Biological and Biomechanical Changes in Muscle Contractures

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Relevant References (references shown in bold attached to handout)

1. **Normal muscle has a stereotypical sarcomere length operating range.**
   
   
   
   
   
   
   
   

2. **Contracture have shortened muscles with highly stretched sarcomeres:**

   
   

3. **Contractures contain muscles with altered extracellular matrix and deranged transcriptional regulation:**


Intraoperative Measurement and Biomechanical Modeling of the Flexor Carpi Ulnaris-to-Extensor Carpi Radialis Longus Tendon Transfer

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Sarcomere length was measured intraoperatively in five patients undergoing tendon transfer of the flexor carpi ulnaris (FCU) to the extensor carpi radialis longus (ECRL) for radial nerve palsy. All measurements were made with the elbow in 20 deg of flexion. Prior to tendon transfer, FCU sarcomere length ranged from 2.84 ± .12 µm (mean ± SEM) with the wrist flexed to 4.16 ± .13 µm with the wrist extended. After transfer into the ECRL tendon, sarcomere length ranged from 4.82 ± .11 µm with the wrist flexed (the new longest position of the FCU) to 3.20 ± .09 µm with the wrist extended, resulting in a shift in the sarcomere length operating range to significantly longer sarcomere lengths (p < 0.001). At these longer sarcomere lengths, the FCU muscle was predicted to develop high active tension only when the wrist was highly extended. A biomechanical model of this tendon transfer was generated using normative values obtained from previous studies of muscle architectural properties, tendon compliance, and joint moment arms. Predicted sarcomere lengths pre- and post-tendon transfer agreed well with intraoperative experimental measurements. The theoretical wrist extension moment-wrist joint angle relationship was also calculated for a variety of values of FCU muscle length. These different lengths represented the different conditions under which the FCU could be sutured into the ECRL tendon. Variation in FCU muscle length over the range 200 mm to 260 mm resulted in large changes in absolute peak moment produced as well as the angular dependence of peak moment. This was due to the change in the region of FCU operation on its sarcomere length–tension curve relative to the magnitude of the ECRL moment arm. These data demonstrate the sensitivity of a short-fibered muscle such as the FCU to affect the functional outcome of surgery. In addition, we demonstrated that intraoperative sarcomere length measurements, combined with biomechanical modeling provide the surgeon with a powerful method for predicting the functional effect of tendon transfer surgery.

Introduction

Tendon transfers in the upper extremity are used to restore function after trauma, peripheral nerve injury, spinal cord injury, brain injury, and neuromuscular disease. Traditional guidelines proposed by surgeons to choose which donor muscle should be used for a particular transfer primarily consider the following factors: muscle availability, functional synergy, route of transfer, and the morbidity caused by loss of a donor muscle. Much less attention has been given to the specific functional and structural characteristics of donor muscles themselves. Studies of upper extremity muscles used in tendon transfer began with the classic presentation by Brand et al. (1981) who presented a description of forearm muscle work capacity based on muscle mass and fascicle length measurements. This work was used to provide guidelines for a number of upper extremity tendon transfers (Brand and Hollister, 1993). Lieber and colleagues quantified muscle properties in terms of fiber length, sarcomere number, and physiological cross-sectional area (Jacobson et al., 1992; Lieber et al., 1990, 1992), and developed a quantitative model for muscle property comparison (Lieber and Brown, 1992). Such muscular comparisons are useful, but neglect the important interaction that occurs between muscles and the joints that they affect. The relationship between moment arm and muscle fiber length determines the sarcomere length range over which a muscle operates, and it has been suggested that this ratio should be matched between donor muscles and substitute muscles (Zajac, 1992).

To provide quantitative information regarding muscle properties pre- and post-tendon transfer, we developed an intraoperative laser diffraction method for measuring human muscle sarcomere length (Fridén and Lieber, 1994; Lieber et al., 1994). This method is noninjurious to the muscle and simply relies on the fact that laser light is diffracted by the striation pattern present in all skeletal muscles (Lieber et al., 1984). Since striation spacing is a direct manifestation of sarcomere length and sarcomere length is a good predictor of relative isometric muscle force, intraoperative sarcomere length measurements would provide the surgeon with the ability to set a muscle intentionally to a prescribed length where function would meet a desired goal.

In this study, we measured sarcomere length in the flexor carpi ulnaris (FCU) muscle prior to and after transfer into the tendon of the extensor carpi radialis longus (ECRL) muscle. While this is a relatively common transfer advocated for the restoration of wrist extension, there are two a priori reasons to
question whether the transfer would provide appropriate substitute function for the lost capabilities of the ECRL. First, FCU fiber length (42 mm, Lieber et al., 1990) is only about half of the ECRL (76 mm, Lieber et al., 1992) suggesting that the FCU muscle may not have sufficient operating range to provide the needed wrist motion. Second, the FCU has a physiological cross-sectional area (3.4 cm²) that is over twice that of the ECRL (1.5 cm²) suggesting that the FCU muscle may be overpowered to perform the tasks of the ECRL. In fact, the FCU and ECRL represent extremes in design of the five prime movers of the wrist (Lieber et al., 1990) and are predicted to have significantly disparate functional properties (Fig. 1). The FCU is suited to high force–low excursion tasks while the ECRL is better suited to low force–high excursion tasks. Based on the compromised FCU operating range relative to the ECRL, it is reasonable to believe that this tendon transfer would be very sensitive to length. Unfortunately, there are not generally accepted objective methods for determining and setting muscle length intraoperatively. This is because measurement techniques do not exist, and even if they did, there is not general agreement on the functional parameter that must be optimized after transfer. Typically surgeons determine muscle length during transfer based on the passive tension generated by the muscle intraoperatively. Unfortunately the relationship between optimal muscle length and passive tension varies considerably between muscles. Thus, the purpose of the study was to measure sarcomere length prior to and following FCU-to-ECRL transfer using standard recommended operative methods in order to understand the potential functional results of the transfer and to discuss specific criteria that might be used to optimize the transfer.

### Methods

**Patient Population.** Five patients were included in this study (three males and two females; aged 32–55 years). All were undergoing tendon transfer of the FCU-to-ECRL for radial nerve palsy. All patients received detailed instruction regarding the experimental protocol and all procedures performed were done so with the approval of the Institutional Review Boards of the University of California, San Diego, and the University of Ōmea. After induction of general anesthesia, the FCU muscle was exposed through a 3 cm longitudinal incision on the ulnar–palmar surface of the distal forearm. After exposure of the FCU by incision of the fascial sheath, a 5 mW He–Ne laser beam was directed into the distal portion of the FCU muscle fibers. With the elbow in 20 deg of flexion and the forearm supinated, FCU sarcomere length was measured with the wrist in full flexion, neutral, and full extension. The FCU was then transferred into the ECRL tendon with the wrist in a neutral position. Wrist joint angle was measured with a goniometer and was not identical in all subjects due to variations in anatomy and intraoperative positioning. After transfer, the wrist, forearm, and elbow were returned to their pretransfer configuration and FCU sarcomere length was again measured at each of the three wrist positions. Preoperatively, the FCU was thus examined as a wrist flexor and postoperatively the FCU was examined as a wrist extensor. Digits were held in the flexed position for all measurements.

Sarcomere length was measured only in the distal portion of the FCU. Based on sarcomere length measurements in fixed cadaveric FCU muscles, we felt that this would be representative of the entire muscle. In pilot studies, sarcomere length and fiber length were measured in nine locations within two FCU muscles: the proximal, middle, and distal region at each of three levels: superficial, middle, and deep. In each location, two separate measurements were made (n = 36—two locations × 2 measurements per location × 2 muscles). Fiber lengths and sarcomere lengths were compared across regions and levels by a 3 × 3 two-way ANOVA. No significant fiber length variation was observed within the FCU as a function of region (p > 0.4) or level (p > 0.8) and no significant sarcomere length difference was seen as a function of region (p > 0.7) or level (p > 0.6). These data support the use of a single sarcomere length as being representative of the entire muscle and thus validates the use of sarcomere length as the variable of choice intraoperatively.

**Biomechanical Simulation.** To predict the muscle force and wrist extension moment generated by the FCU after tendon transfer, the biomechanical model previously described (Loren et al., 1996) was implemented. This model was based on experimental measurement of prime wrist mover muscle architecture (Lieber et al., 1990) and the mechanical properties of each wrist tendon (Loren and Lieber, 1995). Muscle properties were predicted based on architectural values obtained from cadaveric forearms (Lieber et al., 1990) yielding muscle length–force curves scaled to fiber length and physiological cross-sectional area (Fig. 1). Briefly, the model predicts muscle moment as a function of joint angle using the known properties of the FCU muscle tendon and the appropriate moment arms (i.e., the FCU moment arm pretransfer and the ECRL moment arm post-transfer). Sarcomere lengths predicted by the model compare favorably to those obtained intraoperatively from the extensor carpi radialis brevis and FCU muscles (cf. Fig. 7 of Loren et al., 1996). Moment arm (in mm) of the FCU (r_FCU) was given by the expression

\[
 r_FCU = -15 + 0.082\theta
\]

prior to tendon transfer (pre-transfer), where \( \theta \) represents wrist angle (negative angles representing flexion) and the negative sign represents a flexion moment arm. The ECRL moment arm (r_ECRL) was given by the expression

\[
 r_ECRL = 10 + 0.117\theta
\]

post-transfer. The post-transfer moment arm equation was simply that determined for the ECRL and did not include the fact that the FCU was transferred around the ulnar side of the forearm and thus did not have exactly the same line of action as the native ECRL. However, since the ECRL tendon was still placed beneath the extensor retinaculum and the FCU was transferred at about the mid-fossa level, this was not considered a major limitation (see Discussion).

In all cases, the "reference moment" during transfer was the configuration corresponding to neutral wrist extension and 20 deg of elbow flexion. The biomechanical model was used to predict the sarcomere length–joint angle relationship and the

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**Fig. 1** Predicted muscle length–tension relationships for the FCU (dotted line) and ECRL (solid line). Force was predicted from physiological cross-sectional area using a specific tension of 22.5 N/cm² and excursion was predicted directly from muscle fiber length. The FCU is suited for high force–low excursion tasks, while the ECRL is suited for lower force–higher excursion tasks. (Data based on architectural data from Lieber et al., 1990). Small fluctuations in lines are printer artifacts.
Table 1  Intraoperative sarcomere lengths measured from FCU*

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<th>Flexed</th>
<th>Neutral</th>
<th>Extended</th>
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<tr>
<td>Pre-Transfer</td>
<td>2.83±0.11*</td>
<td>3.48±0.170</td>
<td>4.15±0.15**</td>
</tr>
<tr>
<td>Post-Transfer</td>
<td>4.02±0.114</td>
<td>3.99±0.002</td>
<td>3.19±0.097</td>
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*data shown are mean±SEM for 6 of 6 subjects. *p<0.05 comparing post-transfer to pre-transfer with muscle in corresponding position; **p<0.01; abbreviation: FCU, flexor carpi ulnaris.

wrist extension moment–joint angle relationship when the FCU muscle was inserted at various lengths.

Statistical Analysis. Absolute sarcomere length in each of three wrist positions and sarcomere length operating range throughout the range of motion was compared pre- and post-transfer using the Student’s t-test. Significance level (α) was chosen as 0.05 and statistical power (1−β) was calculated to range from 55 to 95 percent using standard equations (Sokal and Rohlf, 1981).

Results

Sarcomere Length Measurements. Sarcomere lengths measured after transfer into the ECRL tendon were systematically longer than those measured prior to transfer (Table 1). For example, maximum sarcomere length was significantly shorter pre- compared to post-transfer (corresponding to the wrist extended pre-transfer and flexed post-transfer) by 0.7 μm (p<0.01). The post-transfer sarcomere length of 4.82±0.11 μm corresponded to a sarcomere length that would actually be well beyond filament overlap (Fig. 2) given the actin filament length of 2.6 μm and myosin filament length of 1.66 μm previously measured in human upper extremity muscles (Lieber et al., 1994). It was noted intraoperatively that with the wrist fully flexed, post-transfer, significant passive tension opposed wrist flexion at these very long sarcomere lengths. Overall, the sarcomere length change throughout the range of motion post-transfer was 1.63±0.09 μm compared to only 1.32±.15 μm, the difference between which was not statistically significant (Fig. 2; p>0.1, β=0.55). This lack of significance was in part due to the fact that the wrist angle corresponding to pre-transfer sarcomere lengths with the wrist at full flexion was less than the wrist angle corresponding to post-transfer sarcomere lengths with the wrist extended. The very long absolute sarco-

mere lengths probably resulted from the elbow being almost fully extended. Pilot experiments suggested previously that 90 deg of elbow flexion resulted in about 1 μm of FCU shortening (cf. Fig. 7(B) of Loren et al., 1996). This result affects the absolute sarcomere lengths measured but has no effect on the change in sarcomere length with joint angle.

Predicted Sarcomere Lengths. Sarcomere length pre-transfer showed a relatively linear increase from ~2.5 μm to ~4.5 μm as the wrist was extended from ~60 deg of flexion to ~60 deg of extension. These length changes were well approximated by the relationship predicted (solid line, Fig. 3(A)) from muscle, tendon, and joint properties (Loren et al., 1996). In fact, the coefficient of determination (r²) between the experimental data and linear model was 0.84 demonstrating explanation of over 80 percent of the experimental variability by theory. After transfer, the sarcomere length predicted was curvilinear and provided a somewhat poorer fit to the data with an r² value of only 0.68 (Fig. 3(B)). Again, however, the basic data form was well approximated by the predicted relationship.

Model of FCU-to-ECRL Tendon Transfer. Given the good fit between experiment and theory, we further predicted all possible wrist extensor moments produced by the transferred FCU as a function of wrist angle and FCU muscle length at the time of transfer (Fig. 4). In general, joint moment increased as the wrist extended. This was largely due to the linear increase in ECRL moment arm with extension as the extensor tendon elevated off of the radius up to the underside of the extensor retinaculum. Superimposed upon this kinematic relationship was the fact that changing FCU length at the time of surgery changed the region of the sarcomere length–tension curve over

Fig. 2  Position on sarcomere length–tension relationship of FCU muscle pre-transfer (dotted lines) and post-transfer (solid lines). The length–tension curve is predicted from measured filament lengths (Lieber et al., 1994) and the sliding filament theory (Gordon et al., 1966). After transfer, the muscle operates at longer sarcomere lengths and over a larger range (cf. Table 1).

Fig. 3  Relationship between wrist joint angle and sarcomere length measured intraoperatively. (A) Pre-transfer. (B) Post-transfer into ECRL tendon. Solid line represents predicted sarcomere length from biomechanical model. Small fluctuations in lines are printer artifacts.
which the FCU operated. For example, if the muscle were inserted at very long lengths (250–260 mm; sarcomere length ~3.9 μm) it operated on the extreme end of the descending limb of the length–tension curve and muscle force increased with extension as the muscle shortened “up” the descending limb. Given the measured intraoperative sarcomere length of 3.89 μm in this joint configuration, this was essentially the situation that described the surgical patients in the current study. At the other extreme, if the muscle were inserted at very short lengths (200–210 mm; sarcomere length ~2.1 μm), it operated on the ascending limb and plateau of its length–tension curve but the overall joint moment was relatively low since, as the muscle developed maximal force, the moment arm decreased to a minimum. The largest joint moments were predicted at intermediate muscle lengths of 220–230 mm (sarcomere length 2.5 μm) since the muscle generated near maximal force on the plateau of the length–tension curve with the wrist extended and moment arm was nearly maximal with the wrist extended.

Intraoperative Decision-Making. The relatively complete description of the biomechanics of this tendon transfer permits a rational choice regarding the setting of muscle length at the time of transfer. This intraoperative decision represents the most important application of the use of these data. Using the data from the biomechanical model (Fig. 4), if the clinical goal is to maximize overall joint moment, the intraoperative decision would be to insert the muscle at a length of ~245 mm (Fig. 5, squares). From a different point of view, high force may be desired throughout the entire range of motion, in which case it would be preferable to optimize median or mean muscle force (Fig. 5, circles and triangles, respectively). If this is the choice, the muscle should be sutured into the ECRL tendon at a shorter muscle length of ~230 mm. Of course, it is not yet possible to answer this question definitively since no prospective clinical trials have yet been performed to quantify the outcome resulting from choice between these possibilities.

Discussion

The purpose of this study was to quantify FCU sarcomere length before and after transfer into the ECRL tendon. This is important not only to obtain objective intraoperative measurements that describe surgical procedures and predict outcome, but to provide an independent test of our ability to predict muscle sarcomere length based on muscle architecture, tendon compliance, and joint kinematics. The results of this study demonstrate that the shorter-fibered FCU acting on an altered wrist moment arm results in longer sarcomere lengths and concomitantly lower muscle forces than would normally be observed for the FCU. These data indicate that overstretch of the FCU will have significant consequences in tendon transfer since it is the shorter-fibered muscle acting at the wrist.

Both the pre- and post-transfer data were well approximated by the biomechanical model previously reported (Loren et al., 1996). It should be emphasized that this model was generated using muscle, tendon, and kinematic data from identical specimens so that the natural complementarity between these structures was maintained. The model was used with these average properties to predict sarcomere length in the current five patients. The only constraint placed on the data was that the predicted sarcomere length in the neutral position was forced to match the average measured sarcomere length. This constraint was used to provide a “scaling factor” between our data obtained from relatively old and atrophied arms to the patients participating in the current study. It is therefore not surprising that the curves fit closely approximate the data near a wrist angle of 0 deg (Fig. 3). However, it is impressive that the model simulates the data even at large flexion and extension angles. This is clearly the case for the pre-transfer data (Fig. 3(A)) and less so with the post-transfer data (Fig. 3(B)). At highly flexed angles, the predicted sarcomere length is slightly underestimated. This is not a function of tendon compliance and muscle activation since at these angles, the muscles are predicted to develop very little force. It is possible that the moment arms generated from cadaveric specimens underestimate the ECRL moment arm in flexion but we do not have a physical explanation for why this would be the case. It is even less likely in light of the excellent fit observed for the FCU pre-transfer and for the extensor carpi radialis brevis (ECRB) previously reported (see Fig. 7(A) of Loren et al., 1996). We suggest that, because the line of action of the transferred FCU is not exactly the same as the native ECRL, the FCU may be slipping off of the ulnar aspect of the forearm with wrist flexion. This possibility can be tested by measuring moment arms on cadaveric specimens after simulated surgical transfers.

![Figure 5](image-url) - Wrist extension moment parameters as a function of FCU muscle length at the time of tendon transfer. Values were determined from the biomechanical model shown in Fig. 4. (■) maximum extension moment, (●) median extension moment, (▲) mean extension moment.
Given the ability to measure sarcomere length intraoperatively and predict function, the key question is, "What is the best choice of sarcomere length that would result in optimal function?" Unfortunately, this question cannot be answered definitively because objective criteria for desired wrist function after FCU-to-ECRL tendon transfer have not been precisely specified. For example, in Green's excellent text on hand surgery (Green, 1993) the procedures described for surgical treatment of radial nerve palsy emphasize the need to maintain adequate strength and range of motion in the joint post-operatively without reference to the moment generated by the muscle at various wrist angles. In Brand's classic textbook (Brand and Hollister, 1993), they explicitly question the use of the FCU since it provides limited range for powering both wrist and finger extension and the FCU is the primary stabilizer of the wrist with greater morbidity after harvest.

Based on our ability to measure sarcomere length and predict function, we suggest several objective outcome measures that could be considered to be the treatment goal (Fig. 5). The first goal is simply to set sarcomere length so that maximum joint moment is generated, regardless of the angle at which it is generated or the moment generated at other angles (Fig. 5, squares). This is the case when the muscle is inserted at a length of about 245 mm, corresponding to a sarcomere length of about 3.1 µm. The reason that moment is maximized in this configuration is that the muscle is operating at its optimal sarcomere length when the ECRL moment arm is also maximized. Note that this is apparently not the case for either the normal wrist flexors or extensors (Loren et al., 1996). A potential problem with simply maximizing joint moment is that maximal moment is obtained in full extension and drops off sharply as the wrist is flexed. An alternate approach would be to maximize the median, mean, or integrated extension moment (Fig. 5, circles and triangles). All of these latter options yield about the same result. To satisfy this criterion, the muscle must be inserted at a length of about 220–250 mm, corresponding to a sarcomere length of about 2.5 µm. In this configuration, moment is maximized in approximately 30 deg of extension and decreases slightly on either side (Fig. 6, thin line). Compared to the actual situation observed for our patients where the sarcomere length at insertion was 3.89 µm (Fig. 6, bold line), this appears preferable. Inserting the muscle at this longer sarcomere length results in a higher peak moment but a dramatic drop in moment with flexion that does not simulate well the natural extension moment of the ECRL (Fig. 6, dotted line). It is simply not clear which if any of these possibilities is preferred. Prospective studies using objective outcome measures are needed to provide definitive answers. The principle consideration will be to balance sarcomere length operating range with changing moment arm throughout the wrist range of motion.

The results of this study should be interpreted with caution. First, all transfers were performed by a single, experienced hand surgeon, but it is possible that this surgeon tended to overstretch the muscle relative to the typical surgical procedure performed. Thus, the absolute sarcomere lengths reported here may not be representative of the results obtained in general by all surgeons. However, we have consulted with other hand surgeons and have concluded that the amount of tension placed on the FCU during transfer was typical. This intraoperative decision is made primarily based on passive tension resulting from muscle length changes. However, since different muscles have passive length–tension curves differentially shifted relative to their active length–tension curves (Labeit and Kolmerer, 1995), passive tension may not be an appropriate indicator of optimal muscle length.

A second limitation of this study is that these data do not take into account the fact that some muscles have the ability to change sarcomere number after transfer (Spector et al., 1980; Tabary et al., 1976; Williams and Goldspink, 1978). It has been demonstrated that an immobilized mouse, cat, or rat soleus muscle adjusts sarcomere number to reset the muscle to its optimal length at the immobilized angle. If such a change were to occur for the increased sarcomere operating range of the FCU, sarcomere number would have to increase by 25 percent, which is in the range of that reported for the soleus muscle experiments. It should be emphasized, however, that this result only applies to the soleus muscle, which is known to be one of the most adaptive skeletal muscle under conditions of altered use (Lieber, 1992). The same investigators have also demonstrated that muscles that contain a higher proportion of fast fibers (e.g., tibialis anterior, medial gastrocnemius) do not adapt as completely as the soleus muscle (Simard et al., 1982; Spector et al., 1982). Thus, it is inappropriate to assume that each muscle will simply "seek" its optimal sarcomere length after tendon transfer. Since the principles that guide sarcomere number remodeling in skeletal muscle are poorly understood, future studies are required to quantify the extent to which such remodeling occurs after transfer.

One of our objectives was to study the FCU sarcomere length operating range. Unfortunately, the relatively extended elbow, that lengthens the FCU considerably, caused a sarcomere length shift to longer values. Our pilot data suggest that this shift is approximately 1 µm but definitive answers to this question await future study.

Finally, the relatively small sample size of this study (n = 5) prevents serious extrapolation to the population at large since age, gender, and body size combinations may affect the data in ways that are not predictable (Table 2). The major driving force to increase sample size is to increase statistical power of a tested hypothesis, thus preventing type II error, which can be considered a "false negative" result (Lieber, 1990). However, this is not a hypothesis-driven study, so that sample size is not a critical factor. Low sample size affects the current study in that there is a relatively large confidence interval to the precise sarcomere lengths measured. Based on the current sample stan-

![Fig. 6 Wrist extension moment as a function of wrist joint angle for FCUs inserted at sarcomere lengths of 2.50 µm (thin line) or 3.89 µm (bold line). These data are taken from the surface data in Fig. 4.](390/ Vol. 119, NOVEMBER 1997 Transactions of the ASME)
standard deviation, the 95 percent confidence interval for the sarcomere lengths presented in Fig. 3 are approximately 0.4 μm.

Since sarcomere length represents the most critical variable under control of the surgeon at the time of the transfer and can be set to a precise value with relatively little additional effort, future studies should be performed that provide specific guidelines for the numerous upper extremity transfers performed. It is clear from this study that it is feasible to perform real-time intraoperative sarcomere length measurements, combined with biomechanical modeling, to place a transferred muscle at a chosen sarcomere length. Future studies are needed to determine whether such choices result in improved patient function.

Acknowledgments

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Novel transcriptional profile in wrist muscles from cerebral palsy patients

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Abstract

Background: Cerebral palsy (CP) is an upper motor neuron disease that results in a progressive movement disorder. Secondary to the neurological insult, muscles from CP patients often become spastic. Spastic muscle is characterized by an increased resistance to stretch, but often develops the further complication of contracture which represents a prominent disability in children with CP. This study’s purpose is to characterize alterations of spastic muscle on the transcriptional level. Increased knowledge of spastic muscle may lead to novel therapies to improve the quality of life for children with CP.

Method: The transcriptional profile of spastic muscles were defined in children with cerebral palsy and compared to control patients using Affymetrix U133A chips. Expression data were verified using quantitative-PCR (QPCR) and validated with SDS-PAGE for select genes. Significant genes were determined using a 2 × 2 ANOVA and results required congruence between 3 preprocessing algorithms.

Results: CP patients clustered independently and 205 genes were significantly altered, covering a range of cellular processes. Placing gene expression in the context of physiological pathways, the results demonstrated that spastic muscle in CP adapts transcriptionally by altering extracellular matrix, fiber type, and myogenic potential. Extracellular matrix adaptations occur primarily in the basal lamina although there is increase in fibrillar collagen components. Fiber type is predominately fast compared to normal muscle as evidenced by contractile gene isoforms and decrease in oxidative metabolic gene transcription, despite a paradoxical increased transcription of slow fiber pathway genes. We also found competing pathways of fiber hypertrophy with an increase in the anabolic IGF1 gene in parallel with a paradoxical increase in myostatin, a gene responsible for stopping muscle growth. We found evidence that excitation-contraction coupling genes are altered in muscles from patients with CP and may be a significant component of disease.

Conclusion: This is the first transcriptional profile performed on spastic muscle of CP patients and these adaptations were not characteristic of those observed in other disease states such as Duchenne muscular dystrophy and immobilization-induced muscle atrophy. Further research is required to understand the mechanism of muscle adaptation to this upper motor neuron lesion that could lead to the development of innovative therapies.
Background
Cerebral palsy (CP) is a disorder in which children experience a non-progressive brain lesion that results in permanent and progressive secondary postural and movement disorders [1]. CP has an incidence of 2.0–2.5 occurrences per 1000 live births in developed nations, making it the most common cause of physical disability in children [2]. There is a spectrum of disease states in CP that affect upper and lower limbs to varying degrees. Since the primary lesion in CP is in the central nervous system, most CP research has been focused on the neurological disorder [3-5]. However, since the secondary effects of CP disrupt posture and movement, most conservative and surgical treatments address the musculoskeletal system [6].

It is clear that skeletal muscles from CP patients are altered secondary to the neurological lesion. There are many neurological symptoms secondary to the brain lesion including dystonia, ataxia, athetosis and particularly spasticity [7,8]. Loss of upper motor neuron (UMN) inhibition on the lower motor neurons (LMN) results in spasticity, altered muscle tone and increased/impaired motor unit firing. Loss of UMN excitation of LMNs leads to negative features of UMN syndrome that include weakness, fatigability, poor balance, and occasionally, sensory deficits. Although the mechanism is unknown, spastic muscle often shortens to create muscle contractures, which is a primary disability of CP that leads to further complications [6]. There are many clinical approaches to managing spasticity to increase function, potentially decrease muscle contractures and most importantly improve quality of life. Oral medications, physical therapy techniques, chemical neuroectomies with phenol or alcohol, chemodenervation using neurotoxins (BTX), and surgical neuroectomies have all been utilized to decrease spasticity in children with CP [9]. Unfortunately, while there has been some success in this management, many children ultimately require orthopedic surgery to lengthen the tendons of contracted muscles so that arm or leg function can be increased. If the adaptation of the muscle tissue were more completely understood, it might lead to novel medical treatments of contractures.

Skeletal muscle from children with CP has been characterized at a variety of levels, with most studies reporting muscle tissue and muscle fiber atrophy, decreased muscle cross-sectional area, muscle shortening, and decreased specific tension [10,11]. All of these changes implicate physiological mechanisms of growth being involved in the pathology of muscle from CP patients. Interestingly, recent intraoperative studies of human muscles revealed abnormally long muscle sarcomere lengths in vivo [12] that were associated with muscle tissue of altered properties. Specifically, muscle fiber sarcomere length under no load (i.e., slack sarcomere length) was significantly decreased while the muscle tissue itself contained a hypertrophic extracellular matrix of poor material quality [11,13]. These changes implicate the mechanical force generating system of the muscle cell as well its extracellular matrix tissue. Muscle has been shown to adapt its mechanical function to neurological input [14], however the mechanism by which UMN lesion could lead to alterations in muscle myogenesis, force generation, force transmission and extracellular matrix properties is unknown. While there is evidence that neurotrophic factors dramatically affect muscle properties [15,16], there is neither mechanistic understanding as to how such factors might alter tissue properties, nor information as to which specific biosynthetic pathways might lead to these changes.

To develop an understanding of the physiological processes altered in spastic muscle secondary to CP, we exploited the fact that muscle tissue from a previous study, in which the clinical severity of the spasticity was clearly established, was available for transcriptional profiling [17]. We used GeneChip technology to contrast the transcriptome from CP patients with age-matched control patients whose muscles were completely normal. We performed a variety of analyses to identify a robust set of genes that were significantly altered in CP and interpreted these genes in their biological context to explain previously defined muscle changes. We also compared our transcriptional data to two other disease states to determine whether spasticity secondary to CP results in a unique muscle disorder at the gene expression level.

Methods

Muscle Sampling
Children were recruited for this study because they were receiving tendon transfers of the flexor carpi ulnaris (FCU) muscle into the extensor carpi radialis brevis (ECRB), the extensor carpi radialis longus muscle, or the extensor digitorum communis muscles [18]. All patients had CP and developed a contracture indicating surgery, despite receiving conservative treatment that included splinting and occupational therapy. Parental consent and child’s assent was obtained in accordance with our institutional review boards. From the original sample size of 23 [18], a subset (n = 6 children, average age 12.8 ± 1.5 years) was selected to cover a range of clinical severities determined from the House [19], Ashworth [20], and Zancollí [21] classification systems as well as characteristics of sarcomere length and range of motion. Control tissue was obtained incidentally (n = 2 children, average age 8.5 ± 2.1 years) from both the FCU and ECRB muscles in children with no previous history of any neural injury who were undergoing surgery for forearm fracture repair. None of the surgeries injured the control muscles in any
way. We suggest that these samples represent true muscle controls for the following reasons: 1) the surgeon verified that the muscles from which biopsies were taken were in pristine condition and showed no signs of damage, 2) surgery was emergent in these children, and therefore, control samples were obtained within 24 hours of fracture, 3) controls showed no significant effect for many of the transcripts associated with trauma or immobilization and were, in fact, often altered in the opposite direction (data not shown) (21, 60). Just prior to harvesting of the spastic muscle biopsies, sarcomere length of the FCU was measured by laser diffraction in vivo. While the wrist was held in neutral, a small fiber bundle was transilluminated with a HeNe light. The sarcomere length could be calculated from the diffraction pattern obtained [18]. CP biopsies were snap frozen in isopentane chilled by liquid nitrogen (-159°C), and stored at -80°C until analyzed (Table 1). No patient had undergone serial casting prior to surgery, two patients (AN and BF) had BTX injections into the FCU several months prior to surgery, and one patient (AQ) had a prior BTX injection in the biceps.

RNA preparation and gene expression profiling
RNA was extracted using a combination of standard Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. Briefly, 30 mg of frozen muscle was homogenized in a rotor-stator homogenizer on ice in 0.5 ml of Trizol; 0.1 ml of chloroform was added to the solution, which was then vigorously vortexed for 15 s followed by centrifugation at 4°C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. After the column was washed, it was incubated with RNAse-free DNase (Qiagen) for 15 min and incubated with RNAse-free DNAse (Qiagen) for 15 min and then washed again three more times before being eluted as described in the manufacturer’s protocol. RNA concentration was determined by the absorbance at 260 nm, and the 260 nm-to-280 nm absorbance ratio was calculated to define RNA purity.

Microarray data analysis
Affymetrix microarrays (*GeneChip* HG-U133A; Affymetrix, Santa Clara, CA) were used for each muscle biopsy (n = 16 chips; 2 muscles × 8 patients) and the data are available [GEO: GSE11686]. RNA processing for the GeneChip, including stringent quality control measures, was performed by the Gene Chip Core at the Department of Veterans Affairs San Diego Health Care System, (San Diego, CA). GeneSpring software (version 7.3; Silicon Genetics, Redwood City, CA) was used to identify those genes that were significantly altered in CP. Initially, a 12.5% (2/16 chips) present call on MAS5 (Affymetrix) was used to filter out poorly performing probe sets in the analyses. Three independent probe set algorithms were used for signal generation and normalization: MAS5, RMA, and GCRMA. Recent reports support requiring concordance among different probe set algorithms as an approach to reduce false positives in data sets [22-24]. Each feature was normalized per chip (to the median of all features on each chip) and per gene (to the median of that feature on all chips). Normalized gene values were subjected to a 2 × 2 Welch ANOVA of muscle type (FCU vs. ECRB) and disease state (CP vs. CTRL) with a required statistical significance (P < 0.05) with a Benjamin and Hochberg False Discovery Rate (FDR) multiple testing correction for present features. Thus 5% of the genes deemed significant for an individual preprocessing algorithm are suspected to be false positives. Features that passed in all three preprocessing algorithms were deemed significantly altered in CP.

The condition tree was created using a Pearson Correlation similarity score and average linkage clustering algorithm for all samples on present features. For severity analysis, a Welch ANOVA for each severity parameter was

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcript</th>
<th>Base pairs</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A2</td>
<td>NM_000089</td>
<td>225</td>
<td>TCCAAAGGAGAGAGCGGTAA</td>
<td>GCCACTTGACCAACGACTA</td>
</tr>
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<td>COL4A2</td>
<td>NM_001846</td>
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<td>CTGGTGCCGAGTGGTTGTG</td>
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</tr>
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<td>COL4A3</td>
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<td>AGAGAATCCAGCCGTGAG</td>
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<tr>
<td>DMD</td>
<td>NM_004019</td>
<td>157</td>
<td>GACAGCCGACACCTCAGCAA</td>
<td>TCACGAGCCGATCAGCAGA</td>
</tr>
<tr>
<td>FBXO32</td>
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<td>GACGACGAGGTGCGAAGT</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>172</td>
<td>TCTGATCTCACAGCGCACC</td>
<td>TGTTCCAGGGTCCTCTCTC</td>
</tr>
<tr>
<td>IFI1</td>
<td>NM_000618</td>
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<td>AGCACTTCTCACAACCTAA</td>
<td>CAGAGACAGCACGCTG</td>
</tr>
<tr>
<td>IFGBP5</td>
<td>NM_005999</td>
<td>339</td>
<td>CAAACACACACCGCATCT</td>
<td>CAGCTCTGATCCTGACCTG</td>
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<tr>
<td>GDF8</td>
<td>NM_002529</td>
<td>167</td>
<td>ATACAGCTCAGCAGGAAAC</td>
<td>GGATCTCAAGGATTCTCTC</td>
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<tr>
<td>MYH1</td>
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<tr>
<td>NKB</td>
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</tr>
<tr>
<td>TRIM63</td>
<td>NM_032588</td>
<td>393</td>
<td>GAGGATCCCGTCGAGTC</td>
<td>AATGGCTCTAGGGCGGCTCT</td>
</tr>
</tbody>
</table>

Table of primers used for QPCR analysis. Genbank accession number and PCR product length given in parentheses after each transcript name.
run on the MAS5 data of the features deemed significantly altered in CP without the control patients and a required statistical significance of \( P < 0.05 \) also with an FDR multiple testing correction.

Promoter sequence analysis was conducted using GeneSpring on the list of genes altered in CP. The upstream sequence from -10 to -1000 base pairs was analyzed for a nucleotide sequence of from 6 to 10 nucleotides long and containing at most 2 N values in the middle. Significance was determined based on the number of times the given sequence appeared in the upstream sequence of all other genes and was corrected for multiple testing. The analysis was performed on the whole list of genes altered in CP and the sub lists of up- or down-regulated.

**Quantitative real-time PCR**

QPCR was performed to validate expression levels of selected genes to the GeneChip data and to provide mRNA expression levels for genes not contained on the HG-U133A chip. After RNA was extracted from the muscle as described previously and diluted 1:5 with DNase/ RNase free water (Invitrogen), 1 µl of each sample was reverse transcribed using standard protocols (Superscript III; Invitrogen). cDNA was amplified with the Cepheid SmartCycler (Sunnyvale, CA) with primers specific to the genes of interest (Table 2). All primers were tested for cross-reactivity with other transcripts using nBLAST and Oligo (version 6.6; Molecular Biology Insights, Cascade, CO). All samples were run at least in triplicate, along with a standard curve. The PCR reaction vessel (25 µl) contained 1× PCR buffer, 2 mM MgCl₂ (Invitrogen), 0.2 mM sense and antisense primers, 0.2 mM dNTP, 0.2× SYBRgreen, and 1 U of platinum Taq polymerase (Invitrogen). Amplification conditions were as follows: An initial hold at 95°C for 2 min was followed by 40 cycles of denaturing at 95°C for 15 s, followed by annealing/extension at 68°C for 40 s. The success of each reaction was deduced based on the observation of a single reaction product on an agarose gel and a single peak on the DNA melting temperature curve determined at the end of the reaction. To express QPCR results, we used the standard curve method with the "cycles to threshold" value representing the number of PCR cycles at which the SYBRgreen signal was increased above the threshold. Each sample's value was measured in triplicate, normalized to the housekeeping gene GAPDH, and then averaged. QPCR data were normalized to the median value of the gene to permit comparison to the GeneChip data.

**Myosin protein content biochemistry**

Myosin heavy chain protein content was measured (Table 2) for comparison to the GeneChip data as previously described [25]. Three bands were identified corresponding to MyHC I, MyHC IIa/fetal and MyHC IIx/embryonic. (Using this methodology, embryonic MyHC cannot be separated from MyHC IIx and fetal MyHC cannot be separated from MyHC IIA.) The gels were scanned in a soft laser densitometer (Molecular Dynamics Sunnyvale, CA, USA). The relative proportion of each MyHC isoform was determined by using a densitometric system (ImageQuant TL Software v 2003.01, Amersham Biosciences, Uppsala, Sweden).

**Table 2: Subject information and clinical evaluations**

<table>
<thead>
<tr>
<th>Sub</th>
<th>Sex</th>
<th>Age</th>
<th>Muscle</th>
<th>SL (µm)</th>
<th>House</th>
<th>Sev</th>
<th>Ash</th>
<th>Zan</th>
<th>PEFF (°)</th>
<th>PESF (°)</th>
<th>AEFF (°)</th>
<th>MyHC gel</th>
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</thead>
<tbody>
<tr>
<td>AI</td>
<td>M</td>
<td>13</td>
<td>FCU</td>
<td>6.36</td>
<td>1</td>
<td>SS</td>
<td>4</td>
<td>3</td>
<td>-5</td>
<td>-90</td>
<td>-70</td>
<td>x</td>
</tr>
<tr>
<td>AN</td>
<td>F</td>
<td>12</td>
<td>FCU</td>
<td>4.56</td>
<td>3</td>
<td>SS</td>
<td>3</td>
<td>2B</td>
<td>50</td>
<td>45</td>
<td>90</td>
<td>x</td>
</tr>
<tr>
<td>AO</td>
<td>F</td>
<td>11</td>
<td>FCU</td>
<td>4.01</td>
<td>5</td>
<td>SM</td>
<td>2</td>
<td>2B</td>
<td>65</td>
<td>60</td>
<td>50</td>
<td>x</td>
</tr>
<tr>
<td>AQ</td>
<td>M</td>
<td>15</td>
<td>FCU</td>
<td>4.01</td>
<td>7</td>
<td>M</td>
<td>2</td>
<td>2B</td>
<td>80</td>
<td>80</td>
<td>50</td>
<td>x</td>
</tr>
<tr>
<td>AT</td>
<td>M</td>
<td>14</td>
<td>FCU</td>
<td>3.57</td>
<td>0</td>
<td>SS</td>
<td>3</td>
<td>3</td>
<td>45</td>
<td>40</td>
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<td></td>
</tr>
<tr>
<td>BF</td>
<td>F</td>
<td>12</td>
<td>FCU</td>
<td>5.31</td>
<td>6</td>
<td>SM</td>
<td>3</td>
<td>2A</td>
<td>85</td>
<td>85</td>
<td>55</td>
<td>x</td>
</tr>
<tr>
<td>BP</td>
<td>M</td>
<td>10</td>
<td>FCU</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>1</td>
<td>0</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>x</td>
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<tr>
<td>AZ</td>
<td>M</td>
<td>7</td>
<td>FCU</td>
<td>-</td>
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<td>0</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>x</td>
</tr>
</tbody>
</table>

Table of the eight subjects used in this study with 2 muscles (FCU and ECRB) per patient with sex and age recorded. Measures of severity were taken to include: intraoperative measurement of sarcomere length (SL) on the FCU, House clinical assessment of activity, Severity (Sev) grouped by House (Severe Severe (SS), Severe Moderate (SM), Mild (M), and Control (C)), Ashworth (Ash) clinical assessment, Zancolli (Zan) classification based on finger extension, passive extension with flexed fingers (PEFF) passive extension with straight fingers (PESF) and active extension with flexed fingers (AEFF). Samples that were available for MyHC SDS-PAGE gels are noted in the final column.
**Gene Ontology analyses**

Gene ontology analysis provides a means of converting a list of differentially expressed genes into a hierarchical list of gene ontologies that are significantly altered. We used the web-based software GOTree [http://bioinfo.vanderbilt.edu/gotm/; [26]) to compare the list of features altered in CP to the list of features present on the HG-U133A chip. In this analysis, a P-value is generated for each ontology based on hyperbolic comparison of the number of genes present in that list to the number of genes expected to be present based on the size of the list. The analysis was performed on the entire list of genes altered in CP, with a required P < 0.01.

**Biological pathway analyses**

To gain understanding into the biological context of transcript changes, we investigated the way in which genes were involved in various muscle pathways. We analyzed pathways from databases including: Ingenuity Pathway Analysis (IPA; [http://www.ingenuity.com]), Kyoto Encyclopedia of Genes and Genomes (KEGG; [http://www.genome.ad.jp/kegg/pathway.html]), and Gene Map and Pathway Profiler (GenMAPP; [http://www.genmapp.org/]). These pathways permit establishment of pathways specific to muscle involving critical muscle functions such as: neuromuscular junction function, excitation-contraction coupling, muscle contraction, extracellular matrix formation, muscle hypertrophy/atrophy, myogenesis, and fiber type switching. Based on the pathway databases and relying heavily our own literature review, we created pathways specific to muscle with particular emphasis on genes altered in CP. To quantify gene expression for pathway analysis, MAS5 data were normalized to the averaged control data within each muscle type and across CP samples. This value is termed the expression ratio.

Finally, to compare the CP transcriptome to other conditions we examined the pathways specific to muscle against transcriptome deposited for three other disease states, Duchenne Muscular Dystrophy (DMD; GSE465; [27]), immobilization (IMB; GSE8872; [28]), and hereditary spastic paraplegia (HSP; GSE1300; [29]). The DMD experiment used muscle from patients age 6–9 years and further details are described in the reference [27], but we compared data only from those U95A chipset. The IMB experiment used medial gastrocnemius muscle from adult patients and further details are described in the reference [28], but we compared only data from voluntary controls and ankle fracture patients immobilized for 4–9 days. The HSP experiment used vastus lateralis muscle from adult patients and further details are described in the reference [29], but we used the U133A chip and controls (1–10) from GSE3307. As these data sets are from subjects of different ages and muscles, and are acute (in the case of 4–9 days of immobilization), direct comparison to our CP dataset is somewhat problematic; however, we are able to investigate whether similar transcriptional trends are present for these muscle conditions. The expression ratio for each feature was taken as the MAS5 ratio of the average disease state:average control state of the particular study so disease values are normalized to their own controls. The genes expression ratio of a pathway for each disease (CP, DMD, IMB, HSP) was log averaged across the pathway with inverse expression values used for inhibitors. We similarly investigated a list of genes involved in satellite cell states of quiescence and activation [30-32].

**Results**

Of the 22,283 probe sets on the HG-U133A GeneChip, 11,312 met the criteria of being "present" on 2/16 GeneChips and were therefore considered for further analysis. The number of genes that were significant for CP (P < 0.05) on the 2 × 2 Welch ANOVA of disease state and muscle (CP vs. CTRL; flexor carpi ulnaris (FCU) vs. extensor carpi radialis brevis (ECRB)) with FDR among the three preprocessing algorithms were: 495 for Microarray Suite Version 5.0 (MAS5), 1,141 for Robust Multiarray Analysis (RMA), and 1,207 for GCRMA. The overlap of these 3 preprocessing algorithms produced a final list of 205 genes (319 features) that were considered significantly altered secondary to CP (Sup. Table 1). Of these, more were up-regulated (143 genes, 220 features) than down-regulated (62 genes, 99 features). Table 3 reports the 72 genes subset of these 205 genes that were considered relevant to specific muscle functions. Genes in the Tables (Table 1; Sup. Table 1) are reported with the P-value for each preprocessing algorithm as well as the expression ratio. The 2 × 2 ANOVA yielded no genes significant (P < 0.05) for muscle type and only one gene with a significant interaction, MYH1. This was due to control ECRB tissue having a very low MYH1 mRNA content. This important result supports our previous contention that, even though these children present with wrist flexion contracture, the FCU and ECRB are equally affected and the wrist flexion simply results from the large size of the FCU [18].

Promoter sequence analysis performed on each gene altered in CP did not reveal any 6–10 base pair sequences that were overrepresented 10–1000 base pairs upstream of the gene. Of course, regulation can occur farther upstream than 1000 base pairs and regulation sequences can be outside of the 6–10 base pair range. Thus, further sequence analysis may reveal significant promoter or enhancer sequences, but none were identified using these criteria.

**Condition tree correlates with clinical severity scores and treatment**

The condition tree resulted in the control patients being clustered together separate from CP patients (Figure 1).
Figure 1 illustrates the condition tree based on all present genes. The tree shows that patients are grouped together in most cases rather than by muscle type suggesting more between-patient than between-muscle variability.

We had hoped that clinical severity \[1,19-21\] would allow us to define transcriptionally, the severity of CP or differences between flexor and extensor muscles. In this way, clinical parameters would be seen as representative of the

<table>
<thead>
<tr>
<th>Table 3: Significantly altered genes in functional categories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENE</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Neuromuscular Junction</td>
</tr>
<tr>
<td>KCNN3</td>
</tr>
<tr>
<td>COL4A3</td>
</tr>
<tr>
<td>LAMB2</td>
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<td></td>
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<tr>
<td>Excitation Contraction Coupling</td>
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<td>CACNB1</td>
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<td>Myogenesis/Fiber type pathways</td>
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</tr>
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<td>IGF1</td>
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<tr>
<td>IGFBS</td>
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<td>COL4A2</td>
</tr>
</tbody>
</table>

Table of genes within function groups related to skeletal muscle. Groups defined by ratio is the expression ratio of CP:CTRL. P-values are listed for the three separate preprocessing algorithms used (MASS, RMA, GCRMA).
Figure 1
Top: Condition tree created using Pearson Correlation for the similarity score and an average linkage clustering algorithm. The tree was created based on all present features. MAS5 data were used with expression values normalized to each features median. Features are ordered from highest expression ratio to lowest. Bottom: Clinical conditions color-coded with values for each sample.
state of the muscle tissue. These parameters were applied to an analysis of all of the 319 features altered in CP (Additional File 1 Table S1) but only sarcomere length and active wrist extension with fingers flexed had features that were significant, containing the same two genes, RBM9 and RHOBTB1. Heat plot of these data reveals that even these genes undergo a much larger change in expression from the control sarcomere length (3.37 μm) to CP sarcomere lengths than they do in CP progression (data not shown). Thus it appears that our study is underpowered to reveal transcriptional correlation with clinical severity scores.

Treatment with BTX was investigated by comparing injected muscles vs. non-injected CP muscles, muscle from patients receiving injection of any muscle vs. non-injected patient muscle, and injected FCU muscles vs. non-injected FCU muscles. None of these analyses yielded any genes that met our requirement for statistical significance, and thus we show no significant transcriptional effect of BTX injection.

RT-PCR results compared to chip results
As a quality control measure, correlation of data between the GeneChip and QPCR was highly significant (P < 0.001) indicating internal consistency. To validate the GeneChip data, 10 genes covering a variety of cellular processes and expression levels were compared directly to transcript levels determined by QPCR on the same cDNA samples (Figure 2). For 9/10 genes studied, the direction of the transcript change (i.e., up- or down-regulation) was confirmed, and there was a good correlation between methodologies in terms of the magnitude of the effect. For two genes, (PVALB, GDF8) expression levels were evaluated relative to GAPDH transcript levels. These genes were selected based on their significant differences on the chip and their relevance to the disease state. Significant positive correlations were observed for both genes (PVALB, r² = 0.924, P < 0.001; GDF8, r² = 0.864, P < 0.001). The QPCR data were also subjected to 2 × 2 ANOVA (CP vs. CTRL; FCU vs. ECRB) and both PVALB and GDF8 were confirmed as significantly up-regulated in CP (Figures 3C and 3D).

Two genes of particular interest that are related to muscle atrophy, MAFbx (FBX032) and MURF1 (TRIM63) were not represented on the chip [28,33]. Their expression was determined in the same manner as the genes described above (Figures 3E and 3F). Both of these genes were down-regulated in CP, but neither reached statistical significance.

Myosin heavy chain protein-mRNA comparison
The GeneChip and QPCR provide only transcriptional data and we wished to determine whether the transcriptional changes resulted in translational changes in the case of myosin heavy chain (MHC) for these samples [25,34]. All of the muscles were of a mixed fiber type, however the control ECRB tissue showed no evidence of type 2X MHC. The spastic muscles had a higher proportion of fast fibers than the controls of the corresponding muscle, with most of the increase in type 2X MHC. Comparison between protein and mRNA was confounded by the fact that MHC expression was normalized as percent of total myosin while mRNA was normalized to the
median of that individual transcript across subjects. In spite of this difference, we still expected to see the same trend across samples, which was the case for type 1 MHC (gene MYH8) and type 2X MHC (gene MYH1) where protein and mRNA levels were significantly correlated (Figures 4A and 4C; P < 0.05) while type 2A MHC (gene MYH4) did not quite reach significance (Figure 4B; P = 0.065). Taken as a whole, these results suggest that, in the case of the MHC, protein levels reflected transcript levels.

**Gene ontology analysis**
Thirty-eight different ontologies were overrepresented based on the 143 up-regulated genes (Additional File 2 Table S2; Additional File 3 Figure S1). The biological processes that stood out as most relevant to the disease state included striated muscle contraction, muscle development, cytoskeletal anchoring, negative regulation of metabolism, protein ubiquitination, and RNA processing. The cellular components of these genes were generally grouped into muscle components and ECM components.
particularly the basement membrane. Twenty-eight different ontogenies were overrepresented based on the 99 down-regulated genes (Additional File 4 Table S3; Additional File 5 Figure S2). The two major functions of the down-regulated biological processes were fatty acid metabolism and transport. This corresponded with the molecular function ontologies involved in fatty acid/acyl CoA binding and also contained cadmium and copper ion binding. Cellular component categories were almost exclusively related to the mitochondria, however it was interesting that sheet forming collagen type IV of the basement membrane also was over represented using this analytical approach.

**Gene pathways related to muscle function**

To understand muscle tissue adaptation to CP from a physiological perspective, we analyzed gene expression ratio patterns within muscle-specific pathways of gene products that interact in a given muscle function.

Because CP is a neurological disorder, a pathway describing the neuromuscular junction (NMJ) was created (NMJ; Figure 5A). No postsynaptic genes were significantly altered in CP including subunits of the nAChR receptor. Collagen type IV subunits\(^{(COL4A3; 2.89 \text{ and } COL4A4; 3.26)}\) and laminin\(^{(LAMB2; 1.74)}\) of the synaptic basal lamina were significantly up-regulated. A Ca\(^{2+}\)-activated K\(^+\) channel\(^{(KCNN3; 12.98)}\) was dramatically up-regulated in CP.

The process of converting the action potential into muscle contraction is referred to as excitation-contraction coupling (ECC; Figure 5B). The \(\beta1\) regulatory subunit of the L-type voltage gated Ca\(^{2+}\) channel was significantly up-regulated in CP\(^{(CACNB1; 1.59)}\). Although the ryanodine receptor responsible for releasing Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) was not altered, the genes \(FKBP1A\) (0.56) and \(PDE4D\) (0.68) that prevent channel leaking, were significantly down-regulated\(^{[35]}\). Myomegalin\(^{(PDE4DIP; 2.10)}\) was altered significantly in CP and is thought to anchor \(PDE4D\) near the SR\(^{[36]}\). Calmodulin\(^{(CALM1; 1.70)}\) was significantly up-regulated. The most drastic change with CP on gene expression was in the up-regulation of muscle-relaxing protein, parvalbumin\(^{(PVALB; 62.6)}\). The process of pumping Ca\(^{2+}\) back into the SR is assisted by triadin\(^{(TRDN; 2.39)}\), which was significantly up-regulated.

**Figure 5**

Pathways specific to muscle analysis of transcription in CP muscle. Pathways A-G involved in muscle function. Color is determined by the expression ratio. Up-regulated genes are red and down-regulated genes are green. Green connectors represent activation and red connectors represent inhibition in the direction of the arrow. Bolded genes represent those that are significantly altered in all three preprocessing algorithms. Italic genes (RAS, MAPK) are sets of genes involved in muscle MAPK pathway in muscle, but are not colored by expression because many individual genes are applicable and not altered in CP. Pathways represented are A: NMJ, B: ECC, C: MC, D: ECM, E: MYG, F: FT, and G: IGF1.
Muscle contraction obviously requires the myosin heavy chain motor and a cytoskeletal framework (MC; Figure 5C). MYH1 (type 2X MH; 8.57) was significantly up-regulated in CP along with MYH4 (type 2B MH; 4.33) a gene not normally expressed in humans [37]. The developmental MHCs, MYH3 (embryonic MHC; 15.74) and MYH8 (perinatal MHC; 7.74) showed large up-regulation. MYH7 (type 1 MHC; 0.72) was unchanged. The MYBP2C (2.16) is a fast isoform of myosin binding protein and was up-regulated in CP. Several sarcomeric structural components were also up-regulated as well: dystrophin (DMD; 2.11), nebulin (NEB; 2.54), and muscle LIM domain binding protein 3 (LDB3; 1.93).

We suspected that ECM transcription would be altered based on previous biomechanical results ([11,13,38]; ECM; Figure 5D). Fibrillar collagens all increased modestly. Interestingly, basal laminar collagen IV was altered with COL4A1 (0.51) and COL4A2 (0.43) decreasing significantly while COL4A3 (2.89) and COL4A4 (3.26) increased significantly. Basigin, (BSG; 0.48) was significantly down-regulated. Various other ECM components were also up-regulated in CP: ECM2 (1.86), KAL1 (2.57), MATN2 (2.24), MFAP5 (3.64), CILP (2.09) and SMC3 (1.88).

Myogenesis describes the pathway that produces muscle growth (MYG; Figure 5E). IGF1 (2.63) was up-regulated along with IGFBP5 (2.48) (IGF1; Figure 5F). Myostatin (GDF8; 3.65), an inhibitor of myogenesis, was also significantly up-regulated. Other significantly up-regulated genes implicated in myogenesis are NEO1 (1.44, [39]), PLCB1 (2.34, [40]), PBX1 (1.86, [41]), and HMBG1 (1.65, [42]), MBNL1 (1.74, [43]), and MAPK6 (1.77, [44]). However, the muscle regulatory factors (MYOD1 1.22; MYF6 0.87; MYF5 1.06; MYOG 1.75) did not show a significant transcriptional change. Mitogen activated protein kinases (MAPK) have been proposed as a major pathway in muscle hypertrophy [45], however our study showed minimal transcriptional affects on this signaling family. Another segment of myogenesis relates to satellite cell activation, proliferation and incorporation into adult muscle fibers. None of the markers for quiescent or activated satellite cells (quiescence: PAX7, FOXK1, MET, CDH15, NCAM1, VCA1, SDC3, SDC4; activation: MYF5, MYOD1, MYOG, MYF6, PCNA, CDKN1A, MYH3, MYH8) were significantly altered in CP suggesting minimal involvement of satellite cells in the disease. Additional cell cycle transcripts were investigated, but did not show a significant change.

Although slow fiber creation is related to myogenesis, there is also a specific pathway for slow oxidative muscle fiber type determination (FT; Figure 5F). Sensing and signaling factors, CALM1 (1.70) and calcineurin (PPP3CA; 1.95) respectively, had significantly increased transcription along with transcription factor MEF2A (1.53), but NFATs and other MEF2 expressions levels were unchanged.

### Cerebral palsy compared to other muscle pathologies

To determine whether the CP transcriptome was unique or simply a secondary adaptation of decreased activity in these children (as might be observed with immobilization (IMB)), or whether the response was a generic muscle pathology (Duchenne Muscular Dystrophy (DMD) being the most-commonly studied), or was similar to spastic muscle in an alternative more developed muscle (Hereditary Spastic Paraplegia (HSP) being a spastic condition with adult subjects) we compared our GeneChip data to these three muscle pathologies for which GeneChip data were available [27-29]. To make these comparisons, the expression ratio values for the pathways were compared amongst the three conditions (Table 4). While averaging over an entire pathway may be misleading (similar scores may result from different gene expression patterns), different scores do emphasize pathways that are unique among disease states. This analysis revealed significant satellite cell activation, as expected, in DMD [27] as well as increased NMJ components (primarily nicotinic acetylcholine receptor subunits) and loss of contractile material as expected in IMB [28]. HSP represents muscle adaptation to altered neuronal input, although there was a negative correlation in most pathways, ECC seemed to be handled in a similar manner. CP was unique relative to the other two pathologies based on the IGF1 pathway increase, slow fiber activation, and increased expression of ECC activators and inhibitors. Thus, the correlation data support the assertion that CP is unique relative to other disease states.

| Table 4: Changes in pathways correlated with other muscle disease states |
|-------------------|--------|--------|--------|--------|--------|
|                  | CP     | DMD    | IMB    | HSP    |
|                  | MEAN   | CORR   | MEAN   | CORR   | MEAN   | CORR   |
| NMJ              | 2.39   | -0.24  | 1.19   | -0.05  | 0.97   | -0.02  |
| ECC              | 0.66   | 0.12   | 1.26   | 0.14   | 1.39   | 0.62   |
| MC               | 2.91   | 0.91   | 0.65   | -0.74  | 1.07   | -0.48  |
| ECM              | 1.63   | -0.23  | 0.69   | 0.44   | 0.66   | 0.02   |
| FT               | 1.35   | -0.21  | 0.92   | 0.37   | 0.91   | -0.79  |
| IGFI             | 1.19   | 1.04   | 0.76   | N/A    | N/A    |
| MYG              | 1.14   | -0.16  | 1.01   | -0.24  | 1.01   | -0.09  |
| SCQ              | 1.19   | -0.67  | 1.11   | 0.66   | 1.23   | 0.07   |
| SCA              | 2.17   | 0.86   | -0.81  | 0.94   | -0.54  |

Quantification of gene pathways in various disease states (CP, DMD, IMB, HSP). Quantification represents MEAN (geometric mean of expression ratios in specific muscle pathways defined in Figure 5 using inverse values for pathway inhibitors) and CORR (correlation of CP with the other disease states, reported with an R value). Satellite cells markers are separated into genes expressed in the quiescent (SCQ) and activated states (SCA).
Discussion
The purpose of this study was to define the muscle transcriptional adaptations in children with cerebral palsy (CP) to gain insights into the cellular mechanisms that might explain muscular adaptation in this neurological condition. We show that the transcriptional profile of CP muscle is fundamentally different compared to normal controls (Figure 1). Previous CP muscle studies of intraoperative sarcomere length [12], in vitro tissue biomechanics [38,46], and immunohistochemical and biochemical assays suggested adaptation of extracellular matrix regulation [11,13], myogenenic pathways [10,38], and fiber type determination pathways [25] in this condition. Our transcriptional analyses provide potential explanations of the cellular bases for these adaptations. Based on a general understanding of muscle physiology and biology, we placed the gene expression patterns into the context of six major muscle physiological systems – the neuromuscular junction (Figure 5A), excitation-contraction coupling (Figure 5B), muscle contraction (Figure 5C), extracellular matrix regulation (Figure 5D), myogenesis (Figure 5E) and fiber type determination (Figure 5F). As will be seen, one feature of CP is that conflicting tendencies occur within and between these various systems.

The initial insult in CP is located in the central nervous system, but this primary insult leads to a secondary effect on the skeletal muscle system. Thus the NMJ, as the nerve-muscle interface, may play a role in CP. Studies have shown disrupted NMJ in that acetylcholine receptors appear outside the NMJ area more often in CP, although they were unable to find any change in transcriptional regulation [47,48]. However, KCNN3 was the 2nd most up-regulated gene on the entire chip and this gene plays a role in causing after-hyperpolarizations which may be a cellular attempt to limit the excessive motor unit firing that has been reported in spastic muscle [49]. Interestingly, KCNN3 is usually expressed in immature muscle and inhibited after innervation, which may indicate a sort of “immature state” of this muscle [50]. Although their localization in these samples is unknown the standard collagenous component of the synaptic basal lamina (COLA3; COLA4A4) was transcriptionally increased, the opposite activity of primary muscle basal lamina collagen (COLA1; COLA4A2) [51]. If these synaptic collagen IV subunits occurred outside the NMJ it would suggest a further degree of NMJ disorganization, alternatively they could be another indicator of muscle in an “immature state.”

We also uncovered significant evidence of altered calcium handling secondary to CP. Our data appear to reflect chronically increased intracellular calcium since the L-type voltage gated Ca2+ channel (CACNB1) was up-regulated (leading to activation of the ryanodine receptor) and leakage through the ryanodine receptor would be increased by down-regulation of two genes that prevent leakage (FKBP1A; PDE4D). Another "attempt" by the muscle to re-regulate [Ca]i can be inferred by the up-regulation of TRDN, which reclaims Ca2+ to the SR by localizing calsequestrin within the SR [52]. Chronically altered calcium levels and subsequent activation of the intramuscular calcium-activated proteases (Calpains) would cause dramatic muscle lesions, although they are not transcriptionally regulated in CP. Indeed, a relatively new class of Calpain-mediated myopathies has recently been described [53,54]. Perhaps in response to this chronic change in [Ca], a huge 63-fold increase in PVALB, a Ca2+ binding protein was induced in order to force muscle relaxation [55]. This dramatic adaptation could have significant effects on the [Ca]i and may even lower it below control levels and alter muscle contractile properties.

Of the proteins involved in calcium induced force generation, MHC isoforms are the most responsive to CP. They are primarily responsible for determining muscle fibers type [56] and undergo a transformation in the direction of a slow-to-fast phenotype. This shift included immature myosins, which saw large increases, although they were only significant in 2/3 algorithms, and lends further evidence to muscle in an "immature state." The many oxidative metabolic genes that are down-regulated in CP (Table 3) support this slow-to-fast transition. The ontology analysis revealed the loss of metabolic and mitochondrial related transcripts represented the majority of down regulated ontologies (Additional File 4 Table S3). Although previous research is mixed on whether spastic muscles become more fast or slow, our data is in concordance with recent research that fast fibers dominate spastic muscle in CP [25,34]. Paradoxically, this transformation occurs despite an overall increase in gene transcription related to the determination of the slow fiber phenotype, particularly calmodulin (CALM1) and calcineurin (PP33CA) [57]. A potential explanation may be that the dramatic PVALB expression actually leads to a decrease in intracellular calcium, thus turning off the initiation of the slow gene program. The validity of the calcineurin/NFAT pathway for transcription of a slow muscle fiber program has also come under question [58,59].

The slow fiber program represents only one segment of myogenesis that is controlled by many other genes. While the majority of the pathway elements (receptors, second messengers, signaling molecules) involved in myogenesis were not changed, two of the most important initial factors were both up-regulated – insulin-like growth factor (IGF1) and myostatin (GDF8). Interestingly they produce opposing effects on myogenesis with IGF1 leading to hypertrophy and myostatin opposing growth [60,61]. What this means for the net level of myogenesis is unclear.
Satellite cells are an important contributor to muscle growth, but their role in CP is difficult for us to discern as neither quiescent nor activated satellite cell markers were altered transcriptionally.

Muscle development was indicated in the ontology analysis (Additional File 2 Table S2) and some genes related to myogenesis were up-regulated (Table 3). While the muscle regulatory factors were not significantly altered, apparently fewer "growth" proteins must be activated since muscle growth in children with CP is decreased [10]. The reduction of parallel growth would lead to decreased muscle strength in CP patients. Reduced longitudinal growth would limit range of motion, and this has been suggested as the cause for extraordinarily long in vivo sarcomere lengths in children with wrist flexion contractures [12]. The increase in GDF8 could be responsible for this lack of growth in spastic CP muscle and thus represent a potential therapeutic target. Other evidence pointing toward muscle degradation is in the expression ontology of protein ubiquitination being increased, based on the up-regulation of 4 related genes (FBXO3, PCNP, RBBP6, and UBE2V2) and supported by an up-regulation of CACYBP, a gene involved in calcium dependent ubiquitination. The opposing actions of IGF1 to increase muscle mass are also controlled by a number of IGF binding proteins and we revealed IGFBP5 was significantly up-regulated in CP, however the effects of IGFBP5 in muscle have been questioned [62,63]. These results make the activation of the IGF1 pathway difficult to decipher at the transcriptional level. Furthermore, the hypertrophic effect of IGF1 is primarily from an increase in translation efficiency, which could have broad effects but would be unobservable in our study.

One of IGF1's broad anabolic effects could be a contribution to the increased ECM in muscle from CP patients [64,65]. While the ECM is altered transcriptionally, it is unclear which components are most affected. The fibrillar components of collagen in muscle are primarily collagen types I and III and each alpha chain of these collagen types were slightly up-regulated. The most dramatic changes were in the collagens of the basal lamina discussed in reference to the NMJ. Overall the basal lamina has been demonstrated as an area of excessive growth, and thus may be important in understanding muscle pathology [13]. Gene ontology analysis revealed a set of genes associated with the ECM that were all significantly up-regulated (Additional File 2 Table S2). This supports the hypothesis of a prolific ECM in spastic muscle of CP patients. The decreased transcription of basigin (BSG; 0.48) could also lead to extensive ECM through the reduced activation of MMPs [66]. Basigin may also imply a disorganized ECM lacking full functionality as MMP activity is usually increased along with increases in ECM production. However TIMP's are the primary MMP inhibitors and did not show a corresponding transcriptional increase [67].

It is important to note the distinct pathology of CP, as spastic muscle does not fit neatly into any of the other "altered use" muscle models [68]. The transcriptional control of muscle in CP was qualitatively different compared to DMD, IMB, or HSP (Table 4). DMD actually showed the most similarity to CP, particularly among contractile genes and satellite cell markers. DMD is known to have activated satellite cells and regenerating muscle and this correlation implicates the same in CP, although no satellite cell activation markers were significantly up-regulated in CP. IMB and HSP had an opposing effect on satellite cells, which shows this is not consistent with all disease states. IMB has been shown to result in muscle atrophy, fibrosis, and a shift from slow to fast muscle fibers. Although we were not able to compare our results to a human overactivity microarray study we clearly did not show the increase in slow fibers and mitochondrial transcripts expected. In fact HSP, which may be expected to the most similar to CP, resulted in little correlation (R < 0.1) with CP in all pathways except ECC. This suggests that ECC alterations may be a defining characteristic of spastic muscle. It is also interesting to note that, in the other cases, IGF1 and GDF8 acted alternatively – IGF1 increased while GDF8 decreased in DMD and conversely for IMB and HSP. This highlights the unique adaptation of CP, where myogenesis is turned on and off simultaneously.

While we are able to demonstrate the transcriptional effects of CP we also investigated this effect on two separate muscles and at different levels of clinical severity. Tendon transfer surgery is relatively common procedure for CP patients and is implicated when there is a muscular imbalance around a joint. It involves transferring the distal tendon of a muscle on the side of a joint considered to have a contracture or relative over activity to a tendon on the opposing side of the joint. Transfer of FCU to ECRB to correct wrist position is one of the common tendon transfer surgeries. Thus FCU is considered the more pathologic muscle and we might have expected a different transcriptional profile. However, we were unable to show any transcriptional differences between the muscles, indicating that both wrist flexors and extensors have a similar adaptation to CP. While the FCU is known to exhibit contractures in CP, we conclude that the contracture is developed due to its architecture, not due to a fundamental difference is secondary adaptation to the altered neuronal input of CP. The FCU is a larger muscle than the ECRB and the larger wrist flexor muscles may simply dominate the disease state based on their size. We were also unable to show significant transcriptional differences among various clinical severity scores in CP patients. This may be because CP transcriptional profiles are either on or off. More likely
our study was unable to resolve a severity effect as the study is biased towards the most severe cases (patients recruited based on corrective surgery) or the study is simply underpowered. We would likely need more patients across the range of clinical severity scores to define the genes most closely correlated with severity. However the low power of the severity analysis is increased in our comparisons of CP vs. control muscle. Further, a discussion of statistical power does not apply to significant differences detected in CP vs. control muscle. We do acknowledge, however, that we are clearly not detecting all transcripts that are altered in CP.

Our study has some inherent limitations, one of which is the small sample size noted above, especially in the case of control patients. As with any human study there is a high degree of heterogeneity among the samples. These patients have been treated in a variety of ways, and it is important to note that our transcriptional profile is not solely based on CP, but includes conservative treatment. We must also point out that this muscle is in a chronic disease state, making it difficult to discern the primary effects of CP from compensatory mechanisms that have taken place. As with any GeneChip study, we discuss only transcriptional control and any observation is subject to post-transcriptional modification.

Despite these inherent limitations we have been able to highlight areas where future work on spastic CP muscle may lead to innovative therapies. Our altered calcium handling data points to chronically elevated calcium levels which are highly dangerous since they may activate endogenous proteases. Fortunately a variety of calcium channel blockers have been developed and tested which could be of use in treating CP. Another potential application of current techniques could come from antifibrotic therapy to combat the increase in ECM components which is suggested by the transcriptomes. Of the most promising may be myostatin inhibitors, currently under investigation, since growth is inhibited in muscle from CP patients and myostatin, a major inhibitor of muscle growth is significantly up-regulated. This transcriptional study helps point the way to these and other areas of protein modifications, cell signaling, and biomechanics where future investigations should be focused.

**Conclusion**

Dramatic transcriptional alterations occur in muscle secondary to CP. These transcriptional changes ultimately lead to derangement of the ECM components of spastic muscle along with alteration of transcripts involved in myogenesis. A number of genes alter their expression in order to create a slow-to-fast transition of MHC isoforms and metabolic profile. GeneChip analysis has also allowed us to demonstrate the many changes in Ca²⁺ handling occur in CP that was not suggested previously. Together we are able to postulate the mechanisms known to affect muscle function in CP and predict new ones. This will aid future research into CP muscle and therapies to treat CP patients.

**Abbreviations**


**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

LRS carried out the RNA isolation, qPCR experiments, genechip analysis, and drafted the manuscript. EP provided the biopsies and assisted in review of the manuscript. YH carried out the myosin heavy chain content experiments. SRW participated in critical review of the manuscript. HC provided expertise on CP and critical review of the manuscript. SS provided expertise on genechip analysis and critical review of the manuscript. All authors have read and approve of this manuscript.

**Additional material**

**Additional file 1**

Significantly altered genes in CP. List of all features with a significant p-value (< 0.05) using each preprocessing algorithm (MAS5, RMA, GCRMA). Ratio of CP/CTRL is determined using MAS5.

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**Additional file 2**

Significantly up-regulated Gene Ontologies in CP. List of all Gene Ontologies significantly up-regulated genes in CP. O: observed genes, E: expected genes, R: ratio of observed/expected, P: p-value.

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Hamstring contractures in children with spastic cerebral palsy result from a stiffer extracellular matrix and increased in vivo sarcomere length

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Non-technical summary Muscle spasticity, due to an upper motoneuron lesion, often leads to muscle contractures that limit range of motion and cause increased muscle stiffness. However, the elements responsible for this muscle adaption are unknown. Here we show that muscle tissue is stiffer in contracture compared to age-matched children, implicating the extracellular matrix (ECM). However, titin, the major load-bearing protein within muscle fibres, is not altered in contracture, and individual fibre stiffness is unaltered. Increased ECM stiffness is even more functionally significant given our finding of long in vivo sarcomeres which leads to much larger in vivo forces in muscle contracture. These results may lead to novel therapeutics for treating spastic muscle contracture.

Abstract Cerebral palsy (CP) results from an upper motoneuron (UMN) lesion in the developing brain. Secondary to the UMN lesion, which causes spasticity, is a pathological response by muscle – namely, contracture. However, the elements within muscle that increase passive mechanical stiffness, and therefore result in contracture, are unknown. Using hamstring muscle biopsies from pediatric patients with CP (n = 33) and control (n = 19) patients we investigated passive mechanical properties at the protein, cellular, tissue and architectural levels to identify the elements responsible for contracture. Titin isoform, the major load-bearing protein within muscle cells, was unaltered in CP. Correspondingly, the passive mechanics of individual muscle fibres were not altered. However, CP muscle bundles, which include fibres in their constituent ECM, were stiffer than control bundles. This corresponded to an increase in collagen content of CP muscles measured by hydroxyproline assay and observed using immunohistochemistry. In vivo sarcomere length of CP muscle measured during surgery was significantly longer than that predicted for control muscle. The combination of increased tissue stiffness and increased sarcomere length interact to increase stiffness greatly of the contracture tissue in vivo. These findings provide evidence that contracture formation is not the result of stiffening at the cellular level, but stiffening of the ECM with increased collagen and an increase of in vivo sarcomere length leading to higher passive stresses.

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Abbreviations ACL, anterior cruciate ligament; CP, cerebral palsy; ECM, extracellular matrix; UMN, upper motoneuron.
Introduction

Cerebral palsy (CP) describes a spectrum of movement disorders caused by upper motoneuron (UMN) lesions that occur in the developing brain (Rosenbaum et al. 2007). CP is the most common childhood movement disorder with a prevalence of 3.6 cases per 1000 in the US (Yeargin-Allsopp et al. 2008). Although the primary UMN insult is not progressive, the resulting muscle pathology does progress (Kerr Graham & Selber, 2003). Pathological muscle in CP is described as spastic, which is a velocity-dependent resistance to stretch due to reduced inhibition of the stretch reflexes (Crenna, 1998). Despite best clinical practices, children with CP often develop contractures that limit their range of motion, decrease their mobility and may be painful. While muscle spasticity and hyper-activity are commonly seen in cerebral palsy, contracture represents a unique muscle adaptation in which the muscle increases passive stiffness such that range of motion around a joint is limited without active force production of the muscle. Thus, muscle contractures represent a major disability to those affected by CP in particular and those with UMN lesions in general (Bache et al. 2003).

The skeletal muscle mechanism by which spasticity results in contracture is not known. Transcriptional data suggest many physiological pathways are altered in contracture (Smith et al. 2009). One consistent finding is that spastic muscles from children with CP are weaker than those of typically developing control children due to a combination of decreased neuronal drive, decreased muscle size and decreased specific tension (Elder et al. 2003; Rose & McGill, 2005; Stackhouse et al. 2005). Previous studies also demonstrated that increased resistance to stretch in spastic muscle has both an active and passive component (Sinkjaer & Magnussen, 1994; Mirbagheri et al. 2001; Lorentzen et al. 2010). However, the passive elements responsible for this increased stiffness have not been identified and these presumably represent the therapeutic targets of physical therapy (Wiart et al. 2008), surgery (Beals, 2001) and neurotoxin injection (Lukban et al. 2009). To date, these treatments do not prevent contracture formation (Tilton, 2006).

It should be noted that the term ‘contracture’ is typically referred to in the muscle physiology literature as an increase in tension of isolated muscles or fibres in response to external activation by caffeine or potassium (Savage & Atanga, 1988). Caffeine induces calcium release from the sarcoplasmic reticulum and potassium depolarizes the muscle as methods to activate the crossbridge cycle that produces muscle active tension (Conway & Sakai, 1960; Hodgkin & Horowicz, 1960). However, the common clinical use of the term ‘contracture’ does not refer to such activation. Rather, a clinical ‘contracture’ represents a condition where a muscle becomes extremely stiff, limiting range of motion, perhaps causing pain, and deforming joints. These contractures often result from upper motoneuron lesions such as those that occur after stroke, head injury or cerebral palsy and represent tremendous challenges to treat (O’Dwyer et al. 1996; Farmer & James, 2001). Often, clinical contractures result from chronic activation of a muscle, referred to as ‘spasticity’ and the net result is a stiff muscle that limits the range of motion around a joint in the absence of any active component of crossbridge cycling (Ferguson et al. 2007).

As muscle architecture is the most important determinant of muscle force-generating capacity and excursion, previous studies have sought to describe the macroscopic structural adaptation of muscle in CP. It has been suggested that contracture results from shortened muscles and thus multiple studies have used ultrasound technology to measure fascicle length in contractured muscle and, while these experiments confirm reduced CP muscle volume, evidence for shortened fascicles is inconclusive (Shortland et al. 2002; Malaya et al. 2007; Mohagheghi et al. 2007, 2008). A major drawback of ultrasound studies is that there is no normalization of fascicle length to sarcomere length so it is conceivable that a CP muscle and control muscle could have exactly the same fascicle lengths, yet have different numbers of sarcomeres in series and correspondingly different functional mechanical properties. This would be invisible to the ultrasound method. Direct measurement of intraoperative sarcomere length revealed that sarcomere lengths are indeed longer in CP muscle, suggesting increased passive stiffness (Lieber & Friden, 2002; Ponten et al. 2007).

Another proposed mechanism for increased passive stiffness in contractured muscle involves alteration of the tissue itself. Previous studies demonstrated that individual fibres from contractured muscles are stiffer than controls, indicating an alteration within the muscle cell (Friden & Lieber, 2003). This increased stiffness from within the fibre was hypothesized to arise from titin, considered the major passive load-bearing protein within the muscle fibre (Prado et al. 2005). Further studies confounded this result showing that bundles of fibres, which include extracellular matrix (ECM), from contractured muscles were more compliant compared to controls, and thus unable to explain the increased stiffness on the whole muscle scale (Lieber et al. 2003). A drawback of our previous mechanical studies is that they studied a variety of human muscles, and we have since shown that healthy human muscles have different passive mechanical properties (Ward et al. 2009b) as was shown for rabbit muscle (Prado et al. 2005).

To avoid complications that arise when making comparisons across different muscles, we have taken advantage of the fact that children who are undergoing
motor function classification system (Palisano et al. 1997) were classified based on clinical measures of the Gross Motor Function Classification System (GMFCS; Palisano et al. 1997) or popliteal angle measurements. The right columns are the number of subjects whose biopsies were used in the various analysis; many biopsies were used for multiple analysis: passive mechanics sample size (Pass Mech N), in vivo sarcomere length (SL), hydroxyproline (OH-pro), myosin heavy chain biopsy analysis (MyHC) and titin biopsy analysis (Titin).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Age</th>
<th>Sex</th>
<th>GMFCS</th>
<th>Popliteal angle</th>
<th>Pass Mech N</th>
<th>SL</th>
<th>OH-Pro</th>
<th>MyHC</th>
<th>Titin</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>19</td>
<td>15.8 ± 1.8</td>
<td>8 M</td>
<td>N/A</td>
<td>N/A</td>
<td>14</td>
<td>N/A</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CP</td>
<td>33</td>
<td>9.6 ± 4.2</td>
<td>23 M</td>
<td>I(2),II(13),III(2),IV(6),V(10)</td>
<td>114 ± 15</td>
<td>17</td>
<td>11</td>
<td>12</td>
<td>6</td>
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</table>

Patient parameters for the control and CP groups; control patients do not have Gross Motor Function Classification System (GMFCS; Palisano et al. 1997) or popliteal angle measurements. The right columns are the number of subjects whose biopsies were used in the various analysis; many biopsies were used for multiple analysis: passive mechanics sample size (Pass Mech N), in vivo sarcomere length (SL), hydroxyproline (OH-pro), myosin heavy chain biopsy analysis (MyHC) and titin biopsy analysis (Titin).

Methods

Muscle biopsy collection

Ethical approval for this study conformed to the standards of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of California, San Diego Human Research Protection Program. After obtaining consent from parents and age-appropriate consent from children, subjects with CP (n = 33) were recruited for this study because they were undergoing hamstring lengthening surgery that involved gracilis and semitendinosus muscles. Control children (n = 19) with no history of neurological disorder were recruited because they were undergoing ACL reconstructive surgery with a hamstring autograft. Muscle biopsies were obtained from the same hamstring muscles from children with CP undergoing surgery. We hypothesized that the passive mechanical properties of spastic muscle are altered in CP and that this could arise across the levels of: protein (titin), cellular (fibre), tissue (fibre bundle including ECM), and/or architecture (sarcomere length). This work will provide further insight into the debilitating mechanism of muscle contracture and drive research on targeted therapies to treat contractures.

Muscle mechanical testing

Biopsies for mechanics were stored in a glycerinated relaxing solution overnight, composed of (mM): potassium propionate (170.0), K3EGTA (5.0), MgCl2 (5.3), imidazole (10.0), Na2ATP (21.2), NaN3 (1.0), glutathione (2.5), 50 μM leupeptin, and 50% (v/v) glycerol. For dissection of fibre or fibre bundle samples, muscles were removed from storage solution and transferred to a relaxing solution at pCa 8.0 and pH 7.1 consisting of (mM): imidazole (59.4), KCH3O2S (86.0), Ca(KCH3O2S)2 (0.13), Mg(KCH3O2S)2 (10.8), K3EGTA (5.5), KH2PO4 (1.0), Na2ATP (5.1), and 50.0 μM leupeptin. Single fibre segments (1.5–3 mm in length) were carefully dissected and mounted in a chamber in a custom apparatus at room temperature (20°C). Fibres were secured using 10-0 monofilament nylon suture on a muscle mechanical testing apparatus (Biopac Inc., Goleta, CA, USA). Force and length were recorded at 20 Hz and filtered at 10 Hz. Force vs. length relations were plotted, and the slope of the initial linear rise was used as an estimate of passive tension (P0).

In vivo sarcomere lengths

Custom muscle biopsy clamps, modified for pediatric use with 0.5 cm jaw spacing were used to determine in vivo sarcomere length (Fig. 1). We previously validated this method against intraoperative laser diffraction (Ward et al. 2009a). After skin incision and prior to lengthening, gracilis and semitendinosus were identified. A small segment of each muscle was atraumatically isolated by blunt dissection. The custom clamp was then slipped over the bundle, with care to prevent undue tension on the muscle. The child’s leg was positioned with 90 deg of hip flexion and 90 deg of knee flexion, and neutral hip abduction–adduction, the clamp was engaged, and the section of muscle within the jaws of the clamp was re-sected and immediately placed in Formalin to fix the biopsy specimen in its in vivo configuration. After 2 days of fixation, muscle bundles were isolated on glass slides and sarcomere length was measured by laser diffraction (see below). For control patients receiving ACL reconstruction, hamstring muscles are not accessible in their in vivo position, which precludes the use of the biopsy clamps to obtain control values. Thus, for estimation of control sarcomere lengths, we extracted these values from our previous musculoskeletal model (Arnold et al. 2010).
one end to a force transducer (Model 405A, sensitivity 10 V g$^{-1}$, Aurora Scientific, Ontario, Canada) and on the other end to a titanium wire rigidly attached to a rotational bearing (Newport MT-RS; Irvine, CA, USA; Supplementary Fig. S1). Segments displaying obvious abnormalities or discoloration were not used. The sample was transilluminated by a 7 mW He–Ne laser to permit sarcomere length measurement by laser diffraction (Lieber et al. 1984). Resolution of this method is approximately 5 nm (Baskin et al. 1979). The system was calibrated with a 2.50 µm plastic blazed diffraction grating prior to experimentation (Diffraction Gratings, Inc., Nashville, TN, USA).

The fibre was brought to slack length, defined when passive tension was just measurable above the noise level of the force transducer. Sample dimensions were measured optically with a cross-hair reticule mounted on a dissecting microscope and micromanipulators on an x–y mobile stage. The fibre was then loaded with strains of approximately 0.25 µm sarcomere$^{-1}$ at 100 fibre lengths s$^{-1}$. Each stretch was held for 2 or 3 min during which stress relaxation was measured, before a sequential stretch was made. Fibres were stretched in total to approximately 100% strain and were saved for titin analysis after mechanical testing. Force data were converted to stress by dividing force by the baseline cross-sectional area value determined assuming a cylindrical sample with an average diameter determined from three separate points along the fibre. Samples were discarded if they did not produce a clear diffraction pattern, if any irregularities appeared along their length during testing, or if they were severed or slipped at either suture attachment point during the test. Muscle bundles were mechanically tested in the same manner as fibres and consisted of approximately 20 fibres and their constitutive ECM.

**Mechanical data analysis**

All analysis was performed using Matlab (Mathworks Inc., Natick, MA, USA). Relaxed stress after 2 or 3 min was used to fit a relaxed stress vs. sarcomere length curve. This curve was fitted with a line for fibres, but with a quadratic for bundles, as there was notable non-linearity in bundle data. For sample fits, sarcomere lengths below slack length are assigned a stress of 0. This produces a ‘toe region’ due to averaging of the fits across the range of slack sarcomere lengths tested in fibres, generally below 2.5 µm sarcomere length. Only the data beyond the toe region in which most fibres are generating tension are depicted (Fig. 2A and C). Tangent modulus was calculated at given sarcomere length by taking the derivative of the relaxed stress vs. sarcomere length fit at that length. Comparisons of tangent
stiffness were conducted with a three-way ANOVA for pathology (CP vs. control), muscle (gracilis vs. semitendinosus; repeated measure), and scale (fibre vs. bundle; repeated measure) with results considered significant at $P < 0.05$. All data are presented in the text as mean ± SEM unless otherwise noted.

**Protein gels**

Titin isoform and MyHC content were analysed on gels from both single fibres after mechanical experiments and from sections of biopsies. Single fibres were stored at $-80^\circ$C until analysed and boiled for 2 min in 10 µl sodium dodecyl sulfate-vertical agarose gel electrophoresis (SDS-VAGE) sample buffer (comprised of 8 M urea, 2 M thiourea, 3% SDS w/v, 75 mM dithiothreitol (DTT), 0.03% bromophenol blue and 0.05 M Tris-Cl, pH 6.8; Warren et al. 2003). For biopsies, a myofibril-rich fraction (~10 mg wet weight) of individual biopsies ($n = 24$ biopsies from 12 patients for titin and MyHC) was homogenized in sample buffer using the Bullet Blender (Next Advance, Inc., Averill Park, NY, USA).

![Figure 2. Passive tension as a function of sarcomere length for fibres and bundles, after stress relaxation](image)

Plots represent the average of the fits from each individual sample ± SEM. The stress vs. sarcomere length fit was linear for fibres with a $R^2$ value of 0.962 ± 0.003 (A and B) and quadratic for bundles with a $R^2$ value of 0.985 ± 0.002 (C and D). A, gracilis fibres show no difference between CP and control. B, semitendinosus fibres show no difference between CP and control. C, CP gracilis bundles show a significant increase in stress at high sarcomere lengths compared to control. D, CP semitendinosus bundles show a significant increase in stress at high sarcomere lengths compared to control.* Inside symbol designates the approximate sarcomere length at 90 deg of hip and knee flexion.
To quantify titin isoforms, the molecular mass of titin in muscle samples was determined using SDS-VAGE. An acrylamide plug was placed at the bottom of the gel to hold the agarose in place. The final composition of this plug was 12.8% acrylamide, 10% v/v glycerol, 0.5 M Tris-Cl, 2.34% N,N′-diallyltartardiamide, 0.028% ammonium persulfate and 0.152% tetramethylethlenediamine (TEMED). The composition of the agarose gel was 1% w/v SeaKem Gold agarose (Lonza, Basel, Switzerland), 30% v/v glycerol, 50 mM Tris-base, 0.384 M glycine and 0.1% w/v sodium dodecyl sulfate (SDS). Titin standards were obtained from human cadaver soleus (3700 kDa) and rat cardiac muscle (2992 kDa). The standard titin molecular masses are based on sequence analysis of the 300 kb titin gene with a coding sequence contained within 363 exons (Labeit & Kolmerer, 1995; Freiburg et al. 2000). These sequences were also homogenized and stored at −80°C until analysis. Before loading onto the gel, a titin standard ‘cocktail’ was created with the following ratio: 1 unit of human soleus standard:3 units rat cardiac standard:6 units sample buffer. Sample wells were then loaded with both biopsy and soleus standard:3 units rat cardiac standard:6 units sample buffer consisting of DTT (100 mmol l$^{-1}$), SDS (2%), Tris-base (80 mmol l$^{-1}$) pH 6.8, glycerol (10%) and bromophenol blue (0.01% w/v). Samples were boiled (2 min) and stored at −80°C. Before loading onto the gel, protein was further diluted 1:15 (0.008 µg µl$^{-1}$) in the same sample buffer to account for the approximately 50-fold greater sensitivity of the silver stain. Ten microlitres of each sample were loaded in each lane. Total acrylamide concentration was 4% and 8% in the stacking and resolving gels, respectively (bisacrylamide, 1:50). Gels (16 cm × 22 cm, 0.75 mm thick) were run at a constant voltage of 275 V for 22 h at 4–6°C. Gels were silver stained (BioRad, Hercules, CA, USA). MyHC bands were identified and quantified with densitometry (GS-800, BioRad). The progression of the band was compared and identified based on its relative molecular weight to that of a human protein standard prepared (as described above) from a normal semitendinosus biopsy that showed all three human MHC bands (IIa, IIx and I).

**Hydroxyproline content**

Collagen percentage was determined using a colourimetric analysis of hydroxyproline content. Briefly, muscle samples were hydrolysed in 6 N HCl for 18 h, neutralized, and samples were treated with a chloramine T solution for 20 min at room temperature followed by a solution of p-diaminobenzenalddehyde for 30 min at 60°C. Sample absorbance was read at 550 nm in triplicate and compared to a standard curve to determine the hydroxyproline content. Hydroxyproline content was converted to collagen using a constant (7.46) that defines the number of hydroxyproline residues in a molecule of collagen.

**Immunohistochemistry**

Biopsies previously snap-frozen in isopentane were used for immunohistochemistry. Cross-sections (10 µm thick) taken from the midportion of the tissue block were cut on a cryostat at −25°C (Microm HM500, Walldorf, Germany). Serial sections were stained with haematoxylin–eosin to observe general tissue morphology. To investigate ECM components sections were labelled with primary antibodies to fibrillar collagen type I (rabbit polyclonal, Rockland, Gilbertsville, PA, USA) and laminin (rabbit polyclonal, Sigma, St Louis, MI, USA). The secondary antibody used for visualization was an Alexa Fluor 594 goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA, USA).

Fibre cross-sectional areas were measured from laminin-stained slides using a custom-written macro in ImageJ (NIH, Bethesda, MD, USA). Filtering criteria were applied to insure measurement of actual muscle fibres. These criteria rejected regions with areas below 50 µm$^2$ or above 5600 µm$^2$ to eliminate neurovascular structures and ‘optically fused’ fibres, respectively. Fibres touching the edge of the field were excluded as they were assumed to be incomplete. Regions with circularity below 0.30 or above 1.0 were excluded to prevent inclusion of fibres that were obliquely sectioned.

**Results**

**Passive mechanics**

Passive mechanical properties were determined for three fibres per muscle and two muscles per subject, for both control children ($n=14$) and children with CP ($n=17$). Fibre diameter was smaller for CP (66.3 ± 2.6 µm) than for control (80.2 ± 2.6 µm; $P<0.001$) as previously described (Fridén & Lieber, 2003). Slack sarcomere length for control (2.31 ± 0.04 µm) fibres was not significantly different from CP (2.31 ± 0.04 µm (SD); $P>0.9$) fibres. The stiffness of CP fibres was not significantly different from control for gracilis (control 21.5 ± 1.8 kPa µm$^{-1}$; CP 21.5 ± 2.4 kPa µm$^{-1}$; Figs 2A and 3A) or semitendinosus...
Passive mechanical properties were determined for three bundles on the same biopsy as for fibres. Fibre bundle diameters were not significantly different between control (366.1 ± 15.0 µm) and CP (354.9 ± 15.2 µm; P > 0.4) bundles, nor were slack sarcomere lengths (control: 2.27 ± 0.03 µm; CP 2.29 ± 0.03 µm; P > 0.5). CP bundles had higher stresses at longer sarcomere lengths for both gracilis and semitendinosus muscle. When comparing the tangent stiffness at 4.0 µm the stiffness of CP bundles was significantly greater than control (P < 0.05) for both gracilis (control 36.1 ± 3.9 kPa µm⁻¹; CP 60.4 ± 11.8 kPa µm⁻¹; Figs 2C and 3B) and semitendinosus (control 25.2 ± 2.9 kPa µm⁻¹; CP 40.7 ± 4.9 kPa µm⁻¹; Figs 2D and 3B).

The three-way ANOVA with muscle (semitendinosus/gracilis; repeated measure), scale (fibre/bundle; repeated measure) and condition (CP/control) on tangent stiffness revealed a main effect of all three independent measures (P < 0.05 for condition and muscle; P < 0.001 scale). The results also showed a significant interaction between muscle and scale (P < 0.05), with post hoc tests revealing gracilis bundles are stiffer than semitendinosus, and a significant interaction of condition and scale, with post hoc tests revealing an effect of CP only at the bundle level for semitendinosus (P < 0.05).

To determine whether the mechanical changes were related to the clinical observations made on the patients, stiffness was correlated with clinical severity score. There was no significant correlation between stiffness and either Gross Motor Function Classification System or popliteal angle at either the fibre or bundle level (Supplemental Fig. 2A). To determine consistency within patients, a correlation was run between gracilis and semitendinosus stiffness within the same patient, or fibre stiffness to bundle stiffness within the same biopsy, but again there were no significant correlations (Supplemental Fig. 2B and C). There was also a concern that there might be an age effect since the control subjects were slightly older than CP subjects (Table 1) but no significant correlation was found, validating the comparison of control to CP subjects with different ages (Supplemental Fig. 2D).

Further, the mechanics were compared from patients who underwent a previous hamstring lengthening surgery or botulinum toxin injection prior to biopsy as these may affect stiffness. No significant difference was observed for prior botulinum toxin injection for bundles (P > 0.3) or fibres (P > 0.8). Only one patient with mechanics measured had undergone a previous lengthening surgery, which was not an outlier among any mechanical measure.
In vivo sarcomere lengths

In vivo sarcomere length from cerebral palsy patients of contractured hamstring muscles \( (n = 22) \) was 3.54 ± 0.14 µm for gracilis and 3.62 ± 0.13 µm for semitendinosus at 90 deg of hip and knee flexion. For control comparison, model results were used as described in Methods (Arnold et al. 2010). Both gracilis and semitendinosus had significantly longer sarcomere lengths at 90 deg of hip and knee flexion than predicted by the model by about 0.5 µm (Fig. 4A, \( P < 0.05 \) for semitendinosus and gracilis). Combining the sarcomere length values with passive mechanical properties demonstrates that CP muscle tissue at these joint angles bears a higher passive load compared to control muscle (Fig. 3C).

To determine whether the sarcomere length was associated with functional changes, clinical measures were correlated to sarcomere length. There was a significant correlation between in vivo sarcomere length and both Gross Motor Function Classification System (\( P < 0.05 \)) and in vivo sarcomere length (\( P < 0.05 \)), indicating that more severely involved patients had longer in vivo sarcomere lengths (Fig. 4B). There was also a significant negative correlation between popliteal angle and in vivo sarcomere length indicating that longer sarcomere lengths were present in joints with more severe contractures (Fig. 4C). Together these correlations provide further evidence that in vivo sarcomere lengths are elevated in CP.

To compare predicted in vivo stiffness of CP muscles compared to control we also evaluated the tangent stiffness at the average in vivo sarcomere length of 90 deg of hip and knee flexion for each muscle and condition from the data above. Combining the in vivo sarcomere lengths with the mechanical data shows that in vivo stiffness is predicted to be much larger for CP muscle (Fig. 3C). As fibres had linear stress–strain relationships, the tangent stiffness does not vary with sarcomere length and is thus the same result as the tangent stiffness at 4.0 µm.

Titin isoforms

To determine if titin size was related to overall muscle stiffness we measured titin molecular mass from a biopsy. The results of a two-way ANOVA showed no significant difference (\( P > 0.05 \)) for CP with mean values of gracilis (control 3588 ± 18 kDa; CP 3667 ± 22 kDa) and semitendinosus (control 3625 ± 19 kDa; CP 3658 ± 26 kDa; Fig. 5) among the samples measured (\( n = 24, 6 \) per muscle condition). The mass of CP titin was actually larger than that of control suggesting, if anything, a more compliant isoform and unable to account for any increased passive stiffness of the muscle as a whole.

The effect of titin isoform was also investigated on single fibres that had previously undergone passive mechanical testing. A two-way ANOVA showed no significant difference (\( P > 0.1 \)) between titin isoform sizes for gracilis (control 3758 ± 24 kDa; CP 3772 ± 36 kDa) or semitendinosus (control 3729 ± 40 kDa; CP 3797 ± 40 kDa) among the single fibres measured (\( n = 55 \)). CP fibres having equivalent titin isoform size to control fibres is consistent with the fact that CP and control fibres have equivalent stiffness. The effect of titin isoform size on the variability in mechanical stiffness of fibres was also investigated, but there was not a significant correlation between titin size and fibre stiffness within single fibres.
(Supplemental Fig. S3). The molecular masses are larger for single fibres than for the whole biopsies, possibly due to modified preparation methods.

Collagen content

Collagen content of the biopsies was measured \((n = 40, 10\) per muscle per condition) as collagen is thought to be the primary load-bearing structure of the ECM within muscle (Purslow, 1989). CP muscles had significantly higher collagen concentrations in both gracilis (control \(8.0 \pm 1.6 \mu g\) (mg wet weight)\(^{-1}\)); CP \(11.2 \pm 2.6 \mu g\) (mg wet weight)\(^{-1}\)) and semitendinosus (control \(4.0 \pm 0.3 \mu g\) (mg wet weight)\(^{-1}\)); CP \(8.8 \pm 0.8 \mu g\) (mg wet weight)\(^{-1}\)) as determined by a two-way ANOVA on muscle and condition (Fig. 6, \(P < 0.05\)). Collagen content was elevated in both gracilis and semitendinosus, although post hoc tests revealed a significant difference only in semitendinosus \((P < 0.001)\). Gracilis also tended to have higher collagen concentrations corresponding to the relationship seen in passive bundle stiffness. The collagen content was not significantly different for patients who underwent a previous lengthening surgery \((P > 0.5)\) or botulinum toxin injections \((P > 0.8)\) prior to biopsy.

Collagen was also visualized by immunohistochemistry. Qualitative results show an increase in fibrillar collagen type I (Fig. 7A–D) in muscle from children with CP, corresponding the hydroxyproline results. There was no apparent mislocalization of collagen; however, an increased frequency of large collagen deposits was observed. Laminin, a component of the basal lamina, also showed marked increase in CP muscle (Fig. 7E–H). These results demonstrate an increase of ECM material that includes, but is not limited to collagen. While histological evidence shows an increase in ECM material in muscle from children with cerebral palsy, there is also a corresponding decrease in fibre cross-sectional area (from \(3141 \pm 375 \mu m^2\) for controls to \(1255 \pm 226 \mu m^2\); \(P < 0.001\) for cerebral palsy) as has been previously reported (Fridén & Lieber, 2003).

Myosin heavy chain

To determine whether any of the single mechanical fibre data might be confounded by systematic differences in muscle fibre type between patient populations, myosin heavy chain isoform content was measured \((n = 35\) fibres). One-way ANOVA comparing tangent stiffness of different fibre types did not produce a significant result for either CP or control fibres \((P > 0.05;\) Supplemental Fig. S4). To determine the distribution of different fibre types for hamstring muscles in CP myosin heavy chain content was measured from a sample of biopsies \((n = 24, 6\) per muscle per condition). CP muscles had increased slow myosin heavy chain expression (gracilis – control \(29.3 \pm 1.9\%\) to CP \(40.0 \pm 2.5\%\); semitendinosus – control \(29.7 \pm 1.7\%\) to CP \(41.0 \pm 3.3\%;\) \(P < 0.001;\) Fig. 8), but there was no significant change in either of the fast isoforms measured (IIa or IIx). Since myosin heavy chain is the primary determinant of fibre type, these results demonstrate a shift to slower fibres in CP muscle. There was no significant difference between gracilis and semitendinosus muscles.

Discussion

The most significant finding of this study is that muscle tissue from children with CP is significantly stiffer compared to typically developing children. This increased
Figure 7. Immunohistochemistry of muscle biopsies show qualitatively increased levels of ECM in CP (B, D, F and H) compared to control (A, C, E and G) children.

Representative images with primary antibody to fibrillar collagen type I in cross section (A and B) and longitudinal section (C and D). Representative images with primary antibody to laminin of the basal lamina, in cross section (E and F) and longitudinal section (G and H). Note that muscle fibers from children with CP are slightly smaller with a great amount of Collagen I and laminin, two of the major components of the extracellular matrix. Scale bars represent 100 µm.

Figure 8. Myosin heavy chain isoforms

There was a significant increase in myosin heavy chain I in CP muscles compared to control suggesting contractured fibres have a slower phenotype. There was no significant difference between muscles. *represents significant difference in MyHC I percentage between control and CP muscles ($p < 0.001$).
passive stiffness is accompanied by an increase in collagen content and is made even more functionally significant in that in vivo sarcomere length of CP hamstring muscles is significantly longer compared to predictions for control children. Taken together, these data provide a mechanistic explanation for the increased joint and muscle stiffness observed in these contracture patients. While fibre bundles were different between CP and control muscle, we found no significant difference in mechanical properties at the single fibre level of muscle and no change in titin isoform size. Thus, we conclude that, for human hamstring muscles, increased passive tension in contracture is due to a change in ECM stiffness and increased in vivo functional sarcomere length rather than any intracellular alteration.

**Bundle mechanics**

While fibres contribute to passive tension of muscle, muscle ECM plays an important role in passive mechanics, especially at longer sarcomere lengths. The results show a significant increase in the tangent stiffness of fibre bundles from CP patients. While fibres were fitted well with a linear stress–sarcomere length relationship, fibre bundles required a non-linear quadratic fit. This non-linearity results in similar tissue stiffness at small strains, but significantly increased stiffness at long sarcomere lengths of the CP muscle tissue. Muscle contractures often limit joint range of motion suggesting that there are large in vivo strains on the muscle. We thus believe that, in vivo, the ECM bears a large portion of the passive muscle load.

Collagen is considered the primary load-bearing structure within muscle ECM (Purslow, 1989). We hypothesized that an increase in collagen content of CP muscle could lead to the increased passive stiffness seen in bundles. Using a hydroxyproline assay to test the hypothesis, the results demonstrate a significant increase in collagen within CP muscle. Although these data are presented in micrograms of collagen per milligram of muscle wet weight, they are similar to previous data presenting collagen as a percent of dry weight (Bendall, 1967), using the assumption that muscle is approximately 80% water (Ward & Lieber, 2005). Increased collagen was also observed by immunohistochemistry along with another ECM component, laminin, a critical component for cellular attachment to the basal lamina. These results are in agreement with a previous study showing increased collagen content within CP muscle (Booth et al. 2001). Many additional factors may be playing a role in the increased ECM stiffness. The organization of collagen, the distribution of collagen types, or the proteoglycan content all could be altered in CP to create a stiffer ECM and represent areas of further investigation. Immunohistochemistry of other muscle proteins (α-actinin, desmin, dystrophin) revealed no obvious differences between patient groups.

One previous study investigated the mechanics of fibre bundles from contractured muscle tissue (Lieber et al. 2003). Despite finding stiffer fibres we found more compliant bundles in CP muscle. This result is difficult to reconcile with the increased passive stiffness of the whole muscle that has been reported. The previous study was conducted on biopsies taken from various muscles that were not matched between populations, which could account for some of these differences and all muscles were from upper extremities, which may respond differently to spasticity. In addition, the ECM from upper extremity muscles was highly deranged in the contractured muscles (see Fig. 2 of Lieber et al. 2003) making area fraction measurements from these specimens difficult. It is possible that the area fraction of ECM was overestimated, resulting in artificially low values for bundle modulus. Finally, the non-linear behaviour of upper extremity muscles was quantified by only fitting data to the linear portion of the sarcomere length–stress curve. The current method represents a more accurate method for handling analysis of the non-linear relationships.

**Sarcomere organization**

Sarcomere length operating ranges of semitendinosus and gracilis muscles are unknown, although muscles are typically believed to operate on the plateau of the length–tension curve (2.5–2.7 μm for human skeletal muscle; Walker & Schrodt, 1974). A previous study demonstrated that spastic muscle operates at longer sarcomere lengths than control (Lieber & Fridén, 2002), which would lead to a larger observed passive stiffness such as that seen in contracture. We measured in vivo sarcomere lengths of patients with CP at a defined joint angle and compared these values to sarcomere lengths of control subjects that were calculated based on in vivo sarcomere lengths, moment arms and muscle–tendon lengths (Arnold et al. 2010). The results showed that the CP sarcomere lengths are significantly longer than those predicted from the model. The lengths measured were also much longer than optimal sarcomere length, lending further evidence to the idea that they are overly stretched in contracture. With CP subjects operating at longer lengths of the passive length–tension relationship, this means that the muscle is experiencing higher stresses not only due to material property changes, but also due to this shift along the passive length–tension curve. This difference becomes more pronounced as the knee extends and the hip flexes, and may limit range of motion for children with contractures.

It is often stated that muscle adds or subtracts serial sarcomeres to optimal sarcomere length in vivo (Williams
& Goldspink, 1973). Long in vivo sarcomeres suggest an inability of the muscle to add sarcomeres in series, which would be exacerbated during growth spurts, which have been associated with the onset of muscle contractures (Janice & Alwyn, 2005). The very long sarcomere lengths observed in vivo clearly imply that muscles from children with CP are under high stress. The source of the force that creates or opposes this stress is not known. However, we have speculated, based on analysis of the transcriptome, that muscles from children with CP are unable to grow serially in response to the stretch imposed by osteogenesis (Smith et al. 2009). It is also possible that muscles would decrease their serial sarcomere number, which would provide a resistive force since the changes could be slow and accompanied by reinforcement of the muscle fibre by the ECM. Muscle contracture is often described as a ‘shortened’ muscle; our finding of increased in vivo sarcomere length corresponds with the notion that muscle shortening is derived from fewer series sarcomeres, not shortened sarcomeres. Longer in vivo sarcomere lengths are an important factor for both passive and active force production of skeletal muscle. Previous research has demonstrated that muscles from children with CP are smaller than those of control children, yet muscle force production is reduced to an even greater extent indicating a dysfunction of active muscle force production in CP (Elder et al. 2003; Moreau et al. 2010). A consequence of having longer in vivo sarcomere lengths for children with CP is the muscle will be working at different portions along its active length–tension curve (Gordon et al. 1966) compared to control subjects. Based on measured human filament lengths (Walker & Schodt, 1974) and the increase in \( \sim 0.5 \mu m \) sarcomere length, the decrease in force from a typically developing child on the plateau of the length–tension curve to a child with CP on the descending limb would be 33%. It is interesting to note that this is on the same scale as the reduction in force that is not accounted for by decreased muscle size in these patients (Elder et al. 2003; Moreau et al. 2010). Thus, perhaps altered in vivo sarcomere length operating range represents a significant functional alteration in muscles from children CP and demonstrates that these muscles are not simply changing sarcomere number to ‘re-optimize’ the muscle after injury.

**Potential mechanisms of contracture formation**

It is possible that the changes in ECM and in vivo sarcomere length take place simultaneously and independently, or that one precedes and directly affects the other. If these two alterations of CP muscle are not causal, they could be a consequence of the same factors within spastic muscle. Previous research showed that myostatin, a negative regulator of muscle growth, also stimulates proliferation of muscle fibroblasts and the release of ECM proteins (Li et al. 2008). Myostatin mRNA has also been shown as significantly increased in CP muscle of the upper extremity (Smith et al. 2009). Alternatively, transforming growth factor-β1 has been shown to induce a shift in satellite cells from a myogenic lineage to fibroblasts (Li et al. 2004). This process also has the potential to limit growth through satellite cell depletion and increase the ECM secreting cell population.

Longer in vivo sarcomeres of CP muscles demonstrate that there is increased sarcomere strain, which has been shown to directly induce skeletal muscle injury (Patel et al. 2004). Repeated strain-induced injuries have been shown to drastically increase collagen content and fibrosis in skeletal muscle (Stauber, 2004). The effects of chronic strain injuries persist for months or even years and could be responsible for the effects of muscle in contracture. Repeated strain-induced injury also results in lower force-producing capacity of muscle, which may provide another explanation for the reduced specific tension of CP muscle (Proske & Morgan, 2001). The increased fibrosis and stiffness of muscle contracture could also be a compensatory mechanism to limit further strain-induced injury.

There is also potential for a fibrosis induced from spasticity to lead directly to a limitation of longitudinal growth. Satellite cells responsible for muscle growth rely on migration across the basement membrane during activation with the release of matrix metalloproteases (Chen & Li, 2009). Skeletal muscle fibrosis could impede muscle regeneration by forming a mechanical barrier to this process (Chen & Li, 2009). Stem cell differentiation is also sensitive to the elasticity of the matrix in which it is embedded (Engler et al. 2006). Our study demonstrated an altered stiffness of the ECM in contracture tissue that could lead to an inhibition of satellite cell activation or proliferation (Boonen et al. 2009; Gilbert et al. 2010) and perhaps even predispose muscle stem cells to differentiate toward the fibroblast lineage. It is also possible that fibrosis and lack of growth create a vicious cycle that leads to muscle contracture.

**Titin isoforms**

Since titin isoform size is related to muscle passive tension (Prado et al. 2005), we hypothesized that shorter titin isoforms would be present in muscle contracture leading to increased stiffness. However, our results show no difference in titin size between CP and control muscles. Thus, we conclude that titin is not altered in CP to cause contracture, at least not in a manner that alters size. Titin isoform changes have been reported in cardiac disease (Neagoe et al. 2003), but literature on titin isoform changes in skeletal muscle is sparse. A previous study investigating...
titin isoform in spastic muscles of stroke patients also found no change in isoform size (Olsson et al. 2006).

**Fibre mechanics**

While titin isoform size contributes to single fibre mechanics, other proteins or organization of fibre material could be responsible for an increased passive tension at the cellular level. Two previous studies did demonstrate stiffer muscle fibres for spastic patients. These studies each had important differences, however. In one the muscle fibres tested were from a range of muscles which was not the same in the spastic and control groups (Friden & Lieber, 2003). This is a confounding issue because it is known that different muscles have different passive mechanical properties (Prado et al. 2005). Another showed increased stiffness only in fast fibres with an increase in the proportion of fast fibres (Olsson et al. 2006). We did not see a corresponding shift to fast fibres of our muscles, and in fact showed a significant increase in type I myosin heavy chain of CP muscles indicating a shift to slower fibres (Fig. 8). Overall, previous studies have shown disagreement on whether spastic muscles gain a faster or slower phenotype, which could be muscle specific (Brooke & Engel, 1969; Scelsi et al. 1984; Jakobsson et al. 1991). However, the most straightforward interpretation of our data is that over-activity resulting from spasticity drives a shift to slower fibres. It is also important to note that the previous study was performed with vastus lateralis muscle biopsies (Olsson et al. 2006), which show much less spasticity and contracture development compared to the medial hamstrings studied here (Damiano et al. 2002; Pierce et al. 2008). Future studies across a wider range of muscles are required to ultimately resolve these ambiguities.

**Study limitations**

One important limitation of this study is the subject heterogeneity. CP is a spectrum disorder and here we have primarily examined only the commonly shared parameters of this range of subjects with spastic CP. A more detailed analysis could be attempted using patient stratification by clinical parameters, Gross Motor Function Classification System, popliteal angle, limbs affected, age and treatment regimens. However, due to limitations of the sample size and the high variability of parameters in working with human subjects, this was not possible. We were able to demonstrate a significant correlation between severity measures and sarcomere length, which helped to mitigate the fact that we are relying on model data for our comparison with in vivo sarcomere lengths control children.

The source of controls for this study is not ideal since the patients had sustained an ACL tear. However, these patients were several months removed from the injury and had normal mobility at the time of surgery. Our approach represents the best available source of normal hamstring muscle from a pediatric population. These subject groups were not perfectly age-matched, although they all came from a pediatric population, as ACL surgeries do not occur prior to the teenage years whereas hamstring-lengthening surgeries often occur much earlier. However, our passive mechanical data did not correlate with age, suggesting that these small age discrepancies did not affect our outcomes. The subjects also underwent varied previous treatments, of which previous hamstring-lengthening surgery and botulinum toxin injections into the hamstrings was tracked. These variables were analysed in relation to mechanical measures and collagen content, but no relationship was found. These treatment effects are further complicated by highly variable times since treatment.

**Summary**

It is known that muscle contractures result from the UMN lesion in CP. Here, using a larger and more controlled study than previous ones, we showed increased passive stiffness of fibre bundles and increased sarcomere length in vivo. Together, these properties create a muscle in CP that experiences much higher stresses with increasing muscle length and clearly contributes to the development of muscle and joint contractures. Future studies are required to understand the mechanistic basis for the sarcomere length change and increased ECM content in CP as these clearly represent targets for therapy.

**References**


Author contributions

Study conception and design: L.R.S., R.L.L., R.L.L. Sample collection: L.R.S., K.S.L., H.G.C. Data analysis: L.R.S. Manuscript preparation: L.R.S., R.L.L. Manuscript editing: L.R.S., K.S.L., S.R.W., H.G.C., R.L.L. All authors approved the final version for publication. This work was completed at University of California, San Diego and Rady Children’s Hospital, San Diego, CA, USA.

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