Evaluation of Skeletal and Cardiac Muscle Function after Chronic Administration of Thymosin β-4 in the Dystrophin Deficient Mouse

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Abstract

Thymosin beta-4 (Tβ4) is a ubiquitous protein with many properties relating to cell proliferation and differentiation that promotes wound healing and modulates inflammatory mediators. We studied the effects of chronic administration of Tβ4 on the skeletal and cardiac muscle of dystrophin deficient mdx mice, the mouse model of Duchenne muscular dystrophy. Female wild type (C57BL10ScSnJ) and mdx mice, 8–10 weeks old, were treated with 150 μg of Tβ4 twice a week for 6 months. To promote muscle pathology, mice were exercised for 30 minutes twice a week. Skeletal and cardiac muscle function were assessed via grip strength and high frequency echocardiography. Localization of Tβ4 and amount of fibrosis were quantified using immunohistochemistry and Gomori’s tri-chrome staining, respectively. Mdx mice treated with Tβ4 showed a significant increase in skeletal muscle regenerating fibers compared to untreated mdx mice. Tβ4 stained exclusively in the regenerating fibers of mdx mice. Although untreated mdx mice had significantly decreased skeletal muscle strength compared to untreated wild type, there were no significant improvements in mdx mice after treatment. Systolic cardiac function, measured as percent shortening fraction, was decreased in untreated mdx mice compared to untreated wild type and there was no significant difference after treatment in mdx mice. Skeletal and cardiac muscle fibrosis were also significantly increased in untreated mdx mice compared to wild type, but there was no significant improvement in treated mdx mice. In exercised dystrophin deficient mice, chronic administration of Tβ4 increased the number of regenerating fibers in skeletal muscle and could have a potential role in treatment of skeletal muscle disease in Duchenne muscular dystrophy.

Introduction

Duchenne muscular dystrophy (DMD) is an inherited X-linked disorder with an incidence of 1 in 3,500 male births that is due to the absence of dystrophin, a large protein linking the intracellular cytoskeleton to the extracellular matrix.[1] The animal model of DMD, the mdx mouse, is genetically similar to the human deletion.[1–3] Although the underlying gene defect is the same in human and the mdx mouse, the clinical picture is quite different. The mdx skeletal muscle undergoes an early acute phase of degeneration at 3–4 weeks of age followed by a successful regeneration phase. The histopathology after this acute phase shows a relatively mild picture, although specific muscles (e.g. diaphragm) and older mice can show more severe pathology consistent with human DMD muscle at presentation (failed regeneration and fibrosis). Commensurate with the pathology, the physical symptoms of the mdx mouse tend to be relatively mild, with muscle weakness more obvious after exercise or lengthening contractions.[4–6] Mdx mice also develop decreased cardiac systolic function slowly over time. This decreased function can be measured at significant levels by non-invasive echocardiography around nine months of age.[7,8]

In order to identify new potential therapeutic agents, studies have looked at skeletal muscle gene expression profiles in mdx mice during disease progression.[9–17] Thymosin beta-4 (Tβ4) was one gene with increased expression in dystrophin deficient skeletal muscle cells and may play a role in compensatory pathways.[11,16–18] Tβ4 is a peptide of 43 amino acids that was first isolated from the thymus gland and subsequently found to be ubiquitous in nature.[19–21] Tβ4 functions mainly as an actin-sequestering molecule regulating cell migration, proliferation and differentiation.[22–25] It also promotes wound healing and
modulates inflammatory mediators.[26,27] Tβ4 was recently shown to promote cardiomyocyte migration, survival and repair in a coronary ligation model.[28]. Based on these mechanisms, we studied the effects of chronic Tβ4 administration on skeletal and cardiac muscle function in exercised dystrophin deficient mice. While we found no significant differences in muscle function, we did see significantly increased skeletal muscle regeneration in Tβ4 treated mdx mice and these regenerating fibers distinctly stained for Tβ4.

Methods
Animal Care
All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the Institutional Animal Care and Use Committee at the Children’s National Medical Center, Washington, DC and the Veterans Administration Medical Center, Washington, DC (Protocol #01079). Eight to ten week old female C57BL/10ScSn-Dmdmlox/P-mdx (mdx) and C57BL/10ScSn (wild type) mice weighing 20–30 grams were purchased from The Jackson Laboratory (Bar Harbor, Ma). All mice were housed in an individually ventilated cage system with a 12 hour light-dark cycle and received standard mouse chow and water ad libitum. All mice were rested at least 10–14 days before starting acclimations and baseline recordings.

Treatment with Thymosin Beta-4
Tβ4 (RegeneRx Biopharmaceuticals Inc., Bethesda, Md) was given via intraperitoneal injection twice weekly over a 6 month period to mdx and wild type mice at a dose of 150 μg in 200 μl PBS. Buffer was given at the same time to mdx and wild type control groups.

Treadmill Exercise
The treadmill exercise uses a common commercially available setup (Columbus Instruments, Columbus, Ohio) which employs a moving belt. We subjected all experimental mice to a 30-minute run on a horizontal treadmill at 12 m/min, twice a week. This test was performed during the morning hours twice weekly during the 6 months except in those days on which functional data was obtained.

Grip Strength Test
Grip Strength was assessed using a grip strength meter consisting of horizontal forelimb mesh and an angled hind limb mesh (Columbus Instruments, Columbus, OH). Five successful hindlimb and forelimb strength measurements within 2 minutes were recorded and normalized to body weight as previously described.[29]

Rotarod Test
Mice were trained on the Rotarod (Ugo Basile, VA, Italy) for two days before collecting data. Each acclimatization session consisted of four training sessions, 2 per day and each session lasting 120 seconds at a speed of 5 rpm. Each trial consisted of placing the mice on the rod at 10 rpm for 60 seconds (stabilizing period) followed by an acceleration from 10 rpm to 40 rpm within the first 25 seconds until the animal falls from the rod or until 180 seconds are reached. If the animals fell during the stabilizing period, they were placed back on the rod to complete the session. The total testing time is 240 seconds (60 sec stabilization time and 180 seconds test time). Each trial was done twice a day (a gap of 2 h interval between sessions) for 3 consecutive days. The latency to fall (seconds) was recorded and all six scores per mouse were averaged and recorded as latency to fall (in seconds) for each mouse.

Echocardiography
Mice were anesthetized with 1–2% isoflurane in 100% oxygen and scanning was performed over 20 minutes using a high frequency ultrasound probe (RMZ 702a, Vevo 660, VisualSonics, Toronto, Canada) as previously described.[8] Qualitative and quantitative measurements were made offline using analytic software (VisualSonics, Toronto, Canada).

Histological Evaluations
At the end of the trial, all animals were euthanized and tissue samples were taken for further testing. Histological evaluations were done by two individuals in a blinded manner using coded H&E stained slides and their results were averaged. The number of tissues examined per group varied based on tissue availability. Quantitative stereology (Olympus C.A.S.T. Stereology System, Olympus America Inc., Center Valley, PA) was used to evaluate the slides. Assessment criteria included: assessment of total fibers present, total fibers with central nuclei, total peripheral nuclei, total central nuclei, regenerating fibers (highly basophilic fibers), degenerating fibers, and inflammation (an interstitial group of more than 10 smaller inflammatory cell dark blue nuclei in a high power field) in five high power (40x) non-overlapping fields in normal and mdx gastrocnemius muscle sections. Fibers intersecting the left and top borders of the field where not counted and nuclei further than one nuclear diameter from the fiber border were deemed “central”.[29]

For immunohistochemistry, tissue slides of untreated mdx gastrocnemius skeletal muscle were deparaffinized and hydrated. For antigen retrieval, slides were immersed in citrate buffer (0.01 M, pH 6.0) and heated twice in a microwave (700 W or high) for 5 min. The slides were quenched with endogenous peroxidase by incubation with 3% hydrogen peroxide solution for 5 minutes and washed three times in PBS for 5 minutes. Slides were then immunostained with rabbit polyclonal antibody to thymosin β4 (1:2000 dilution; ALCPCO Diagnostics, Windham, NH, USA) at 4°C overnight. After primary antibody incubation, slides were washed three times in PBS for 5 minutes and incubated with secondary antibody for 1 hour. Then, slides were washed four times in PBS for 5 minutes each and the color reaction was developed with DAB and slides were counterstained Meyer’s hematoxylin (DAKO, Carpinteria, CA, USA) for 20 seconds, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Quantification of Fibrosis
Using gastrocnemius, diaphragm and cardiac muscle tissue from treated and untreated wild type and mdx mice, five paraffin embedded sections for each group were stained with hematoxylin and eosin (H&E) (Sigma, St. Louis, Mo) and Gomori’s Tri-Chrome stain containing: fast green FCF, chromotrope 2R, and phosphotungstic acid (Sigma, St. Louis, Mo). The tissue was imaged under a light microscope at 10X and digital images were obtained using computer software (Olympus C.A.S.T. Stereology System, Olympus America Inc., Center Valley, PA). The digital images were copied into NIH Image J program and threshold set to separate blue staining collagen from red staining muscle tissues. The total area of blue staining collagen was then expressed as a percent of total tissue area in the image.
Creatine Kinase (CK) Determination

Blood was obtained by heart puncture immediately after euthanasia. 250 μL of blood was collected into eppendorf tubes, allowed to clot and kept at room temperature to allow clot-contraction prior to centrifugation and serum collection. CK determination was performed according to the manufacturer’s instructions using standard spectrophotometric method with enzyme-coupled assay reagent from Fisher Scientific (CK10).[30] Absorption at 340 nm was measured every min for 2 min at 37°C to calculate enzyme activity. Duplicate measurements were done on each serum sample and the data was expressed as U/L.

Statistical Analysis

Measurements between wild type and mdx mice were compared at each time point using an analysis of variance with Sidak adjustment for multiple comparisons (body weight, GSM, Rotarod, echocardiography, percent collagen). Normality of each quantitative measurement was confirmed prior to analysis and those not conforming to normality underwent data transformations. Histology measurements (degeneration fibers, regenerating fibers, inflammation, calcification, and central and peripheral nuclei) were first compared between two investigators to determine their consistency. Comparisons were then made using Poisson regression or using negative binomial regression where the Poisson model did not fit the data due to over dispersion.

Results

Both wild type and mdx mice were treated with Tβ4 for 6 months and the behavioral data was collected at baseline (3 months of age), mid trial (5–6 months of age), and end of the trial (9 months of age). All results are presented as mean ± standard deviation except those in figures 1 and 2.

Body Weight

No significant differences were seen in body weights between treated and untreated mice within the same strain. When comparing mdx and wild type mice, there was a significant increase in the body weight of mdx mice at baseline and 6 months but not at 9 months of age (Table S1).

Grip Strength

Untreated mdx mice had significantly decreased normalized forelimb grip strength (kilogram force per kilogram; KGF/kg) compared to untreated wild type mice at 3, 6 and 9 months of age. Comparing the normalized hindlimb grip strength of these same groups showed a significant decrease in mdx mice at 3 and 6 months but not at 9 months of age. There were no significant differences in normalized forelimb or hindlimb grip strength between treated and untreated mice within the same strain during the trial (Table S1).

Rotarod

There were no significant differences in latency time to fall on the Rotarod apparatus between wild type and mdx mice. There were also no differences between treated and untreated mice within the strain, but treated mdx mice showed significantly lower performance in comparison with treated wild type mice at 9 months of age (Table S1).

Echocardiography

High frequency echocardiography found decreased cardiac function, measured as percent shortening fraction, in untreated mdx (27.9±1.86%) mice compared to untreated (30.6±2.6%) and treated (32.0±5.2; p = 0.045) wild type mice (Figure 1). Treated mdx (26.2±3.1%) mice also showed significantly decreased cardiac function compared to treated (p<0.01) and untreated (p<0.05) wild type mice. There were no significant differences between treated and untreated mice within the same strain. No significant differences were found in measurements of left ventricular chamber size or wall thickness between mdx and wild type mice, showing no dilation in the hearts of mdx mice with decreased function.

Figure 1. Significantly decreased cardiac function (mean ± SEM) measured as percent shortening fraction (%SF) in mdx mice is seen after 6 months of treatment with thymosin-beta 4 compared to wild type mice. There is no significant difference between treated and untreated mdx mice. doi:10.1371/journal.pone.0008976.g001

Figure 2. Significantly increased percent collagen (mean ± SEM) for cardiac (n = 3 for treated and untreated mdx mice and untreated wild type, n = 4 for treated wild type; panel A), diaphragm (n = 5 for all groups; panel B), and gastrocnemius (n = 3 for all groups; panel C) is seen in mdx mice compared to wild type. There was no significant difference between treated and untreated mdx mice. doi:10.1371/journal.pone.0008976.g002
function. No significant differences were found in heart rates or Doppler measurements of aortic, pulmonary, tricuspid or mitral blood flow velocities between treated and untreated mdx mice (Table S2).

**Skeletal Muscle Histology**

Evaluation of the gastrocnemius skeletal muscle histology found significantly increased number of regenerating fibers in treated mdx mice (11.6±13.5) compared to untreated mdx mice (2.6±1.1; p=0.03). Both treated and untreated mdx mice had increased central nuclei, central nuclei per fiber and central nucleated fibers compared to treated and untreated wild type. Untreated mdx mice showed significantly increased total peripheral nuclei compared to treated mdx mice (p=0.014). There was also significantly increased inflammation (3+) between mdx and wild type mice that was not significantly altered in the treated groups (Table S3).

**Quantification of Fibrosis**

Using Gomori’s tri-chrome staining, an analysis of percent collagen showed significantly increased collagen found in the left ventricles of untreated (3.83±0.9%) and treated (4.39±1.2%) mdx mice compared to both untreated (1.6±1.1%) and treated (1.82±0.9%) wild type controls (all p values<0.05) (Figures 2 and 3). The diaphragm also showed significantly increased percent collagen in the untreated (22.9±7.5%) and treated (25.0±6.6%) mdx mice compared to untreated (7.4±0.8%) and treated (7.9±1.3%) control mice (all p values<0.01). The gastrocnemius also showed significantly increased percent collagen in untreated mdx mice (6.25±1.7%) compared to untreated control mice (3.0±1.1%; p<0.05) (Figure 2). There were no significant differences in percent collagen between treated and untreated groups within the same strain.

**Serum Creatine Kinase**

There was a significant increase in serum creatine kinase in both treated (5205±1785 U/L, n=8) and untreated (5788±2494 U/L, n=10) mdx mice compared to treated (141±108 U/L, n=14) and untreated (85±75 U/L, n=13) wild type controls (p<0.001). There was no significant difference between treated and untreated mdx mice.

**Tβ4 Localization Using Immunohistochemistry**

Staining of untreated wild type and mdx skeletal muscle (gastrocnemius) with anti-Tβ4 antibody shows localized staining in regenerating fibers. Sequential slides were stained for desmin, a marker for regenerating fibers, and this staining corresponded to Tβ4 staining. There was no staining of fibers with either anti-Tβ4 or anti-desmin antibodies in wild type tissue (Figure 4).

**Discussion**

We completed a six month trial using Tβ4 in exercised mdx and wild type mice. We found no significant improvement in treated mdx skeletal or cardiac muscle function compared to untreated mdx mice. However, we did find significantly increased regenerating fibers in treated mdx skeletal muscle and these fibers convincingly stained for Tβ4. While Tβ4 led to increased regeneration in mdx skeletal muscle, it did not improve fibrosis in the cardiac, diaphragmatic or skeletal muscle of treated mdx mice. This study shows that chronic Tβ4 administration is beneficial for skeletal muscle fiber regeneration in dystrophin deficient mice.

Previous gene profiling experiments showed increased Tβ4 expression in skeletal muscle of mdx mice. Tseng et al. (2002) showed that in 16 week old mdx mice, the gastrocnemius muscle showed a two-fold increase in Tβ4 mRNA expression. Boer et al. (2002) showed that another member of the thymosin family with similar properties, thymosin beta-10, showed a 4-fold increased expression in 13–15 week old mdx gastrocnemius muscle compared to wild type.[9] Nakayama et al. (2004) found up-regulation of Tβ4 in 2 month old mdx hindlimb skeletal muscle cell culture and showed that it was not altered after the addition of micro-dystrophin to the

![Figure 3. Gomori’s tri-chrome stained slides of cardiac tissue showing increased fibrosis in mdx mice. A) Untreated wild type cardiac tissue showing minimal collagen staining (light blue color) corresponding to a percent collagen of 1.82±0.5%. B) Untreated mdx cardiac tissue showing diffuse fibrosis in the LV and RV ventricular walls corresponding to a percent collagen of 3.83±0.5%. C) Tβ4 treated wild type mice showing few increased areas of collagen staining corresponding to a percent collagen of 1.6±0.5%. D) Tβ4 treated mdx cardiac tissue showing large areas of collagen staining in the LV and RV walls corresponding to a percent collagen of 4.39±0.7%. (LV – left ventricle, RV – right ventricle). doi:10.1371/journal.pone.0008976.g003](#)

![Figure 4. Peroxidase staining of regenerating fibers using anti-Tβ4 antibodies in skeletal muscle (gastrocnemius). A) Mdx muscle treated with anti-Tβ4 antibody shows peroxidase staining of regenerating fibers (*); B) Mdx muscle treated with anti-desmin antibody shows staining in regenerating fibers (#), the same as in plate A; C) Wild type muscle treated with anti-Tβ4 antibodies shows no staining; D) Wild type muscle treated with anti-desmin antibodies shows no staining. doi:10.1371/journal.pone.0008976.g004](#)
increased cardiac and renal perivascular fibrosis.[38] Pokharel et al. (2004) also showed that in rats over-expressing angiotensin-converting enzyme, which decreases levels of Ac-SDKP, there was increased cardiac collagen content.[39] Thus, the chronic exposure of cardiac and skeletal muscle to Tβ4 may lead to a down-regulation of Tβ4 expression or receptor activity and a decrease in Ac-SDKP.

Although we did not directly measure Tβ4 levels in treated mice, a previous study showed significantly increased levels in the hearts and skeletal muscle of mice after treatment with 400 micrograms of Tβ4 via intraperitoneal injection.[40] Chronic exposure of Tβ4 could potentially lead to the development of anti-Tβ4 antibodies. These antibodies could neutralize Tβ4 and prevent any beneficial effects on cell survival and decreased fibrosis. The presence of any antibodies was not assessed in this study.

This study found a significant increase in Tβ4 positive regenerating fibers in the skeletal muscle of exercised mdx mice. There were no beneficial effects of chronic Tβ4 treatment on muscle function or fibrosis. This study provides histological correlation for previous gene expression studies showing the importance of Tβ4 in skeletal muscle regeneration.

Supporting Information
Table S1 Body weight, normalized grip strength, and Rotarod latency to fall measurements in treated and untreated wild type (BL10) and mdx mice after 6 months of treatment with thymosin beta-4. Found at: doi:10.1371/journal.pone.0008976.s001 (0.05 MB DOC)
Table S2 Cardiac M-mode and spectral Doppler echocardiography measurements in treated and untreated wild type (BL10) and mdx mice after 6 months of treatment with thymosin beta-4. Found at: doi:10.1371/journal.pone.0008976.s002 (0.05 MB DOC)
Table S3 Gastrocnemius skeletal muscle histology measurements in treated and untreated wild type (BL10) and mdx mice after 6 months of treatment with thymosin beta-4. Found at: doi:10.1371/journal.pone.0008976.s003 (0.04 MB DOC)

Author Contributions
Conceived and designed the experiments: CFS EH KN. Performed the experiments: CFS HJC AS GP EP ADG EH KN. Analyzed the data: CFS HJC AS GP EP ADG EH KN. Contributed reagents/materials/analysis tools: CFS HJC GP EP HGD. Wrote the paper: CFS ADG HGD KN.

References
1. Hoffman EP, Brown RH Jr, Kunkel LM (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51: 919–928.
2. Ryder-Cook AS, Sicinski P, Thomas K, Davies KE, Worton RG, et al. (1988) Localization of the mdx mutation within the mouse dystrophin gene. EMBO J 7: 3017–3021.
3. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, et al. (1989) The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science 244: 1575–1580.
4. Anderson JE, Bresalier RH, Ocalle WK (1988) Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. J Muscle Res Cell Motil 9: 499–515.
5. De Luca A, Pierro S, Lantiono A, Cetrone M, Camerino C, et al. (2003) Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. J Pharmacol Exp Ther 304: 453–463.
6. Lefaucheur JP, Pastoret G, Sebille A (1995) Phenotype of dystrophinopathy in old mdx mice. Anat Rec 242: 70–76.
7. Quinlan JG, Hahn HS, Wong BL, Lorenz JN, Wernich AS, et al. (2004) Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings. Neuromusc Diord 14: 491–496.
8. Spurny CF, Knochel S, Pirotta EE, Nagaraju K, Martin GR, et al. (2008) Dystrophin-deficient cardiomyopathy in mouse: expression of Nox1 and Lox are associated with fibrosis and altered functional parameters in the heart. Neuromusc Diord 18: 371–381.
9. Boer JM, de Meijer EJ, Mank EM, van Ommen GB, den Dunnen JT (2002) Expression profiling in stably regenerating skeletal muscle of dystrophin-deficient mdx mice. Neuromusc Diord 12 Suppl 1: S118–124.
10. Marotta M, Ruiz-Roig C, Sarria Y, Peire JL, Nuñez F, et al. (2009) Muscle genome-wide expression profiling during disease evolution in mdx mice. Physiol Genomics 37: 119–132.
11. Nakayama Y, Nara N, Kawakita Y, Takeshima Y, Arakawa M, et al. (2004) Cloning of cDNA encoding a regeneration-associated muscle protease whose
expression is attenuated in cell lines derived from Duchenne muscular dystrophy patients. Am J Pathol 164: 1773–1782.

12. Porter JD, Khamma S, Kaminis HJ, Rao JS, Merriam AP, et al. (2002) A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. Hum Mol Genet 11: 263–272.

13. Porter JD, Merriam AP, Leathy P, Gong B, Feuerman J, et al. (2004) Temporal gene expression profiling of dystrophin-deficient (mdx) mouse diaphragm identifies conserved and muscle group-specific mechanisms in the pathogenesis of muscular dystrophy. Hum Mol Genet 13: 257–269.

14. Porter JD, Merriam AP, Leathy P, Gong B, Khamma S (2003) Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (mdx) mice. Hum Mol Genet 12: 1813–1821.

15. Rouger K, Le Cunff M, Steenman M, Potier MC, Gibelin N, et al. (2002) Global/temporal gene expression in diaphragm and hindlimb muscles of dystrophin-deficient (mdx) mice. Am J Physiol Cell Physiol 283: C773–784.

16. Tseng BS, Zhao P, Patterson JS, Gordon SE, Granchelli JA, et al. (2002) Regenerated mdx mouse skeletal muscle shows differential mRNA expression. J Appl Physiol 93: 537–545.

17. Turk R, Sterrenburg E, de Meijer EJ, van Ommen GJ, den Dunnen JT, et al. (2003) The thymosins. Prothymosin alpha, parathymosin, and beta-thymosins: structure and function. Vitam Horm 66: 257–296.

18. Weber A, Nachmias VT, Pennise CR, Pring M, Safer D (1992) Interaction of thymosin beta4 with muscle and platelet actin: implications for actin sequestration in resting platelets. Biochemistry 31: 6179–6185.

19. Oates K, Goldstein A (1995) Thymosin. In: DeVita VT, Hellman S, Rosenberg SA, eds (1995) Biologic Therapy of Cancer. Philadelphia: JB Lippincott. pp 841–852.

20. Pantalone D, Carlier MF (1993) How profilin promotes actin filament assembly in the presence of thymosin beta 4. Cell 75: 1007–1014.

21. Reti R, Kwon E, Qiu P, Christopherson PL, Wheeler M (2007) Thymosin beta 4 increases hair growth by activation of hair follicle stem cells. FASEB J 18: 385–387.

22. Safer D, Elzinga M, Nachmias VT (1991) Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem 266: 4029–4032.

23. Safer D, Golla R, Nachmias VT (1990) Isolation of a 5-kilodalton actin-sequestering peptide from human blood platelets. Proc Natl Acad Sci U S A 87: 2536–2540.

24. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

25. Spurney CF, Gordish-Dressman H, Guerrou AD, Sali A, Pandey GS, et al. (2009) Preclinical drug trials in the mdx mouse: Assessment of reliable and sensitive outcome measures. Muscle Nerve 39: 591–602.

26. Mora CA, Baumann CA, Paino JE, Goldstein AL, Badamchian M (1997) The thymosins. In: DeVita VT, Hellman S, Rosenberg SA, eds (1995) Biologic Therapy of Cancer. Philadelphia: WB Saunders Co. pp 682–689.

27. Porter JD, Merriam AP, Leathy P, Gong B, Khamma S (2003) Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. Invest Ophthalmol Vis Sci 45: 1095–1100.

28. Reti R, Kwon E, Qiu P, Wheeler M, Safer D (2008) Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. Exp Eye Res 72: 605–608.

29. Safer D, Elzinga M, Nachmias VT, Pennise CR, Pring M, Safer D (1992) Interaction of thymosin beta 4 with muscle and platelet actin: implications for actin sequestration in resting platelets. Biochemistry 31: 6179–6185.

30. Tietz N (1982) Fundamentals of clinical chemistry. Philadelphia: WB Saunders Co. pp 682–689.

31. Sosne G, Siddiqui A, Kupkakis-Wheater M (2004) Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. Invest Ophthalmol Vis Sci 45: 1095–1100.

32. Safer D, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

33. Sosne G, Siddiqui A, Kupkakis-Wheater M (2004) Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. Invest Ophthalmol Vis Sci 45: 1095–1100.

34. Safer D, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

35. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

36. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

37. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

38. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

39. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.

40. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, et al. (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res 74: 293–299.