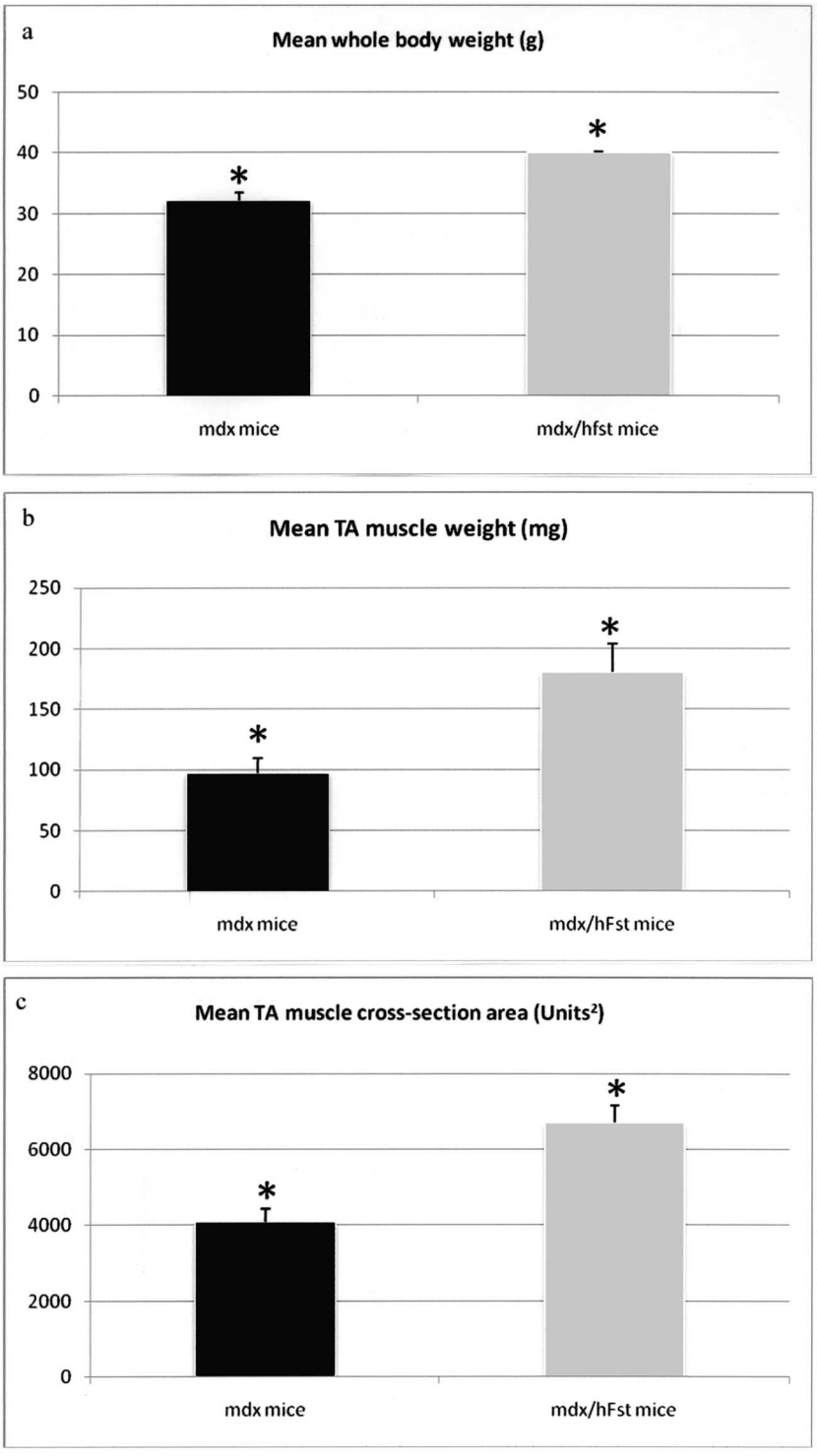

Figure 1. Consequences of the overexpression of the human follistatin in *mdx* mice on whole body weight, TA weight, and on fiber hypertrophy. (a) Means and SDs of whole body weights (in grams) of *mdx* and *mdx*/hFst mice (*$p \leq 0.05$). (b) Means and SDs of TA muscle weights (in mg) of *mdx* and *mdx*/hFst mice ($p \leq 0.05$). (c) Means and SDs of TA muscle cross-section area (in units^2) of *mdx* and *mdx*/hFst mice (*$p \leq 0.05$). (d) Immunohistochemistry of caveolin-3 on TA muscle sections of *mdx* and *mdx*/hFst mice (original magnification 200×). (e) Distributions of TA single fiber cross-section area (in units^2) of *mdx* and *mdx*/hFst mice. (f) Means and SEMs of *mdx* and *mdx*/hFst TA muscle single fiber cross-section area (in units^2) (*$p \leq 0.05$). $n = 4$.

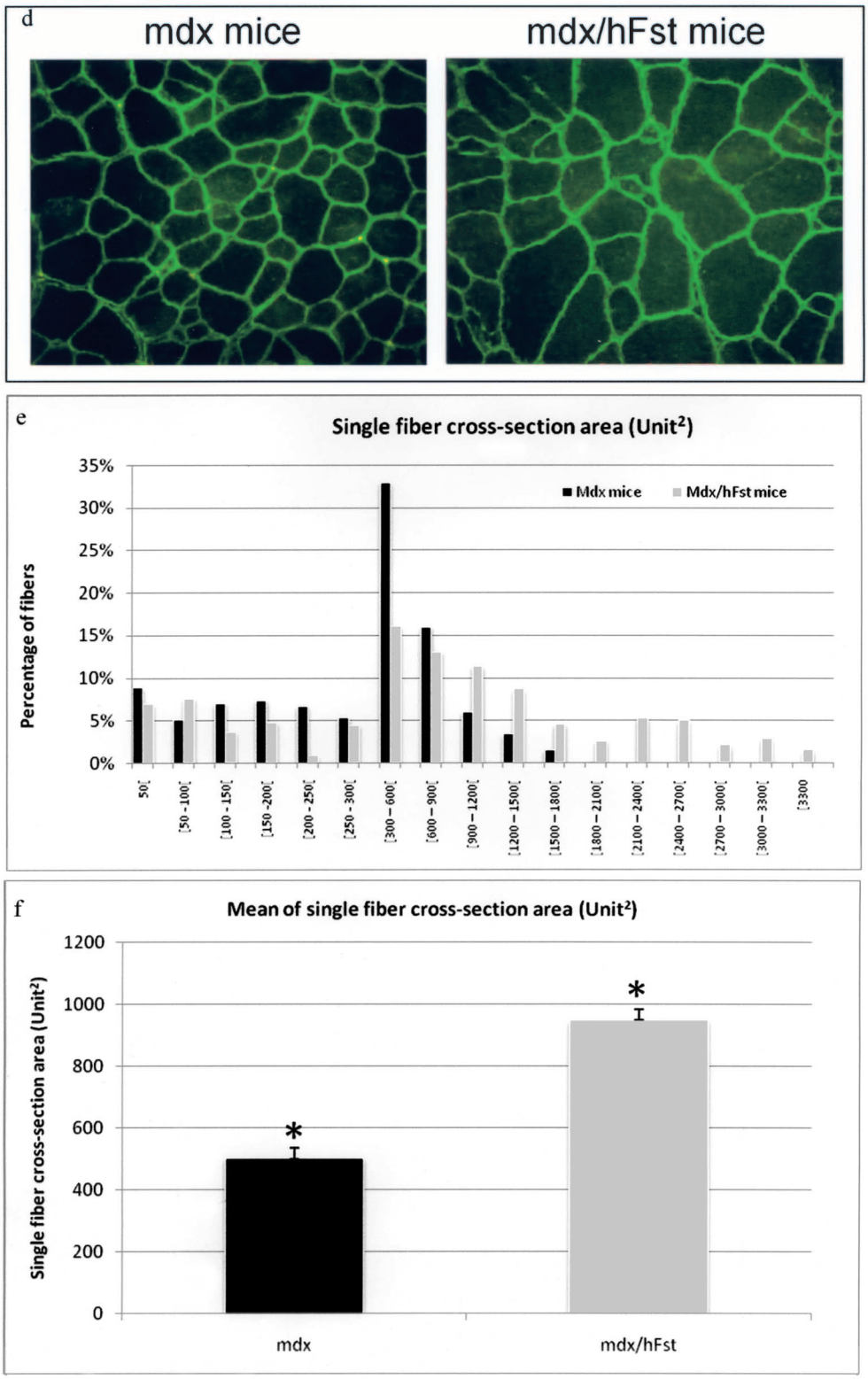

Figure 1. (*continued*)

Improvement of Transgenic Mouse Performance in the Rota Rod and Force Grip Tests

As shown in Figure 2, the *mdx*/hFst mice remained on the rotating rod longer than the *mdx* mice. Indeed, *mdx*/hFst mice fell from the rod after 15 s, while *mdx* mice fell after 7.5 s. This shows that follistatin overexpression in muscles enhanced the endurance of dystrophic mice. A force grip test was also performed on the same animals to assess their whole body strength. Figure 2b shows that *mdx*/hFst mice performed better than control *mdx* mice. Indeed, the total peak force generated by transgenic mice was about 2.4 N, while it was about 1.4 N for control mice.

Improved Myoblast Transplantation Success in Transgenic Dystrophic Mice

The number of dystrophin-positive fibers formed after normal myoblast transplantation was 2.6-fold higher in TA muscles of hFst transgenic dystrophic mice compared to TA muscles of control mice (Fig. 3). This can be explained by the fact that myostatin, normally present in skeletal muscle, is inhibited by follistatin overexpression in transgenic mice, and thus its negative effect on myogenic cell proliferation and fusion is abolished allowing an increased proliferation and fusion of the transplanted cells, thus leading to the formation of more dystrophin-positive hybrid fibers.

Efficiency of the Electrotransfer Technique

Four weeks after the electrotransfer of pCMV-β-Gal, the presence of β-galactosidase-positive fibers was verified and the results (data not shown) demonstrated that the technique was effective and safe for the animals. However, the number of β-galactosidase-positive fibers was not equal in all electroporated muscles, indicating that the effectiveness of the technique was variable. This may be due to the variability of the plasmid injections as well as the behavior of the animal during the following electric shock. Western blots against follistatin were also made on protein extracts from muscles electropora-

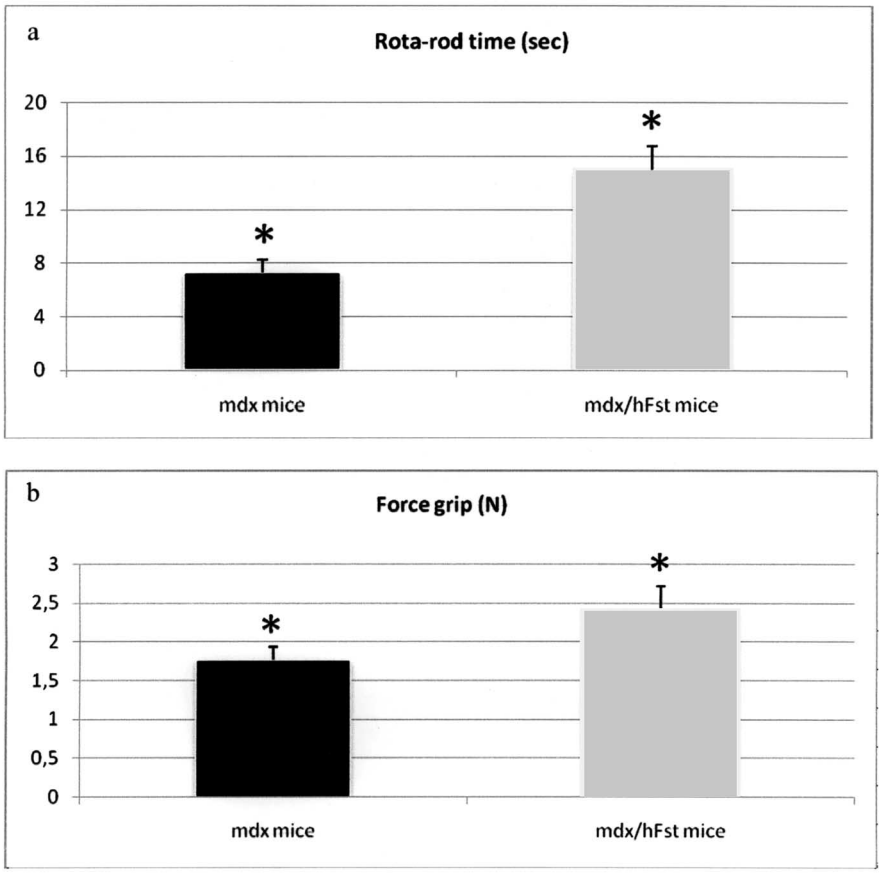

Figure 2. Improvement of transgenic mouse performance in the rota rod and force grip tests. (a) Means and SDs of endurance times (in seconds) on a rota rod of *mdx* and *mdx*/hFst mice (*$p \leq 0.05$). (b) Means and SDs of whole body strength (in Newtons) of *mdx* and *mdx*/hFst mice in a force grip assay (*$p \leq 0.05$). $n = 4$.

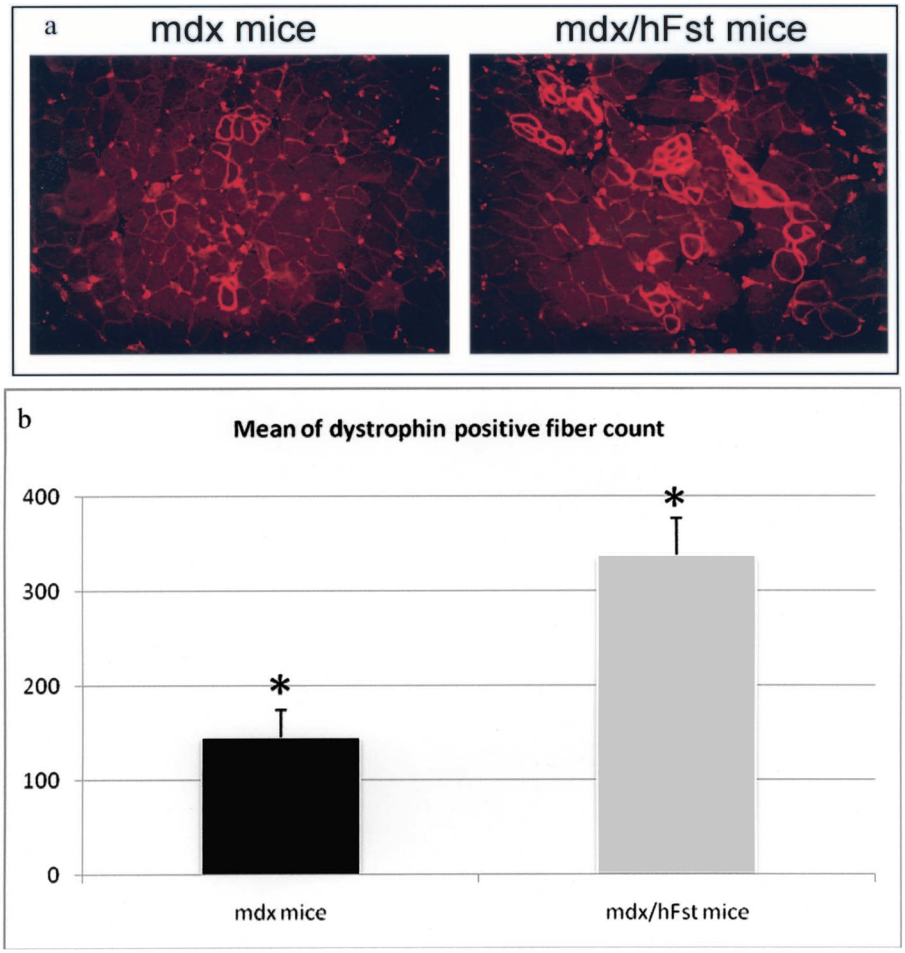

Figure 3. Enhanced myoblast transplantation success in *mdx*/hFst mice. (a) Immunodetection of the dystrophin in transplanted TA muscle sections of *mdx* and *mdx*/hFst mice (original magnification 100×). (b) Means and SDs of dystrophin-positive fiber count in transplanted TA muscle sections of *mdx* and *mdx*/hFst mice (*$p \leq 0.05$). $n = 8$.

ted with either pCMV-β-Gal or pCMV-hFst using a mAb that reacted with both mouse and human follistatin. The blots (data not shown) showed that the follistatin was clearly more abundant in the pCMV-hFst electroporated muscles than in those electroporated with pCMV-β-Gal. However, the success of the electrotransfer was variable in different muscles.

Increased Whole Body Weight, TA Skeletal Muscle Mass, and Single Fiber Cross-Section Area in Dystrophic Mice Electroporated With a Follistatin Plasmid

Overexpression of the follistatin following electrotransfer of a follistatin plasmid directly in the TA muscles of *mdx* mice induced a 20% increase of the whole body weight compared with those of mice electroporated in their TA muscles with pCMV-β-Gal plasmid (Fig. 4a). This treatment also induced a 60% increased weight of the TA muscles (Fig. 4b). An immunohistochemistry against the caveolin-3 sarcolemmal protein permitted to illustrate the presence of larger fibers in pCMV-hFst electroporated muscle sections than in the pCMV-β-Gal electroporated muscle sections (Fig. 4c). This phenomenon was confirmed by a histogram distribution and the average size of the muscle fiber area (Fig. 4d, e).

Improved Myoblast Transplantation Success Following Electrotransfer With the Follistatin Plasmid

The results in Figure 5 show that the transplantation success was improved in follistatin electroporated TA muscles. The success of the transplantation was improved by 94% after the follistatin plasmid electrotransfer compared with the control (β-galactosidase plasmid electrotransfer). Indeed, 274 dystrophin-positive fibers

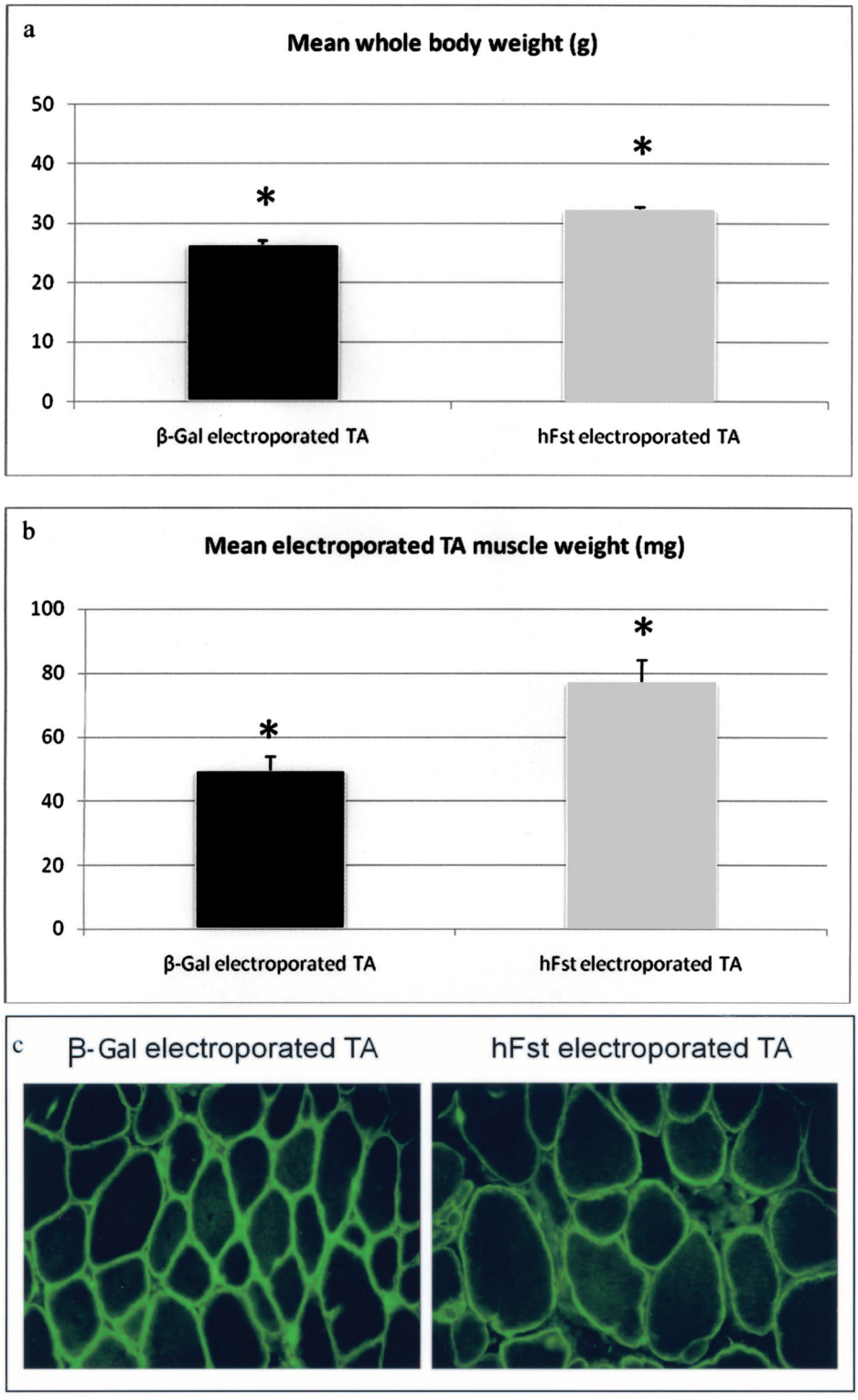

Figure 4. Increased whole body weight, TA muscle weight, and single fiber cross-section area in *mdx* TA muscles electroporated with pCMV-hFst. Mdx mice were electroporated with a plasmid coding either for β-galactosidase or for human follistatin. (a) Means and SDs of whole body weights (in grams) ($*p \leq 0.05$). (b) Mean weights (in mg) of the TA muscles ($*p \leq 0.05$). (c) Immunohistochemistry of caveolin-3 in TA muscle sections (original magnification 400×). (d) Distributions of TA muscle fiber cross-section area (in units^2). (e) Means and SEMs of the muscle fiber cross-section area (in units^2) ($*p \leq 0.05$). $n = 6$.

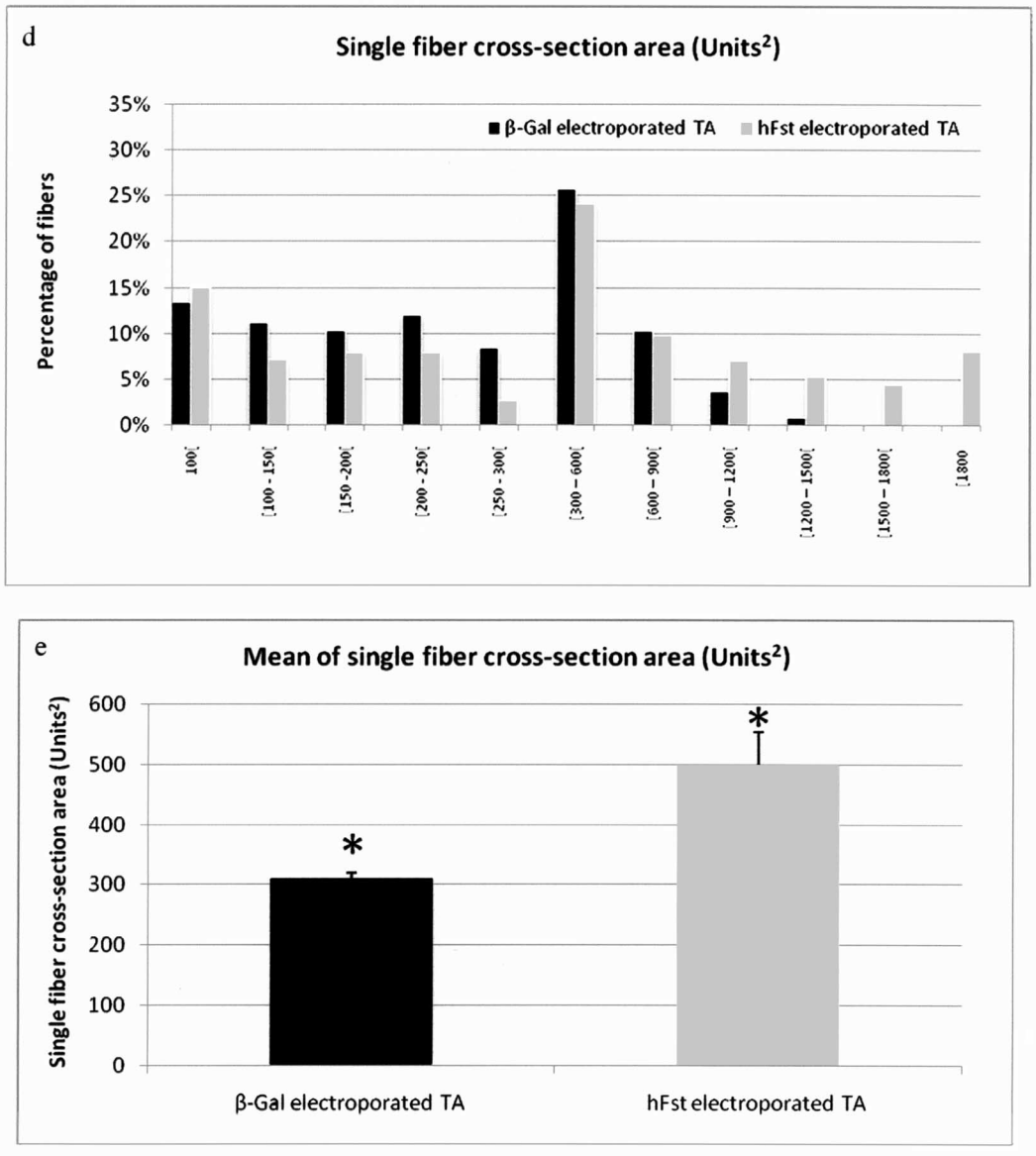

Figure 4. (*continued*)

were present in follistatin electroporated muscles, and only 142 were present in β-galactosidase electroporated muscles.

Increased Fiber Cross-Section Area in TA Muscles of mdx *Mice Treated With Trichostatin A*

Histological evaluation of TA muscles from untreated and TSA-treated *mdx* mice illustrates an increase in muscle fiber cross-section area (Fig. 6a). Frequency histogram of fiber cross-section area illustrates the presence of larger muscle fibers in TSA-treated *mdx* mice than in untreated control *mdx* mice (Fig. 6b). In addition, the mean fiber cross-section area was also slightly increased, as shown in Figure 6c.

Improved Myoblast Transplantation With the Trichostatin A Treatment

Immunohistochemistry against dystrophin permitted to evaluate the success of myoblast transplantation with and without TSA treatment (Fig. 7a). Dystrophin-positive fibers were counted in each condition. Figure 7b shows that the myoblast transplantation success was increased by 30% with TSA treatment compared to the control condition. Indeed, with TSA treatment 200 dys-

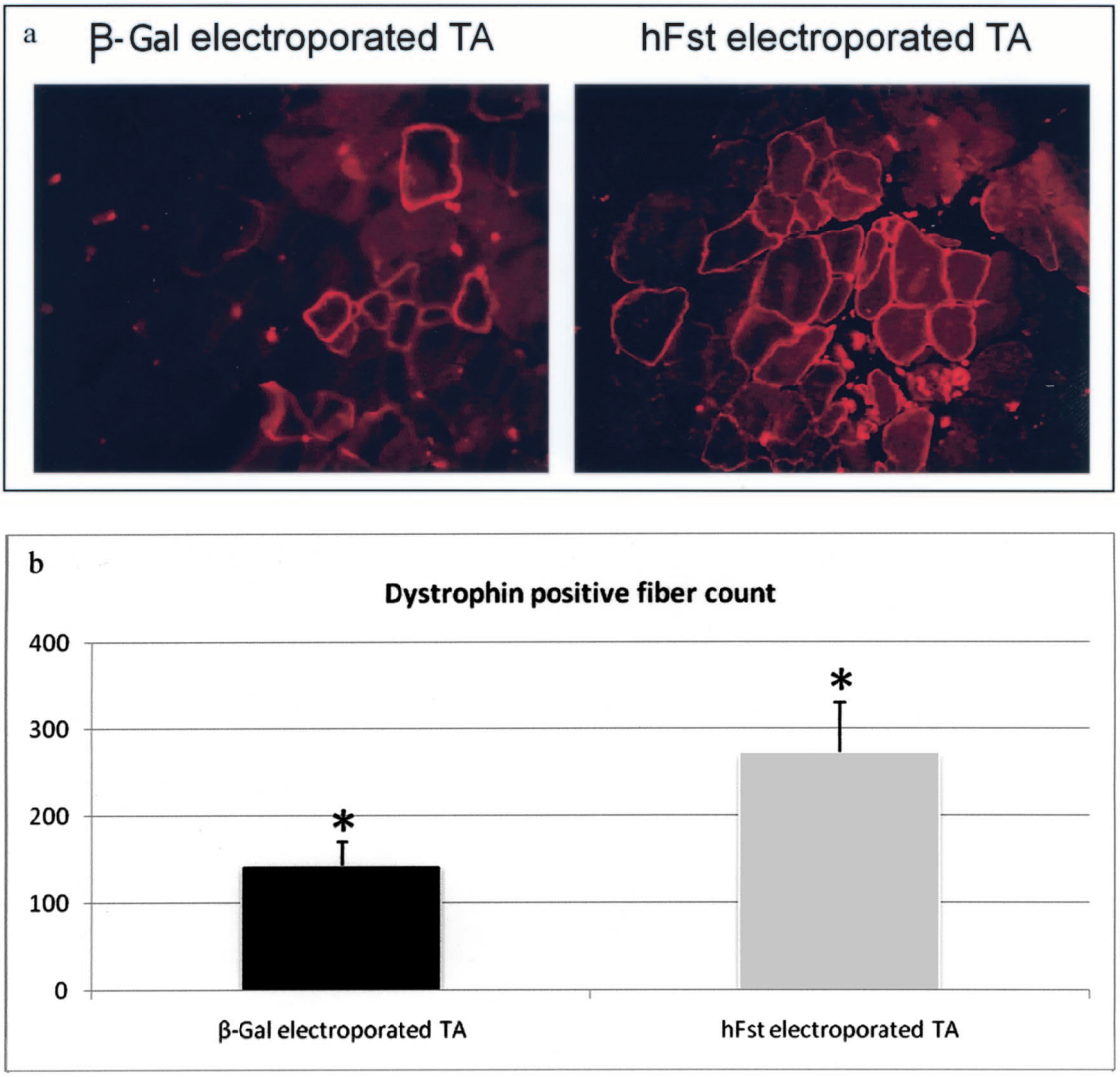

Figure 5. Improved myoblast transplantation success in *mdx* TA muscles electroporated with the pCMV-hFst plasmid. (a) Immunodetection of the dystrophin on TA muscle sections of *mdx* mice electroporated in that muscle with pCMV-β-Gal or pCMV-hFst (original magnification 200×). (b) Means and SDs of the numbers of dystrophin-positive fibers in sections of *mdx* TA muscles electroporated with pCMV-β-Gal or with pCMV-hFst plasmid (*$p \leq 0.05$). $n = 6$.

trophin-positive fibers were counted while only 150 were counted in control muscle sections.

DISCUSSION

Our results demonstrated that blocking endocrine myostatin by the induction of follistatin secretion in dystrophic mice—either by generating transgenic dystrophic mice, by a follistatin plasmid electrotransfer in transplanted muscles, or by an indirect follistatin induction in muscles via a TSA treatment—not only increased the whole body weight and the muscle mass, but also increased the success of normal myoblast transplantation.

Indeed, in the first part of the article concerning transgenic dystrophic mice overexpressing the follistatin in their muscles, we have demonstrated first that both whole body and individual TA muscle weights were increased in those transgenic dystrophic mice compared with control dystrophic (nontransgenic) mice. We think that this observation is due to the inhibition of locally present myostatin and probably other members of TGF-β family such as GDF-11, which is highly related to

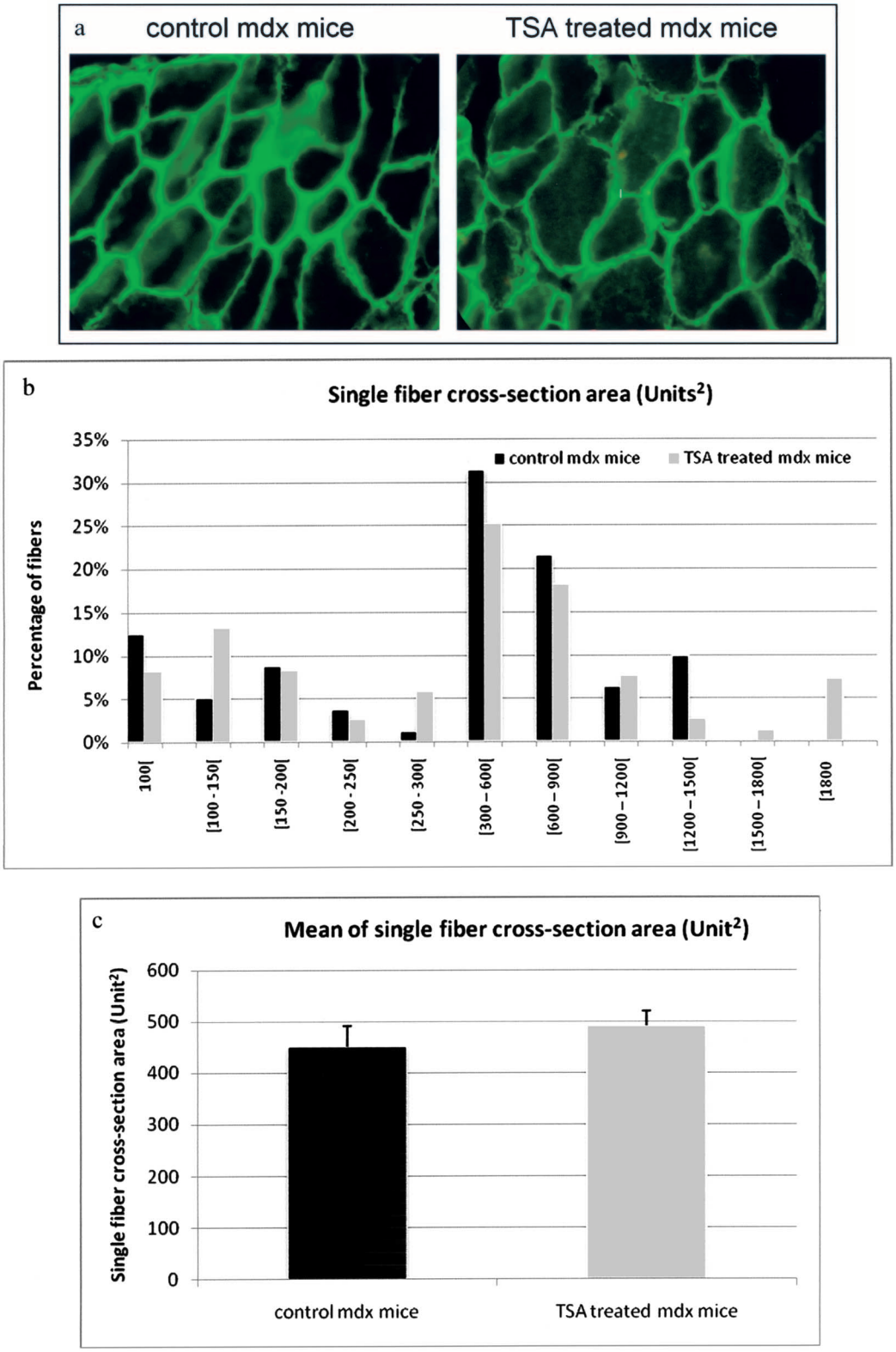

Figure 6. Effects of TSA treatment on fiber cross-section area: (a) Immunohistochemistry for caveolin-3 on TA muscle sections of control and TSA-treated *mdx* mice (original magnification 400×). (b) Distributions of TA muscle fiber cross-section area (in units^2) of control and TSA-treated *mdx* mice. (c) Means and SEMs of the results illustrated in (b). $n = 4$.

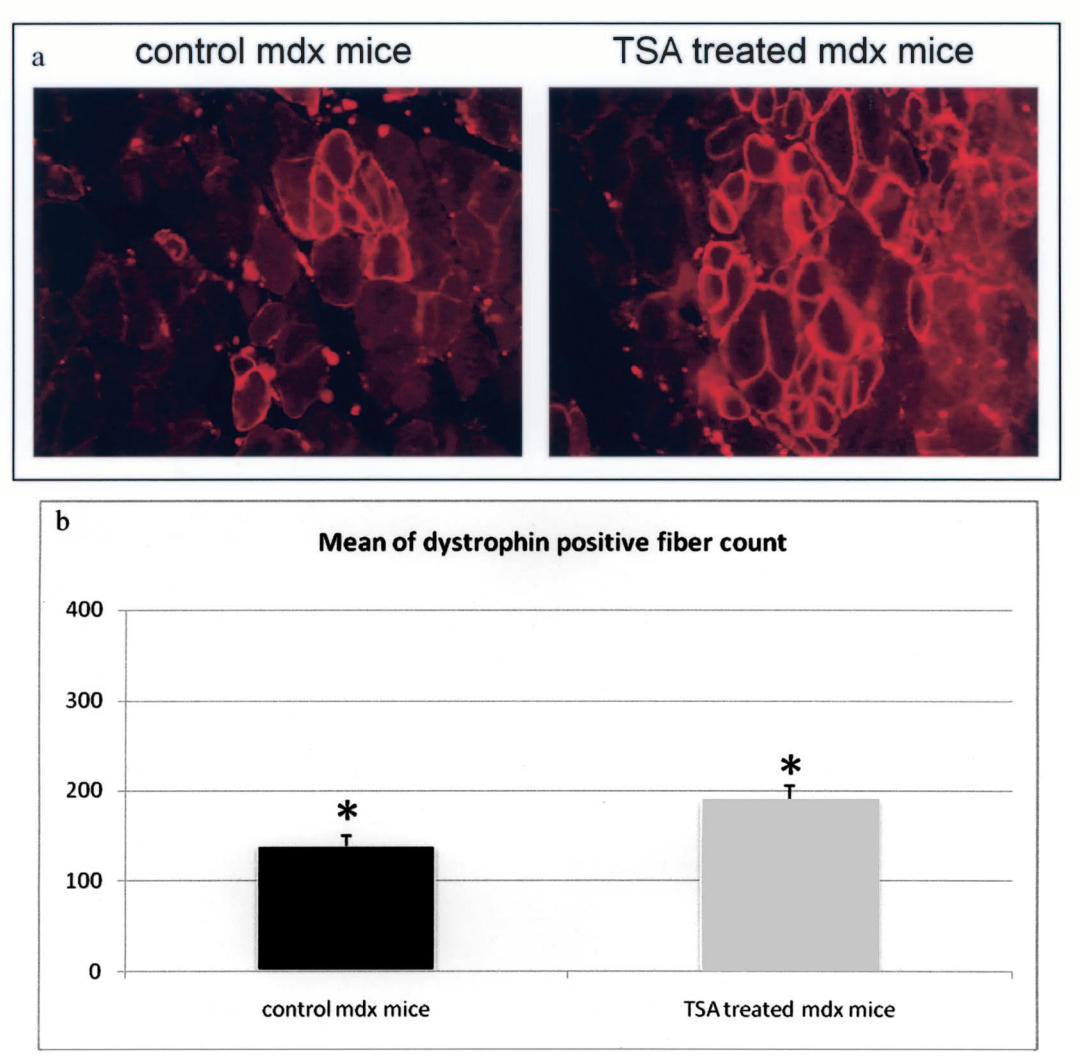

Figure 7. Improvement of the success of myoblast transplantation in *mdx* mice TA muscle with TSA treatment. (a) Dystrophin immunodetection on TA muscle sections of control and TSA-treated *mdx* mice (original magnification 200×). (b) Number of dystrophin-positive fibers on TA muscle sections of control and TSA-treated *mdx* mice. (c) Mean numbers and SDs of dystrophin-positive fibers in TA muscle sections of control and TSA-treated *mdx* mice ($*p \leq 0.05$). $n = 4$.

myostatin, by the overexpressed follistatin (15). Follistatin is known to antagonize several members of the TGF-β family, including myostatin, by its association with the active C-terminal dimer of myostatin blocking its ability to bind to receptors (13).

This augmentation in whole body and TA muscle weight is also explained in part by an hypertrophy of myofibers due to reduced myostatin suppression of the stem cell proliferation and/or differentiation (17). As transgenic mice expressing follistatin under the control of a myosin light chain promoter/enhancer (15), the follistatin transgenic dystrophic mice have an increased number of muscle fibers because there is a reduced myostatin activity early during embryonic development. We also showed that the follistatin transgenic dystrophic mice performed better in both rota-rod and force grip assays. Indeed, as *mdx* mice treated with myostatin blocking antibodies (4), *mdx* mice overexpressing the follistatin protein performed better than control mice, probably due to their increased muscle mass and strength. In addition, the grip strength of these transgenic dystrophic mice was also increased compared to control mice. Such results were also observed in *mdx* mice lacking myostatin (28).

These results demonstrate that blocking myostatin attenuates the severity of muscular dystrophy in dystrophic mice, and improves the muscle function. Finally, our results in transgenic hFst/*mdx* mice also demonstrated that follistatin overexpression in *mdx* mice enhanced the success of normal myoblast transplantation.

This result can be explained by the fact that myostatin bound to follistatin is unable to exert its negative effect on transplanted myoblast proliferation and/or differentiation. Thus, transplanted myoblasts can proliferate more in vivo before their fusion with each other or with damaged host myofibers.

The second part of our experiments concerns the study of the consequences of the induction of the follistatin in dystrophic mouse muscles by the electrotransfer technique. Follistatin electrotransfer induced an increase in whole body and TA weights in all mice compared to the control mice, and larger myofibers were observed in follistatin electroporated muscles. Again this is due to the improved proliferation and/or differentiation of transplanted myoblasts and of host activated satellite cells, in the absence of functional myostatin. Fiber hypertrophy occurs when existing fibers (in particular damaged fibers) recruit the fusion of myoblasts. This increases the number of nuclei per fiber and also the sarcoplasm volume. We also demonstrated that the success of normal myoblast transplantation was improved in TA muscles electroporated with a follistatin plasmid; the mechanism of the effect would be the same as in the follistatin transgenic mice.

The third part of our experiments treats the consequences of the administration of TSA in *mdx* mice. We did not observed any increase in the whole body weight or in individual TA muscle weight. This can be explained by the fact that the TSA treatment was not very long. However, fibers with bigger cross-section area were founded in TSA-treated mice than in control untreated mice. This concurs perfectly with the result obtained by Minetti et al., who showed that deacetylase inhibitors increased muscle cell size by promoting myoblast recruitment and fusion through induction of follistatin (20). In addition, it has been proven that recombinant myostatin blunted the TSA treatment effect, and that follistatin resumes the ability of TSA to induce myoblast recruitment in the presence of myostatin. These results demonstrate that TSA, and thus follistatin, favors the myoblast recruitment by inhibiting myostatin (9). Moreover, TSA was demonstrated to be unable to induce follistatin expression in noninjured muscles, indicating that TSA exerts its pharmacological biological activity only on activated satellite cells (9). Thus, the induced success of myoblast transplantation in *mdx* mice treated with TSA is explained by the increased proliferation and fusion index of transplanted myoblasts, because the TSA-induced follistatin blocked myostatin inhibition.

On the basis of all of the above results, we conclude that inhibition of the myostatin by its potent antagonist follistatin in combination with myoblast transplantation is a promising novel therapeutic strategy to improve the success of the treatment of genetic muscle diseases such as Duchenne muscular dystrophy as well as urinary incontinence by myoblast transplantation (16).

ACKNOWLEDGMENT: We thank Dr. Lee for giving us transgenic mice and the original human follistatin plasmid.

REFERENCES

1. Amthor, H.; Nicholas, G.; McKinnell, I.; Kemp, C. F.; Sharma, M.; Kambadur, R.; Patel, K. Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis. Dev. Biol. 270(1):19–30; 2004.
2. Benabdallah, B. F.; Bouchentouf, M.; Tremblay, J. P. Improved success of myoblast transplantation in mdx mice by blocking the myostatin signal. Transplantation 79(12): 1696–1702; 2005.
3. Blau, H. M.; Webster, C.; Pavlath, G. K. Defective myoblasts identified in Duchenne muscular dystrophy. Proc. Natl. Acad. Sci. USA 80(15):4856–4860; 1983.
4. Bogdanovich, S.; Krag, T. O.; Barton, E. R.; Morris, L. D.; Whittemore, L. A.; Ahima, R. S.; Khurana, T. S. Functional improvement of dystrophic muscle by myostatin blockade. Nature 420(6914):418–421; 2002.
5. Emery, A. E. Duchenne muscular dystrophy—Meryon's disease. Neuromuscul. Disord. 3(4):263–266; 1993.
6. Gonzalez-Cadavid, N. F.; Bhasin, S. Role of myostatin in metabolism. Curr. Opin. Clin. Nutr. Metab. Care 7(4): 451–457; 2004.
7. Hoffman, E. P.; Brown, Jr., R. H.; Kunkel, L. M. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell 51(6):919–928; 1987.
8. Iezzi, S.; Cossu, G.; Nervi, C.; Sartorelli, V.; Puri, P. L. Stage-specific modulation of skeletal myogenesis by inhibitors of nuclear deacetylases. Proc. Natl. Acad. Sci. USA 99(11):7757–7762; 2002.
9. Iezzi, S.; Di Padova, M.; Serra, C.; Caretti, G.; Simone, C.; Maklan, E.; Minetti, G.; Zhao, P.; Hoffman, E. P.; Puri, P. L.; Sartorelli, V. Deacetylase inhibitors increase muscle cell size by promoting myoblast recruitment and fusion through induction of follistatin. Dev. Cell 6(5): 673–684; 2004.
10. Inouye, S.; Ling, N.; Shimasaki, S. Localization of the heparin binding site of follistatin. Mol. Cell. Endocrinol. 90(1):1–6; 1992.
11. Kambadur, R.; Sharma, M.; Smith, T. P.; Bass, J. J. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 7(9):910–916; 1997.
12. Kirk, S.; Oldham, J.; Kambadur, R.; Sharma, M.; Dobbie, P.; Bass, J. Myostatin regulation during skeletal muscle regeneration. J. Cell Physiol. 184(3):356–363; 2000.
13. Lee, S. J. Regulation of muscle mass by myostatin. Annu. Rev. Cell Dev. Biol. 20:61–86; 2004.
14. Lee, S. J.; McPherron, A. C. Myostatin and the control of skeletal muscle mass. Curr. Opin. Genet. Dev. 9(5): 604–607; 1999.
15. Lee, S. J.; McPherron, A. C. Regulation of myostatin activity and muscle growth. Proc. Natl. Acad. Sci. USA 98(16):9306–9311; 2001.
16. Liu, Z.; Wu, Y.; Chen, B. G. Myoblast therapy: From bench to bedside. Cell Transplant. 15(6):455–462; 2006.
17. McCroskery, S.; Thomas, M.; Maxwell, L.; Sharma, M.; Kambadur, R. Myostatin negatively regulates satellite cell activation and self-renewal. J. Cell Biol. 162(6):1135–1147; 2003.

18. McCroskery, S.; Thomas, M.; Platt, L.; Hennebry, A.; Nishimura, T.; McLeay, L.; Sharma, M.; Kambadur, R. Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. J. Cell Sci. 118(15):3531–3541; 2005.
19. McPherron, A. C.; Lawler, A. M.; Lee, S. J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387(6628):83–90; 1997.
20. Minetti, G. C.; Colussi, C.; Adami, R.; Serra, C.; Mozzetta, C.; Parente, V.; Fortuni, S.; Straino, S.; Sampaolesi, M.; Di Padova, M.; Illi, B.; Gallinari, P.; Steinkuhler, C.; Capogrossi, M. C.; Sartorelli, V.; Bottinelli, R.; Gaetano, C.; Puri, P. L. Functional and morphological recovery of dystrophic muscles in mice treated with deacetylase inhibitors. Nat. Med. 12(10):1147–1150; 2006.
21. Partridge, T. A.; Morgan, J. E.; Coulton, G. R.; Hoffman, E. P.; Kunkel, L. M. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature 337(6203):176–179; 1989.
22. Qu-Petersen, Z.; Deasy, B.; Jankowski, R.; Ikezawa, M.; Cummins, J.; Pruchnic, R.; Mytinger, J.; Cao, B.; Gates, C.; Wernig, A.; Huard, J. Identification of a novel population of muscle stem cells in mice: Potential for muscle regeneration. J. Cell Biol. 157(5):851–864; 2002.
23. Roth, S. M.; Walsh, S. Myostatin: A therapeutic target for skeletal muscle wasting. Curr. Opin. Clin. Nutr. Metab. Care 7(3):259–263; 2004.
24. Schuelke, M.; Wagner, K. R.; Stolz, L. E.; Hubner, C.; Riebel, T.; Komen, W.; Braun, T.; Tobin, J. F.; Lee, S. J. Myostatin mutation associated with gross muscle hypertrophy in a child. N. Engl. J. Med. 350(26):2682–2688; 2004.
25. Skuk, D.; Goulet, M.; Tremblay, J. P. Use of repeating dispensers to increase the efficiency of the intramuscular myogenic cell injection procedure. Cell Transplant. 15(7): 659–663; 2006.
26. Skuk, D.; Tremblay, J. P. Myoblast transplantation: The current status of a potential therapeutic tool for myopathies. J. Muscle Res. Cell Motil. 24(4–6):285–300; 2003.
27. Trollet, C.; Bloquel, C.; Scherman, D.; Bigey, P. Electrotransfer into skeletal muscle for protein expression. Curr. Gene Ther. 6(5):561–578; 2006.
28. Wagner, K. R.; McPherron, A. C.; Winik, N.; Lee, S. J. Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. Ann. Neurol. 52(6):832–836; 2002.