Muscle Excursion Does Not Correlate with Increased Serial Sarcomere Number after Muscle Adaptation to Stretched Tendon Transfer

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ABSTRACT: Chronic skeletal muscle stretch typically increases serial muscle fiber sarcomere number. Since serial sarcomere number correlates with functional excursion in normal muscle, observed changes in sarcomere number are often extrapolated to their new assumed function. However, this has not been well demonstrated experimentally. Thus, we measured the functional properties of muscles stretched due to tendon transfer surgery. Muscle active and passive length–tension curves were measured 1 week and 4 weeks after surgery, and then each muscle was further examined to determine structural adaptation as well as single fiber and fiber bundle passive mechanical properties. We found a disconnect between the functional and structural muscle properties. Specifically, muscle excursion was significantly lower in the transferred muscle compared to controls, even though serial sarcomere number had increased. Furthermore, maximum tetanic tension was significantly reduced, though the two groups had similar physiological cross sectional areas. Passive tension increased in the transferred muscle, which was deemed to be due to proliferation of extracellular matrix. These data are the first to report that muscle morphological adaptation after chronic stretch does not accurately predict the muscle's functional properties. These data have significant implications for examining muscle physiological properties under surgical interventions. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 30:1774–1780, 2012

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The sarcomere is the functional unit of muscle contraction and its length determines isometric tension because sarcomeres generate tension via the interactions between thick and thin filaments. Classic morphological experiments demonstrated that skeletal muscles can adapt to chronic length change by altering serial sarcomere number. In one of the most widely cited models in which the soleus was stretched by immobilization in ankle dorsiflexion, serial sarcomere number increased by ~20%, which allowed maximum tension to be generated in the position of immobilization (dorsiflexion).[1,2] These results were interpreted to suggest the general concept that muscles adjust serial sarcomere number to reset sarcomere length to the length of the immobilization.

While soleus muscle adaptation appears to be robust, it may not generally apply to all muscles that experience chronic length changes. Indeed, muscles are chronically stretched in various orthopedic surgeries, such as tendon transfers and limb lengthening. Intraoperative measurements revealed that, during tendon transfer surgeries, transferred muscles are overstretched on average to the point where the muscles produce <30% of their maximum active tension.[3] Furthermore, limb lengthening may occur to such a degree that muscle fibers might be stretched several times longer than their initial length.[4] Surgeons assume that muscles will adapt to the stretch leading to functional recovery. However, no functional measurements after muscle adaptation have been reported. Therefore, investigating muscle functional adaptation to chronic length changes has profound clinical significance.

For normal skeletal muscle, strong support exists for the idea that serial sarcomere number is proportional to muscle excursion[5] and/or contraction velocity,[6] while physiological cross-sectional area (PCSA) is proportional to maximum tetanic tension (P_o).[7–9] However, these relationships were experimentally measured in normal muscles. To address the relationship between serial sarcomere number adaptations and adaptations in muscle function, the purposes of this study were to determine the correlation between serial sarcomere number adaptation and muscle function in a previously validated model of tendon transfer[10,11] and to understand the structural basis for muscle passive tension after transfer.

MATERIALS AND METHODS

Experimental Tendon Transfer

All experiments were performed in accordance with the Institutional Animal Care and Use Committee of the University of California and Veterans Administration, San Diego. Randomly assigned hindlimbs of male New Zealand White rabbits (body mass = 2.54 ± 0.31 kg, $n = 20$) were used for tendon transfer operations; the contralateral hindlimb served as a control. Surgical procedures were performed as previously described.[11] Briefly, the extensor digitorum muscle to the second toe (EDII) was exposed, and its distal tendon was transferred to the ankle extensor retinaculum (Fig. 1). The EDII was stretched to the specific sarcomere length (L_s) of 3.7 μm that was chosen to be outside the functional L_s range. It is also close to the end of the descending limb of the L_s-tension curve in rabbit muscle,[12] similar to the L_s typically chosen in clinical tendon transfer surgery.[3] The L_s was measured intraoperatively using a laser diffraction device

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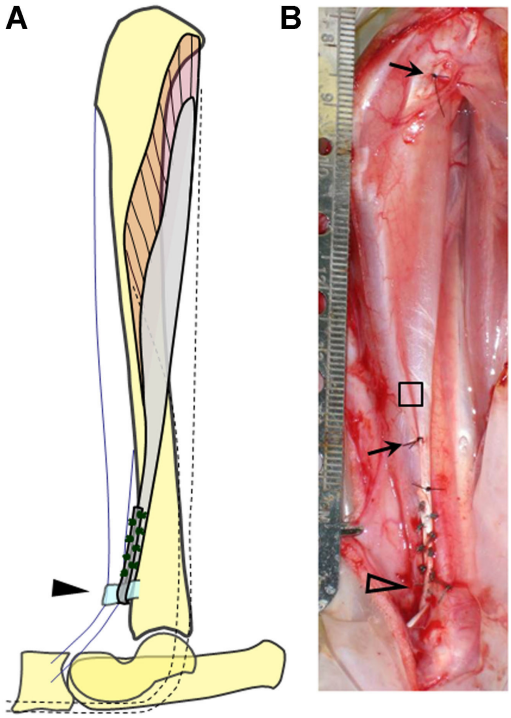

Figure 1. (A) Drawing of the tendon transfer operation. The distal tendon of the extensor digitorum muscle of the 2nd toe (normal course shown in dotted lines) was translocated into the extensor retinaculum (solid lines with arrowhead). (B) Surgical field after completion of the transfer. The EDII was transferred at the muscle length where sarcomere length was intentionally set to 3.7 μm, which was measured intraoperatively at the most distal fiber bundle (square). Suture markers (arrows) allowed tracking length changes of the muscle.

(Myogenesis, Inc., Del Mar, CA). To permit stress–relaxation of the muscle and tendon, the L_s measurement was performed 1 min after the applied stretch. The diffraction pattern was converted to L_s using the equation: $n\lambda = L_s \sin\theta$, where λ, θ, and n are the laser wavelength (632.8 nm), diffraction angle, and diffraction order, respectively.[13] The diffraction order was measured with a digital caliper, and caliper measurement error (0.05 mm) corresponded to a sarcomere length resolution of ~0.01 μm.

Muscle Active and Passive Tension Measurement

Animals were re-anesthetized 1 week ($n = 11$) or 4 weeks ($n = 9$) after transfer. After exposing the EDII muscle, the hindlimb was secured with Steinmann pins to a custom-made jig. Then, the distal tendon of the EDII muscle was transected and clamped to a servomotor (Cambridge Model 310B, Aurora Scientific, Ontario, Canada) at the distal muscle-tendon junction. The clamp was aligned with the force-generating axis and adjusted at the initial muscle length measured prior to tenotomy at 90° of both knee and ankle joints. Muscle tetanic contraction was evoked using a 650-ms train of 100-Hz stimulation via a cuff electrode placed on the proper nerve branch. Passive tension was measured as the resting muscle tension at each length measured during the 100-ms time period prior to muscle contraction. Muscle temperature was maintained at 37°C with a care to keep the muscle moist. Two-minute rest intervals were interposed between contractions to minimize fatigue effects. Tension measurements were initiated at the initial muscle length and were carried out alternating between shorter and longer muscle lengths in ~0.5-mm increments to minimize order effects. Muscle length and tension were recorded at each length using a data acquisition board (610E series, National Instruments, Austin, TX) and a custom-written LabView program (Fig. 2). Active tension was calculated by subtracting the passive tension from the corresponding total tension. Upon completion of bilateral testing, animals were euthanized and both muscle samples were processed for further analyses.

Determination of Serial Sarcomere Number and PCSA

A fresh muscle bundle ~2-mm wide and 10-mm long was excised from the middle of each EDII muscle for fiber bundle and single fiber measurements (see below). The remainder of each muscle was fixed in 10% buffered formaldehyde, while initial muscle length was maintained. Muscle architecture was determined as described previously.[14,15] Fiber lengths were measured on six samples per muscle. Sarcomere length of each fiber bundle was determined by laser diffraction. Serial sarcomere number was calculated by dividing fiber length by sarcomere number for each fiber. PCSA (cm^2) was calculated according to the equation: $(M \cos\theta)/(\rho L_{f(N)})$, where M, θ, ρ, and $L_{f(N)}$ represent muscle mass (g), fiber pennation angle, muscle density (1.056 g/cm^3), and fiber length (cm) normalized to a sarcomere length, respectively.

Fiber Bundle and Single Fiber Measurements

The fresh muscle bundle taken from the EDII was immediately immersed in chilled relaxing solution[16] to measure passive mechanical properties of fiber bundles and single fibers ($n = 9$ for week 1 and $n = 8$ for week 4). Fiber bundle and single fiber segments were dissected from the muscle bundle in the chilled solution under a microscope (Leica MZ8, Heerbrugg, Switzerland). The dissected segment was

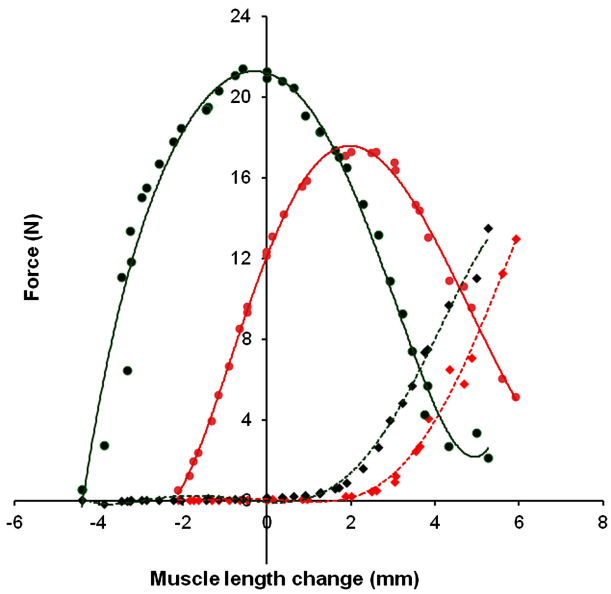

Figure 2. Representative active (solid lines) and passive (dashed lines) muscle length–tension curves from the transferred (red) and contralateral (black) muscles. Zero muscle length corresponded to the initial length.

secured on each side to 150-μm-diameter titanium wires using 10-0 silk suture loops. One wire was attached to an ultrasensitive force transducer (Model 405, Aurora Scientific) and the other to a micromanipulator as previously described.[16] Mounted segments were first set to "slack length," which is the length where the segment did not have a curved appearance in any plane and the tension measured was within a standard deviation of the noise level of the force transducer. Major and minor diameters were measured to compute cross-sectional area (CSA) assuming an elliptical cross section. The segment was stretched in 250-μm increments with a 2-min stress relaxation period after each stretch; then L_s and tension were recorded. Segments were elongated until mechanical failure. Elastic modulus was calculated as the slope of the segment's stress–strain relationship, and raw data were converted into elastic modulus to account for CSA. The elastic moduli of segments were presented within the predetermined L_s range (2.5–4.0 μm), which excluded extreme sarcomere lengths. Mechanical measurements from fiber bundles reflect the properties of both the fiber itself and the extracellular matrix (ECM) between fibers, which is mostly endomysium.[17] Measurements were performed on three different segments for both fiber bundles and single fibers from each muscle.

Data Analysis

For active and passive muscle length–tension curves, each data set was plotted and fit with quintic polynomials (Fig. 2, all r^2 values were >0.95). P_o was defined as the maximum value calculated from the polynomial equation for active tension. The muscle length where the muscle generated P_o was defined as optimal muscle length. Then, each muscle length–tension curve was expressed relative to the individual P_o. Functional muscle excursion was determined as a range of muscle lengths where the muscles were mathematically predicted to generate active tension >50% of P_o.[5] The elastic modulus of the muscle was calculated as the slope of the muscle length–stress curve up to 4 mm longer than the optimal muscle length.

Paired *t*-tests were used for comparison between transferred and control muscles. One-way ANOVA followed by post-hoc *t*-test was used in analysis of fiber bundle and single fiber experiments. Linear regression was used for correlation between serial sarcomere number and isometric contractile data. All data except body mass are expressed as mean ± standard error of means (SEM).

RESULTS

Entire Muscle Active and Passive Length–Tension Relationships

The shape of the active muscle length–tension curve demonstrated an ascending limb, plateau, and descending limb. P_o for transferred muscles (12.34 ± 1.01 N) was significantly lower than controls (18.38 ± 1.00 N, $p < 0.001$, Fig. 3A) 1 week after transfer. Four weeks after transfer, P_o of the transferred muscle (17.37 ± 1.32 N) was still significantly lower than the control (20.70 ± 0.78 N, $p < 0.05$, Fig. 3B), despite some improvement from week 1. Active length–tension relationships normalized to P_o enabled comparison of muscle excursions between the muscles. The excursion of transferred muscle (5.53 ± 0.19 mm) was significantly shorter compared to control muscle (6.66 ± 0.16 mm, $p < 0.001$, Fig. 3C) 1 week after transfer and 4 weeks after transfer (5.89 ± 0.14 mm for the transferred muscle compared to 6.63 ± 0.15 mm for the control muscle. $p < 0.001$, Fig. 3D).

The passive muscle length–tension curves were shifted to shorter muscle lengths with a steeper inclination in the transferred muscle both 1 week and 4 weeks after transfer compared to the control muscle (Fig. 3A,B). Moreover, passive tension of the transferred muscle began to rise at shorter than the optimal muscle lengths 1 week after transfer. Passive tensions in the transferred muscle were larger at 1 week than at 4 weeks, indicating that passive tension was increased within the first week and then decreased in the following period. The transferred muscle's elastic modulus was >5 times stiffer compared to control muscle at 1 week (772.6 ± 106.3 kPa vs. 148.3 ± 26.5 kPa, $p < 0.001$) and >3 times stiffer at 4 weeks (513.0 ± 64.4 kPa vs. 160.5 ± 22.7 kPa, $p < 0.001$).

Correlations between Function and Serial Sarcomere Number and PCSA

Surprisingly, although mechanically measured muscle excursion of the transferred muscle *decreased*, the transferred muscle actually *increased* serial sarcomere number by ~20% 1 week after transfer (4,192 ± 142 for the transferred muscle vs. 3,543 ± 61 for the control, $p < 0.001$, Fig. 4A) and ~13% 4 weeks after transfer (4,127 ± 149 for the transferred muscle vs. 3,670 ± 89 for the control, $p < 0.01$). Regression analysis revealed no significant correlation between serial sarcomere number and excursion ($r = 0.373$, $p = 0.10$ for control. $r = 0.246$, $p = 0.30$ for transferred, Fig. 4B).

Whereas P_o was lower in the transferred muscle, PCSA of the transferred muscle did not differ from that of control (Fig. 4C). P_o was linearly correlated with PCSA in both the control ($r = 0.458$, $p < 0.05$) and transferred ($r = 0.473$, $p < 0.05$) muscle although these relationships were not strong. Specific tension (P_o/PCSA) of the transferred muscle (194.9 ± 12.6 kPa) was about 30% lower compared to control muscles (282.9 ± 12.5 kPa, $p < 0.001$), indicating decreased intrinsic force-generating capacity of transferred muscles.

Fiber Bundle and Single Fiber Measurements

In agreement with whole muscle passive properties measured, elastic properties of fiber bundles from transferred muscles were stiffer compared to controls muscles both 1 week and 4 weeks after transfer. Elastic modulus of fiber bundles from transferred muscles was ~90% higher 1 week (280.3 ± 32.6 kPa) and ~70% higher 4 weeks after transfer (310.4 ± 24.8 kPa) compared to control muscles (146.9 ± 16.7 kPa and 179.0 ± 17.4 kPa, respectively, Fig. 5A, $p < 0.001$). No differences were found in bundle elastic

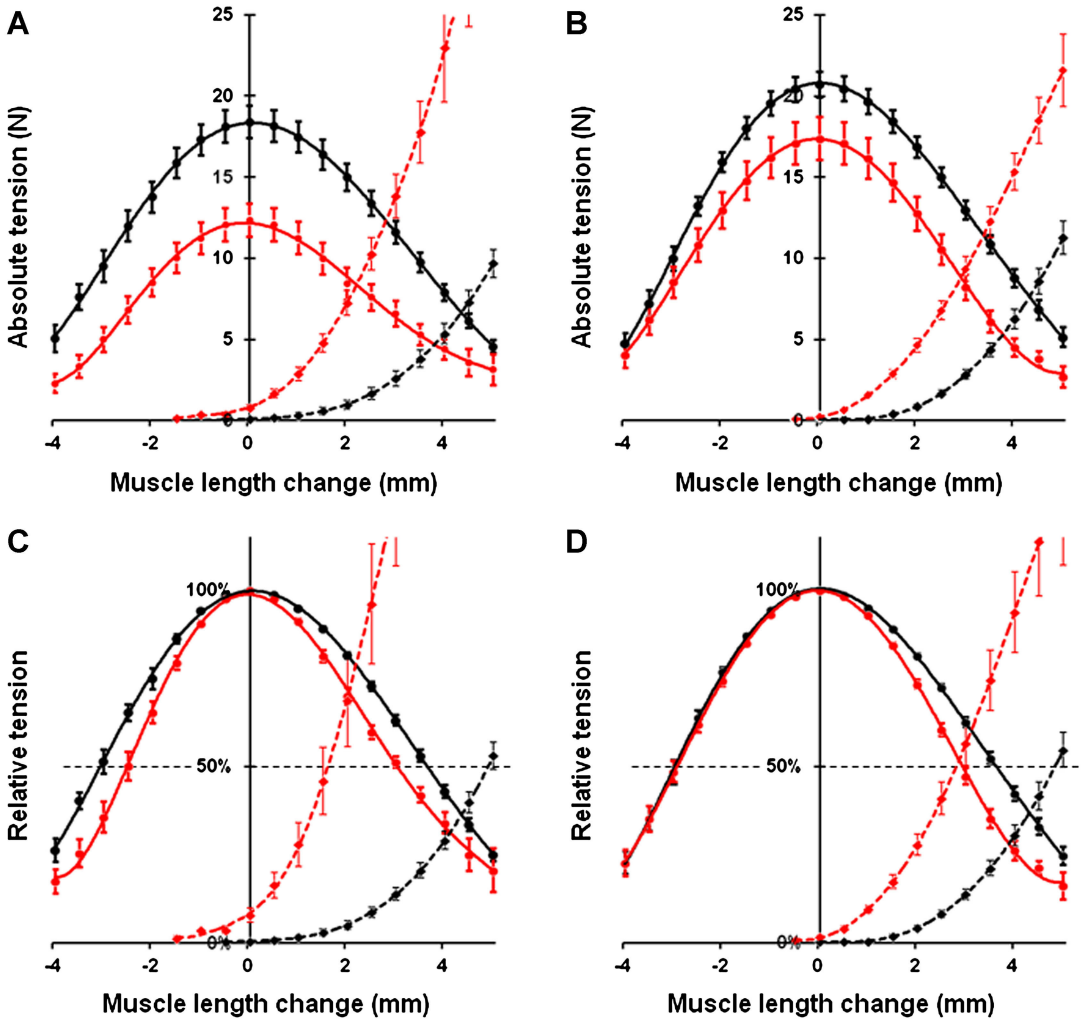

Figure 3. Average active (solid lines) and passive (dashed lines) length–tension relationships for the transferred EDII muscle (red lines) and the control (black lines) at 1 week (A) and 4 weeks (B) expressed on absolute scale. *x*-axes indicate muscle length change from the optimal muscle length (mm); *y*-axes designate absolute tension (*N*). Error bars represent SEMs among animals. Length–tension relationships at 1 week (C) and 4 weeks (D) expressed relative to P_o. Muscle excursions were compared using widths of the length–tension relationships, where the muscle could generate >50% of P_o. Error bars represent SEMs among relative tension curves.

modulus between the two times within either transferred or control muscle. In contrast to the elastic properties of fiber bundles, the properties of single fibers in transferred muscles varied less between groups. The elastic modulus of single fibers from transferred muscle (206.4 ± 12.0 kPa) was significantly higher at 1 week compared to the corresponding control muscle (141.0 ± 11.1 kPa. Fig. 5B, $p < 0.001$) but not at 4 weeks (178.5 ± 13.4 kPa for transferred and 156.0 ± 14.2 kPa for control). As a control to make sure specimen size was not a confounding effect, we found that neither single fiber CSA nor fiber bundle CSA, the latter of which is totally depending on the dissection procedure, were statistically different between groups. Thus, measurement of elastic properties was not confounded by differences in segment CSA among muscle groups.

DISCUSSION

Our most important finding was that, despite the dramatic serial sarcomere number *increase* induced by tendon transfer, muscle excursion *decreased*. In addition, P_o decreased despite no change in muscle PCSA. These results are surprising in light of general teachings suggesting that serial sarcomere number and PCSA correlate linearly with muscle excursion and P_o, respectively.[7–9] Our results may alter thinking regarding muscle adaptation because muscle adaptation is typically measured morphologically, and then morphological adaptations are used to infer function.

It is difficult to explain the mechanistic basis for these results. Since the EDII muscle produced new sarcomeres in series within such a short period of time, one possible explanation for the lower than

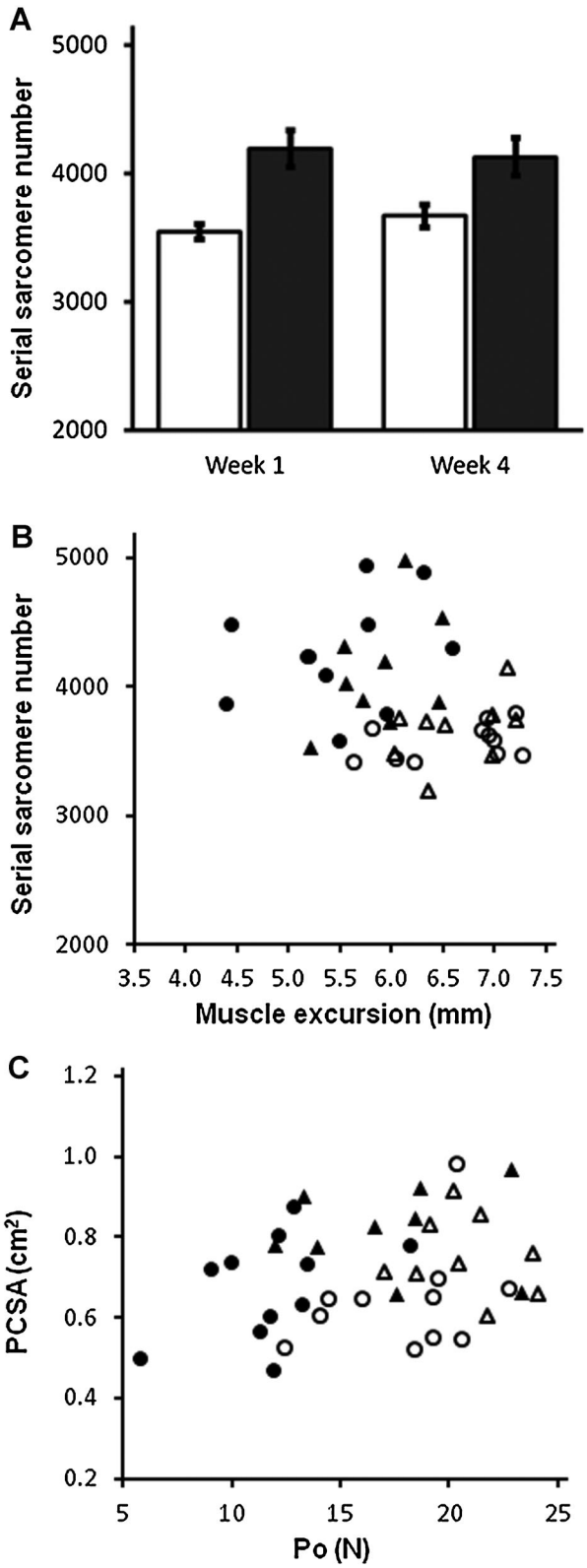

Figure 4. Serial sarcomere number of the control (open bars) and transferred muscles (filled bars) at two time points determined by muscle architectural study (A). Serial sarcomere number (*y*-axis) plotted against muscle excursion determined according to the range of length–tension relationship more than half P_o (*x*-axis) for the control (open symbols) and transferred (filled symbols) muscles at 1 week (circles) and 4 weeks (triangles) after transfer (B). Scatter plot of P_o (*x*-axis, N) versus PCSA (*y*-axis, cm^2; C). Symbols are the same as in (B).

expected excursion is that the newly synthesized sarcomeres were not yet functional, and these less-functional sarcomeres might negatively affect the entire muscle contractile function. Similarly, the process of sarcomere synthesis and integration may require local increases in stiffness and stability that negatively impact excursion and increase passive mechanical properties. In addition, this study demonstrated that muscle excursion was still shorter at 4 weeks after transfer, at which serial sarcomere number was still larger. Dramatic intramuscular transformation and reorganization, such as myosin isoform transition, occurs by 4 weeks after the onset of altered muscle use.[18–20] Since additional intramuscular alterations during the following period were also described, contractile properties might have improved at still later time points. Further experiments are needed to understand the mechanism of muscle structure–functional adaptation after sarcomerogenesis and whether this model represents a stable functional adaptation.

We also demonstrated that muscles became stiffer after transfer, during the time sarcomerogenesis was occurring. Interestingly, it took only 1 week for muscle to increase passive tension, which was nearly maintained by 4 weeks. Considering our results, the passive tension increase of the transferred muscle was likely provided mainly through proliferation of ECM between muscle fibers, which is formed by the collagen mesh network,[21,22] and perhaps through alteration in intracellular components. The elastic modulus of the entire muscle was much higher than that of the fiber bundle for the transferred muscles, but about the same for control muscles. This indicates that perimysium and epimysium, which are not incorporated in the fiber bundle preparation, became stiffer in the transferred muscles. Comparing the elastic moduli of single fiber, fiber bundle, and the entire muscle, the contribution of intracellular components to the entire passive tension increase appears to be negligible. Proliferation of ECM is observed in muscles undergoing surgical limb distraction,[23] in the regenerating muscle after injury[24] and in dystrophic muscle,[25] during which time the structural alterations exacerbated muscle contractile function. Though we did not directly measure ECM content, the biological processes underlying our study may be the same as those in other studies—muscle regeneration including sarcomerogenesis might concomitantly stimulate proliferation of ECM resulting in muscle fibrosis. The increased passive tension might be beneficial to protect muscle fibers from further injury produced by the applied stretch. Restoration of passive tension to the pre-operative level would take a long time because the ECM that consists of chemically stable collagen is remodeled less readily.[26] We speculate that the rapidly produced ECM has a less organized collagen fibril network whose roles are to provide elasticity to accommodate muscle fiber and stiffness to transmit tension, and that this ECM impairs both the muscle fiber contraction itself and

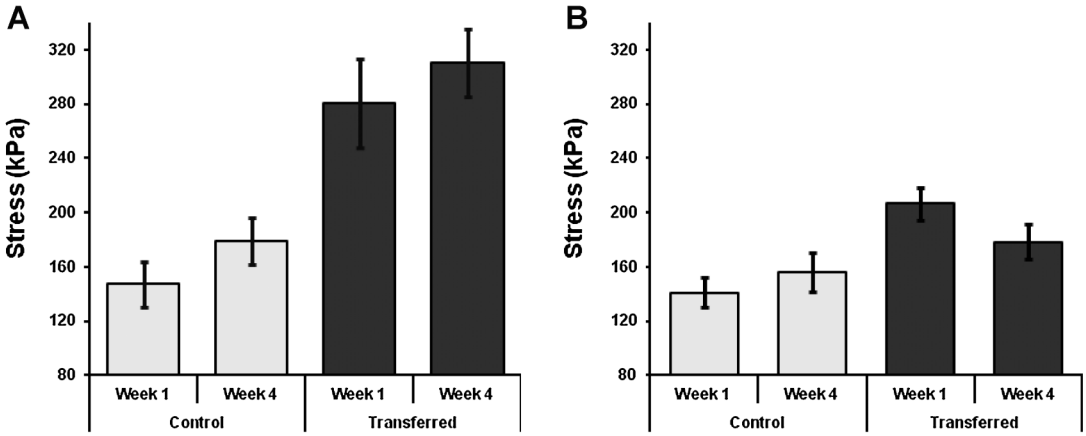

Figure 5. Elastic modulus of fiber bundles (A) and single fibers (B). Values from the control and transferred EDII muscles are expressed by open and filled bars, respectively. *y*-axes represent stress value (kPa).

force transmission, resulting in both decreased muscle excursion and reduced P_o.

Limb reconstructive surgeries, which force the muscles to stretch and adapt to new functional demands, are commonly practiced clinically. However, functional outcomes in terms of muscle contractile properties are not always satisfactory in those procedures.[27,28] Although morphological muscle adaptation was observed in various experimental conditions and even clinically,[29] our study suggests that this does not necessarily predict functional muscle adaptation. If this type of adaptation occurs in humans, it will be important to control the mechanical changes in the transferred muscles. Reduction in intramuscular fibrous tissue is associated with better functional recovery in muscle injury and muscle dystrophy.[30–32] Increased expression of ECM components is correlated with a decrease in myogenic differentiation in vivo,[33] and the presence of collagen interferes differentiation of myogenic cells in vitro.[34] Thus, modulation of ECM may lead to functional muscle adaptation that correlates with ECM morphology.

In conclusion, we present the first report of muscle morphological adaptation after applied stretch that does not predict its functional adaptation, specifically for the relationship between serial sarcomere number and muscle excursion. Increased passive tension, caused by proliferation of ECM was a characteristic feature of the transferred muscle. The proliferation of ECM may be related with impaired tension generating capacity and reduced muscle excursion, and ultimately represent a target for therapeutic intervention to optimize functional muscle adaptation.

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