Myosin Heavy Chain Isoforms and Asymmetrical Hybrid Fibers in Rat Skeletal Muscle

By

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Introduction

Muscle Structure

Skeletal muscle tissue is best considered to be a sum of multiple functioning parts, working together toward achieving contraction. Within a whole muscle, which is surrounded by a connective tissue layer called the epimysium, there are smaller units called fascicles, which are encased by another connective tissue layer; the perimysium. The muscle fascicles are comprised of bundles of muscle fibers, which are the individual muscle cells. However the fibers themselves are not the smallest unit of function within the muscle. Within the muscle fibers are myofilaments; composed of myosin and actin which act as motors during contraction. The actin and myosin filaments are anchored to Z-lines by the protein titin, and this area between Z-lines is referred to as a sarcomere. Sarcomeres are the smallest unit of contraction, and are organized in series of hundreds of sarcomeres throughout the entire length of the whole muscle. This patterning is what gives skeletal muscle its striated appearance. Sarcomeres are essential to muscle contraction, because when eliciting a contractile response, the Z-lines contract toward each other as the sarcomere shortens, per action potential that is sent to the motor neuron controlling those specific muscle fibers (Bottinelli and Reggiani, 2006)

Regulation of Contraction

Action potentials are electrical impulses that are sent from the brain when movement is desired. The motor neuron receives the impulse and is stimulated to release neurotransmitter, acetylcholine, into the synaptic cleft. The neurotransmitter then binds to binding sites on the nicotinic ACH receptor within the cleft, stimulating the release of

sodium into the muscle fiber. The increase in sodium concentration creates a depolarization of the sarcolemma which is coupled to opening of ryanodine receptors (RyR) within the sarcoplasmic reticulum, which the releases Ca^{2+}. Ca^{2+} binds to troponin, a protein complex connected to the tropomyosin protein. In the resting state, tropomyosin physically blocks the myosin motors from attaching to actin to elicit contraction. However, upon Ca^{2+} binding to troponin, tropomyosin moves to the side and allows myosin to bind to the actin protein which is in very close proximity. Myosin has a head end and a tail end; the tail is where it's anchored and the head is where it can bind to 'pull' against the actin in a motion that is referred to as the power stroke. This power stroke resembles walking in the sense that the myosin heads binds and release actin along its length for the duration that the contraction is sustained. As these proteins are sliding against each other, and because of the fact that they are both anchored to the Z-lines, this causes the shortening of the sarcomeres during contraction. After contraction has been achieved, Ca^{2+} pumps embedded in the SR membrane remove Ca^{2+} from the cytoplasm. This causes Ca^{2+} to unbind from troponin, causing myosin, actin, and tropomyosin to resume their original positions. So, for each action potential delivered from a motor neuron, the muscle fiber undergoes a process of contraction, followed by relaxation. This process is called a muscle 'twitch' (Bottinelli and Reggiani, 2006).

Whole muscles are comprised of a number of motor units, which are a single motor neuron and all of the individual fibers it innervates. Individual motor units are generally assumed to be comprised of identical fiber types, each containing the same myosin heavy chain isoform. This leads to a whole muscle that is derived of several motor units, acting separately or in a coordinated fashion. Recruitment of these motor units proceeds in an

orderly pattern so that muscle force is appropriately matched to the task. So, if one were to lift a pencil, the slower motor units would be activated through their respective motor neurons. However, if one were to lift an entire box of pencils, this heavier load would result in the recruitment of the moderate-speed motor units. The last of the motor units to typically be recruited are those corresponding to the largest and fastest fibers. These would be recruited, for example, if one were to try to lift 50 boxes of pencils. The motor units within a whole muscle work as a unique division of labor, in that they tend to work together to generate the appropriate force; some being activated while others are not until the load is heavy enough that it is necessary. It is important to understand that while the contraction is "all or nothing" meaning that the action potential will either fire (when contraction is desired) or not fire (the skeletal muscle is at rest,) the physiology of the muscle contraction is not—only the motor units that are necessary are recruited (Bottinelli and Reggiani, 2006).

To fully understand muscle fibers and their functioning, it becomes necessary to look at the mechanism behind muscle contraction. As these muscles are stimulated to contract, which can be replicated by stimulating an electrical pulse, the muscle shortens as the myosin and actin filaments slide against each other during contraction, and relaxes as ATP is unbound from the actin and the temporary bond between the two filaments is broken. However, when prolonged contraction occurs, an example of which would be during weightlifting, several of these pulses are submitted by the motor neuron, stimulating the muscles to contract, often before the first contraction is allowed to relax completely. This addition of pulses is a phenomena referred to as summation, where the pulses begin to decrease in length of time sustained as they begin to increase in force.

Complete summation or sustaining contraction is referred to as tetanus of the muscle, where it is in its most contracted, shortened state. Tetanus is a threshold beyond which the muscle cannot further contract. Relating back to the recruitment of motor units, the beginning of the contraction where individual pulses occur is an example of where single, type IIX motor units would be recruited. A greater number of motor units are recruited in summation, representing a state where types IIX and IIA would be recruited. For tetanus, all motor units are recruited; types IIX, IIA, and I. Relaxation of the contraction follows tetanus in normal incidences of contraction (Bottinelli and Reggiani, 2006).

Myosin Heavy Chain Isoforms Define Different Fiber Types

Due to the fact that myosin is so important to contraction, muscle physiologists are particularly interested in the working parts and properties of muscle fibers that generate contraction and force. Myosin has been found to occur in four different isoforms; type I which is known to have a slow contractile speed, or shortening velocity, relative to the other three isoforms. The next fastest of the isoforms is type IIa, followed by IIx, which differ slightly in composition and are of moderate contractile velocity. The fastest of the pure isoforms is type IIb, which is usually only present in small quadrupeds (like mice and rats) that typically perform quick, repetitive revolutions of their limbs. Each individual fiber is characterized by the expression of a specific myosin isoform, but they also differ in metabolic properties. The type I fibers are known to be oxidative, as they have proven to efficiently utilize oxygen in sustaining contraction via aerobic respiration. These fibers typically present in marathon runners or other high endurance athletes that compete for extended periods of time. On the other hand, type IIb is known to be glycolytic; quickly

expending its oxygen supplies and converting to anaerobic respiration. These fibers are used for quick, strength-requiring activities, such as sprinting. As in contractile velocity, types IIa and IIx are moderate in oxidative capacity as well.

Hybrid Muscle Fibers

These pure isoforms are not the only forms that myosin can be present in; there is a presentation known as hybrid myosin heavy chain fiber type, in which two or more of the pure isoforms are expressed. This phenomenon can actually be more prevalent than the incidence of pure fiber types; it has been found that approximately 55-60% of muscle fibers are actually composed of hybrid fiber types in certain rat muscles (Caiozzo et al., 2003).

Hybrid fibers are particularly significant in studying the muscle fibers due to the fact that the co-expression of two fiber types can often indicate a point of transition. The hybrid fiber has not been studied in depth until the 1990's, so while science seems to understand a lot behind their occurrence, there are still questions to be answered and this adds to the necessity to study hybrids in greater depth. They were not studied thoroughly because they were written off as being indicative of injury or disease. However, it has since been found that hybrids are seen in whole muscle hypertrophy, maturation, decrease or increase in neuromuscular activity, and depletion of resources (mimicking drastic diet change) in addition to injury or disease. Hybrids have since been found to exhibit a variety of characteristics, having the ability to have a wider breadth of functionality and filling more roles (Stephenson et al., 2001).

Asymmetric Hybrid Fibers

Asymmetric hybrid fibers are fibers that express one isoform at one location along the length or width of a single fiber, and transition to another isoform further down its' length. The presence of asymmetries has been suggested in a number of research studies (Lutz et al., 2001, McLoon et al., 2011, Sakuma et al., 1995, Zhang et al., 2010). It is thought that there is a link between hybrids and asymmetries, in that hybrid character can be found at locations where fiber type is changing from one isoform to another, making them more likely to be found on individual fibers at sites where an asymmetry is present. One technique used to identify fiber type asymmetries through dissection of individual fibers which are segmented and the resulting fragments are run on an SDS-PAGE gel. The separated proteins are stained and each band is identified and its density is determined through densitometry. The proportion of one isoform to another within the same individual fiber sample is then converted to a ratio. Due to the fact that the segmented fibers are run sequentially, the ratios of each portion can be plotted on a graph. A changing ratio along the length of any one fiber lends proof to asymmetric fibers, because it can be shown that the fiber makeup and quantity of each isoform are changing. (Zhang et al., 2010). Again, the question is raised as to how this may affect the functioning of the muscle as a whole, if it is comprised of isoforms that would not contract uniformly in speed.

Knowing what we do about asymmetric hybrid fibers, it becomes important to studying asymmetric hybrids because they are defined as a change in isoform along the fiber's length. An example of an asymmetry would include where a pure fiber type then begins to co-express another fiber type; transitioning from one pure fiber type to a hybrid. Pinpointing hybrids either through PCR, immunohistochemistry, or SDS PAGE may, as a

result, help to discover areas of asymmetry. Also referred to as polymorphic fibers, hybrids are also significant because they present an interesting question: if hybrids are a co-expression of two fiber types that are not "nearest neighbors," shouldn't it be functionally unsound during contraction for different portions of the fiber to contract at different velocities? A paper by Dammeijer et. al. (2000) reflects on an instance of hybrid expression in the stapedius muscle, which is located in the inner ear. The stapedius utilizes MHC isoform co-expression to actually prevent injury by stabilizing the auditory receptors and decrease the potential for injury by intense acoustics above the 80 decibel threshold. The main conclusion drawn from this study was that the hybrid phenotype allows the muscle to respond and change to adapt to a stimulus. This finding lends support to the idea that hybrid fibers are a transitional state in transforming to a better fit for the muscle's load or function. It seems that while the hybrid fiber makeup seems counterintuitive to proper muscle functioning, the phenomena actually helps, in some cases, for proper physiological performance (Stephenson et. al., 2001).

Hypothesis

Based on the fact that a large number of hybrids have been reported (Caiozzo et al., 2003) and that hybrids are most common in juvenile animals (Brummer et al., 2013), I have composed a three-part hypothesis. First, I hypothesize that young rats (age 3 & 6 weeks) will show the greatest number of hybrids within their fibers, and correspondingly, the greatest number of asymmetries compared to the older rats. As is such, the second portion of my hypothesis is that old rats (ages 9 & 12 weeks) will have fewer hybrids and asymmetries within their fiber makeup. Finally, I hypothesize that hybrid and

asymmetrical character is a normal component of muscle growth and development, not as a result of disease or injury.

Methods

Whole Muscle Removal

Prior to experimentation, our methods were approved by the Institutional Animal Care and Use Committee (IACUC). Three female Fisher rats each of ages 3, 6, 9, and 12 weeks, were purchased from Taconic, Hudson NY, and euthanized upon arrival. Their total body mass was recorded. A small incision was made at the posterior ankle in order to reflect the skin above the knee. The gastrocnemius muscle was removed from the body by detaching both proximal heads from their origins on the femur, and their distal insertion at the calcaneal (commonly known as Achilles) tendon. The whole soleus was removed by bisecting the calcaneal tendon; its distal insertion, and detaching the muscle from its proximal origin on the posterior tibia. This procedure was repeated on the opposite leg. Both the soleus and gastrocnemius from each animal was also weighed to the nearest 0.01 g. (Refer to Table 1 for data.) The whole muscles were then placed into centrifuge vials containing an aliquot of relaxing solution and stored at -20 °C until use.

Preparation of Relaxing Solution

Muscles were placed in a relaxing solution (Caiozzo et al. 2003) to preserve the fibers for later dissection. To generate 100 mL relaxing solution, 50 mL (50%) glycerol was combined with 0.076 g (2 mM) EGTA, 0.02 g (1 mM) MgCl2, 0.22 g (4 mM) ATP, 0.069 g

(10 mM) imidazole, and 0.746 g 100 mM KCl. The solution was adjusted to pH 7.0 using an electrode and refrigerated until use.

Obtaining Precise Measurements of Femur and Tibial Length

Following removal of the whole muscles from the rats, the left femur was dissected from its joint with the pelvis. Each left leg from rats 1-12 was placed in a 50 mL conical vial with an aliquot of 1 M sodium hydroxide to fully submerge the limb. After one week, the sodium hydroxide was poured off leaving the bones fully exposed. The legs were then rinsed initially with deionized water and progressive dehydration was performed using increasing daily concentrations of ethanol. Following 24 hours in 99% ethanol, the liquid was poured off and the remaining, dried bones were measured for femoral length and tibial length using calipers. (See values in table 2.)

Whole Muscle Cross-Sectional Analysis

Cryosectioning

The right shank complex, composed of the soleus, gastrocnemius, and plantaris muscles was used for cryosectioning made using a Leica cryostat. They were first mounted onto wooden sample holders, using embedding medium (O.C.T. Compound, Tissue Tech) and next they were rapidly frozen by immersing them in isopentane cooled liquid nitrogen. Care was taken to position the complex so the Achilles tendon was facing upward, and 9 um sections were made and collected onto glass slides which were stored at -20 °C .

ATPase Histochemistry

The cross sectioned samples were rinsed before the ATPase stain was pipetted onto each individual section. Buffered calcium chloride (10 µL) was added and the solution was added to the slides, which had been traced around using a pap pen. ATP (5 mg) was dissolved in an aliquot of deionized water, pipetted onto the sections, and incubated for 30 minutes at 37 °C. Following incubation, the slides were rinsed three times; the first in 1% calcium chloride for a duration of six minutes, the second in a 2% cobalt chloride solution for two minutes, and then the prior rinses were removed by a generous rinse with deionized water. The slides were then developed by being submerged in 1% ammonium sulfide for 30 seconds. As a result of this staining reaction, fast fibers stain dark, while slow fibers only stain lightly.

Following the slide development, they were progressively dehydrated using increasing concentrations of ethyl alcohol. Dilute permount was used to fix the cover slip permanently to each slide.

Immunohistochemistry of Cross Sections

Both whole muscle cross sections and individual muscle fibers were analyzed using fluorescence-based immunohistochemistry. To obtain cross sections to label, whole muscle cryosectioning was performed using a cryostat and the samples were collected onto microscopic slides. The samples were kept frozen at -20 °C until staining or labeling.

TBS (Tris-Buffered-Saline)(20 mm) was first made, by adding 4.84 g Tris and 58.4 g sodium chloride to 1500 mL deionized water with continuous stirring. After the Tris and sodium chloride had been completely dissolved, the pH was adjusted to 7.6 with a pH

meter (Accumet Excel.) The volume was increased to 2 L, and 1 mL 0.05% Tween-20 was added. The solution was then stored at 4 °C. The resulting TTBS was used to wash the slides between steps. To 100 mL TTBS, 2 g bovine serum albumin (BSA) was added with constant stirring. Following the completion of dissolving BSA into the TTBS, an aliquot of sodium azide was added to prohibit microbial growth. The resulting BSA solution was used to block the slides prior to antibody labeling.

The slides were prepared for antibody labeling by blocking 45 minutes in a solution of 2% BSA in TTBS. The slides were then washed with constant agitation in a solution of TTBS for approximately 10 minutes. The primary antibody solution (prepared as a 1:10 dilution of the desired antibody into the 2% BSA) was then pipetted onto the slide, which was outlined using a PAP Pen, creating a hydrophobic barrier within which to contain the solution. The primary antibody solution was left to bind for a minimum of 2 hours at 25 °C. Alternately, the solution was left overnight at 4 °C. Following primary antibody labeling, the slides were washed, again in TTBS for approximately 10 minutes, and the secondary antibody solution was applied. This solution consisted of a 1:100 dilution of the desired secondary containing a fluorescent "tag" into 2% BSA. (Refer to table 5 for information pertaining to antibody selection.) The secondary antibody was left to bind for approximately 1 hour. Following secondary labeling, the slides were washed again in TTBS. Most samples were dual labeled, so this procedure was repeated with a different primary and different secondary antibody. At the end of the procedure, the sections were covered in mounting medium (Fluoromount) and covered with a glass cover slip.

Single Fiber Analysis

Sample Collection

Muscle fibers from the left soleus were covered in glycerination solution and stored at -20°C until they were individually dissected by hand using foreceps under a stereomicroscope. Once an individual fiber has been removed, it is placed into a centrifuge vial with 30 µL urea buffer. (See below for procedure to generate urea buffer.)

Preparation of Urea Buffer

Sample buffer was prepared as previously described (Blough et al. 1996). To produce 250 mL buffer, 120.12 g (8 M) urea and 38 g (2 M) thiourea are dissolved over heat, to which 25 g mixed bed resin is added with constant stirring for 15 minutes. Following the filtration and discarding of the resin, 1.51 g (0.05 M) Tris, 2.89 g (3%) sodium dodecyl sulfate, and 7.5 g (0.004%) bromophenol blue are added. The solution was adjusted with litmus paper to pH 6.8 and frozen until use.

Resolution of MHC Isoforms in Single Fibers

We used sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) of proteins extracted from single fibers to unambiguously identify muscle fiber types, including hybrid fibers. To make the resolving gel, 12 mL 30% 200:1 acrylamide to bis N,N'-methylene-bis-acrylamide, 4.8 mL (100%) glycerol, 18 mL (1.5 M) Tris at pH 8.8, and (2.45 mL) deionized water were combined in a 50 mL test tube and vortexed to mix thoroughly. Following the mixing step, 0.4 mL (10%) sodium dodecyl sulfate was added, and the entire tube was placed within a vacuum for 20 minutes to remove excess gas present in the gel. After the

degasing period, 32 µL N'N'N'N'-tetramethylethylene-diamine (TEMED) and 320 µL (10%) ammonium persulfate were added to the gel which is pipetted into the plates within the Hoefer (model 600SE) apparatus. A layering buffer was applied immediately following pipetting, which is composed of the same solutions as the resolving gel, but lacking glycerol, TEMED, or ammonium persulfate. The gel is given a minimum of 60 minutes to polymerize before the stacking gel is poured.

To make the stacking gel, 1.33 mL (30%) 20:1 acrylamide to bis was combined with 2.5 mL (0.5 M) Tris at pH 6.8 0.1 mL (10%) sodium dodecyl sulfate and 6 mL deionized water in another 50 mL test tube. This tube was also degased within the vacuum for twenty minutes. Following the degasing, the layering buffer was poured off and 0.75 mm combs were inserted between the plates on the Hoefer apparatus, which will form the wells for sample to be loaded into. TEMED (5 µL) and 100µL (10%) ammonium persulfate were added, and inverted to mix, to prepare the stacking gel. This solution was pipetted into the plates of the Hoefer 600SE apparatus, avoiding introducing any bubbles. These gels were given a minimum of sixty minutes to polymerize as well.

The running buffer within the Hoefer electrophoresis unit was prepared using a concentration of 25 mM Tris, 192 mM glycine, and 0.1% sodium dodecyl sulfate in deionized water. The unit was filled halfway with buffer, which uses approximately 2 L running buffer. To produce the upper buffer which was layered on top of the stacking gel, samples, and around the electrode of the Hoefer electrophoresis unit, 1 L of running buffer was used, to which 800 µL 2-mercaptoethanol was added. Both buffers were chilled in the refrigerator before application.

Following the 60 minute polymerization period of the stacking gel, the combs were removed and the resulting wells were filled with chilled upper buffer. Each sample was heated and vortexed to mix thoroughly before 8 µL of each sample were pipetted into the wells. The electrode was placed on top of the plates for the Hoefer apparatus, filled to the top with upper buffer, and the entire apparatus was placed within the Hoefer electrophoresis unit. The unit was run at 15 mA and 250 V for 40 hours at 7-8 °C .

After approximately 40 hours, the gels were removed from between the plates; the stacking gel was discarded and the resolving gel was soaked for a minimum of sixty minutes in 250 mL 50% methanol with 250 µL 37% formaldehyde.

Staining Gels

Proteins in the gels were visualized using silver stain according to the procedures of Wray et al. (1981). To stain, the gels were first rinsed in deionized water, after pouring off the 50% methanol and formaldehyde solution. The gel was gently agitated while rinsing, for a maximum of five minutes.

During the rinsing of the gel, 0.6 g silver nitrate was combined with 4 mL deionized water into a 15 mL test tube. This was added drop wise to a solution of 21 mL (0.36%) sodium hydroxide and 1.4 mL ammonium hydroxide, with constant stirring. The silver nitrate may precipitate out of solution, in which case ammonium hydroxide can be added drop wise until the solution becomes clear again. Once the silver was entirely added, the resulting solution was increased to a total volume of 100 mL with deionized water. The deionized water that the gel was washing in was poured off, and the solution was added within five minutes of it having been mixed. The gel was left in the solution to stain for

approximately 30 minutes. Developer was prepared during staining by adding 2.5 mL (1%) sodium citrate and 0.25 mL (37.5%) formaldehyde to an Erlenmeyer flask and increasing the total volume of the solution to 500 mL with deionized water.

After the 30 minute staining period has passed, the solution was poured off and the gel was rinsed in deionized water, while still being agitated, for 1 minute or less. The rinsing water was poured off and one-third to half of the developer was added, maintaining agitation constantly. Bands began to appear after approximately 3 minutes, at which point the current developer was discarded and fresh developer poured on. Once the bands in the gel appeared dark enough to be easily seen with the naked eye, the developer was replaced with 50% methanol which stopped the reaction. The gel was then refrigerated in this solution, which shrinks them in size.

Mounting Gels

The shrunken gel was removed from the refrigerator and the 50% methanol was replaced with 2% glycerol. The gel was agitated within this solution, which caused it to enlarge, and absorb the glycerol due to the porous nature of the gel. Once the gel had reached the desired size, it was mounted, clamped between plastic plates, surrounded between thin sheets of plastic, and laid horizontally to allow the water to evaporate out of the gel.

Scoring Gels

MHC isoform migration patterns were compared to standards obtained from our own research as well as work from publications to determine which isoform each

banding/migratory pattern represents. A soleus standard of known composition, type I/IIA, was used as a reference on each gel.

Standards were produced by obtaining a homogenate of soleus fibers which were into pieces, and heated in the urea buffer. This allowed the breakdown of the fibers into a representation of the sample as a whole, which for soleus is largely type I and type IIa fiber type composition.

Immunohistochemistry of Single Fibers

Individual fibers were analyzed using a unique method involving dissecting out individual fibers by hand onto a microscope slide. The samples were stored in a relaxing solution, which was allowed ten minutes to dry at room temperature before being rinsed with deionized water, after which the sample was allowed another few minutes to dry. The PAP Pen was used to trace a hydrophobic barrier around the samples and a 0.1% solution of Triton X in TTBS was then applied and allowed to sit for 30 minutes. The purpose of this solution is to remove the remaining plasma membrane layers that may still be attached to the fibers and prohibit antibody binding. The slides were then washed in TTBS with agitation for 10 minutes and the same antibody labeling procedure was used as was used in the cross sectional samples, (see pages 11-12) starting with blocking with BSA for 45 minutes. (Refer to Table 1 for information regarding antibodies used.)

Results

In order to determine the rate of growth and where it is most significant during the lifespan, the rats and their muscles were weighed to the nearest 0.01g prior to analysis of

any of the tissue. (Refer to Appendix A for values.) We found that for both measurements, the rats increased growth rapidly through ages 3, 6, and 9 weeks, and began to level off between weeks 9 and 12 (Fig. 1, Fig. 2). Following removal of the muscle tissue, the entire leg was detached from the body at the hip joint, and the remaining tissue was dissolved using sodium hydroxide in order to expose the bone for caliper measurements of length. (Refer to Appendix B for measurements.) Again, the observed trend was the largest amount of growth was below 9 weeks of age, and nearing a plateau between 9 and 12 weeks. The youngest rats demonstrated the highest character of hybrid fibers; this age group contained the largest proportion of fibers, and it is unlikely that this is due to disease or injury as the numbers were very similar within individuals of the same age. Based on this observation, supporting literature (Wigston et al., 1992, di Maso et al., 2000) and that we found the largest percentage of hybrid fibers to be within the 3 and 6 week old rats, we were able to confirm that this group demonstrates the most rapid growth. This pattern supports the hypothesis that hybrids and asymmetries are normal components of growth and not as a result of injury or disease.

Overall, we analyzed more than 1,600 fibers via SDS PAGE to determine their MHC isoform content. While prior research has indicated a hybrid fiber content of over 50% in rat muscles in general, my research found hybrids in 25% of samples and pure fibers in 75% (Adapted from data in Appendix C & D). When broken down by specific muscle, the gastrocnemius was found to have approximately three times the percentage of hybrids as the soleus, from 44.0% versus 15.8% respectively. There is some room for error in that the gastrocnemius fiber sampling in that care was taken to sample from the deep medial gastrocnemius. Using this measure to sample the red-appearing portion of the whole

muscle doesn't guarantee that each rat's gastrocnemius is sampled from the same location. The gastrocnemius is known to have an outer cortex on the most external, superficial portion of the muscle that is largely composed of type IIb fast isoforms. Deeper within the muscle, the fibers are more likely to be of hybrid character, as several of the slow to intermediate speed isoforms are co-expressed. As far as age is concerned, the gastrocnemius was found to always have a higher proportion of hybrids than the soleus. The 3 week old rats' muscles had a higher percentage of hybrid fibers than the 12 week old rats as well. (Refer to Table 2 for values.)

Bivariate soleus versus age statistics have been obtained by plotting the average number of hybrids in the soleus muscles (refer to Appendix C for data) against the age of the rats. The fit of the line to the data follows the equation:

$$\log(soleus\ hybrids) = 4.6321491 - 1.1015635 * \log(age)$$

and the regression of the line is 0.784967. The decrease in the number of hybrids as the rats age was found to be significant, at $p < 0.05$ (Fig. 4).

Bivariate gastrocnemius versus age statistics have been obtained by plotting the average number of hybrids in the gastrocnemius muscles (refer to Appendix D for data) against the age of the rats. The fit of the line to the data follows the equation:

$$gast.hybrids = 46.5 - 2.8333333 * age$$

and the regression of the line is 0.62491. This decrease in hybrids as the rats age was found to be statistically significant, where $p < 0.05$ (Fig. 5).

Through immunohistochemistry, we were able to determine fiber type with certainty, as a result of the specific binding of monoclonal antibodies to individual MHC isoform in the sample. Red (TRITC) and green (FITC) fluorescent secondary antibodies

were used, which allowed visualization and image capture via light microscope. Co-expression of isoforms was also seen, where both antibodies bound and a yellow-appearing label was observed (Fig. 9). A continuum of coloration was seen as a result, due to the fact that co-expression of MHC isoforms varies between fibers. Future research could demonstrate asymmetric character in these samples, by reconstructing the labeled cross sections through the use of software, and detecting changes along the length of individual fibers.

Finally, we were able to demonstrate asymmetries in muscle fibers using immunohistochemistry. Fibers that change along their length suggest that the multiple nuclei are uncoordinated in the manner under which they express their MHC isoform phenotypes. (Zhang et al. 2010) Due to this phenomenon, we found that the asymmetries were present in varying degrees, or varying monoclonal antibody labeling along their length (Fig. 16, Illustrative Figs. 14 & 15). We were able to demonstrate asymmetries in the young age group (3 & 6 weeks) of rats. The inability to find asymmetries in the older rats (9 & 12 weeks) does not necessarily mean that they are not present in our whole muscles, as the prevalence of asymmetries has yet to be quantified in literature.

Discussion

We were able to confirm through careful measurements and performing a regression analysis that while there is a change in mass of the whole muscles, this is mostly due to longitudinal growth of the muscles, which grow in correspondence to the lengthening shank muscles (Fig. 3). Also accounting for the increase in mass is the fact that muscle fibers undergo hypertrophy, or increase in diameter of a single fiber as they grow.

Because this type of growth is taking place as opposed to hyperplasia, or an increase in the number of fibers, we know that the fibers are changing from one fiber type to another. Specifically, we have found that the incidence of faster fiber types decreases as they change to the type I MHC isoform with increasing age (Fig. 6, Fig. 7). Using ATPase histochemistry to classify fiber types, we found that over 93% of the fibers in the 12 week old rats were type I, compared to 62% in the 3 week old rats (Fig. 9). This conversion to the pure type I isoform supports the hypothesis that older rats have fewer hybrids than younger rats, specifically in the soleus.

We found that the muscles studied within the deep portion of the shank, the soleus and deep medial gastrocnemius, possess a significant number of hybrid fibers. While the soleus has a lower percentage overall of hybrid fibers compared to the gastrocnemius in every age group, (Fig. 4, Fig. 5) and both muscles show a decline in proportion of hybrids with increasing age, (Fig. 6, Fig. 7) the hypothesis is supported that young rats have fewer hybrids in comparison with those that are older. Hybrids are indicative of changing muscle fiber phenotype due to the largely plastic nature of muscle fibers. While the most common isoforms found in the soleus were type I and IIa, we observed fast isoforms in the young 3 week old rats' soleus' such as IIa/IIx and the neonatal isoform that were not observed in the old 12 week old rats. The older rats showed a high proportion of triplet banding patterns, where three isoforms we co-expressed in the single fibers: type I, IIa, and IIx. This phenotype was seen only in the gastrocnemius, and very infrequently in rats younger than 9 weeks of age, including a 0% incidence in 3 week old rats (Appendix D). This pattern is consistent with other studies' findings that the gastrocnemius will express a large proportion of hybrid fibers, especially in comparison to the soleus (Caiozzo et al., 2003).

Using immunohistochemistry with highly specific monoclonal antibodies, we confirmed the common occurrence of hybrid fibers in both the soleus and deep regions of the gastrocnemius (Fig. 9, Fig. 17). Within all sampling techniques: SDS-PAGE, ATPase histochemistry, and monoclonal antibody labeling, we found that there are a larger proportion of hybrid fibers in the younger rats as opposed to the older rats. Our research has consistently shown that hybrid proportions are high in our young rats and lower in our older rats (Fig. 4, Fig. 5). After finding that hybrids often point to asymmetries, and that the hybrid isoforms drastically decrease to 7% or less as growth tapers off, my data supports the hypothesis that hybrid character is a normal component of healthy muscle growth and development. This corresponds to trends that are also documented in the Brummer et al. 2013, di Maso et al. 2000, and Zhang et al. 2010 papers.

Muscle fibers are known to be plastic, meaning that they change to meet the needs of the musculoskeletal system (Bottinelli and Reggiani, 2006). These changes typically are in response to a change in the strain or load placed on the muscle, and the metabolic capacity required by the muscle. These changes occur as the muscle breaks down and rebuilds itself, expressing a different gene coding for a different MHC isoform when it is rebuilt. This was demonstrated in prior experiments where electrical stimulation was used to deliver impulses to denervated muscles. Based on the pattern of the firing of electrical stimulation; low frequency versus high, the muscles changed properties as a response. Muscle transformation from one fiber type to another is not just at random or at large scales, most often it occurs between "nearest neighbor" isoforms (Staron and Pette, 1987). This would mean that type I would become the hybrid fiber type I/IIa, before becoming the even faster type IIa, instead of type I immediately becoming a type IIb fiber, for example.

Changing muscle fibers can often be identified because they co-express one increasing muscle fiber type as another is phasing out. Due to this co-expression, hybrid fibers become of interest in studying asymmetries. As they are indicative of muscle plasticity, they can also indicate an area where asymmetries may be occurring. This creates a link between the observation of hybrid fibers and the likelihood of seeing asymmetries in muscle fibers (Bottinelli and Reggiani, 2006).

In the soleus muscle, we found that the proportion of slow fibers increased at the expense of either the fast fibers or type I/IIa hybrids (Fig. 8). As the rats age, the number of fibers present in the whole muscles do not change. They are, in fact, predetermined by genetics and fixed from an early stage of development. As is such, any changes to the character of the fibers must be due to the transformation of the fiber itself. Our ATPase histochemical results showed a corresponding pattern as our immunohistochemistry and single fiber analysis, in that the proportion of fast fibers in the soleus declined with age (Fig. 8, 9, 15, 16, 17). A sample of the fibers was counted for ATPase staining at each age group, and found that 62.8% of fibers were found to be slow in the 3 week old rats, 69.0% in the 6 week old rats, 78.5% in the 9 week old rats, and 90.5% in the 12 week old rats. This supports our hypothesis that fiber type shifts toward a slower, type I fiber with increasing age. This pattern suggests that the type I/IIa hybrids are being converted into 'pure' type I fibers in the soleus.

Through our research, we were able to find evidence of asymmetric hybrid fibers. Asymmetries are a specific type of hybrid fiber, where the MHC isoform expressed or co-expressed changes along the length or width of the fiber. Asymmetries are of interest within muscle studies because their dynamics seem to be physiologically unsound, as it

seems counterintuitive to have portions of the muscle fiber contracting at different rates for the integrity of the muscle tissue. The fact that multiple studies in different model species have repeatedly reported fiber type asymmetries raises the question as to why they exist while being of seemingly poor design (Lutz et al., 2001, Sakuma et al., 1995, Zhang et al., 2010). We know that asymmetric hybrids can be found in different proportions throughout the fiber (Zhang et al., 2010), and that the differential expression leads to differences in the properties of the fiber and its contraction (Edman et al., 1985.) We were able to use immunohistochemistry to identify fiber type asymmetries in a number of soleus fibers; both in mouse hybrids during preliminary research, (Figs. 12, 13, 14) and in rat fibers in multiple age groups (Figs. 15, 16, 17). In all of these images, difference in monoclonal antibody labeling is observed along the length of the fibers. Due to the specificity of the antibodies, we can conclude that each of these images (Fig. 16 & Fig. 17) shows an example of asymmetry in MHC expression. The prevalence of these instances of asymmetric hybrids suggests that this phenomenon may be more common than is currently believed. Through our experimentation we were able to find a number of asymmetries in muscle fibers. While they were typically found in the younger rats, suggesting that our hypothesis would be supported, there is not enough data in the scientific community to determine the rate of the phenomenon and thus the hypothesis cannot be accepted simply due to the absence of asymmetries in our older rats.

In summary, through using a number of complementary techniques, we were able to support all three portions of the hypothesis: that younger rats have a large number of hybrid fibers, that older rats have a lower hybrid and asymmetric proportion and a high number of pure fiber types, and that hybrids and asymmetries are a normal part of growth

and development. While some published studies have reported hybrid proportions to be higher in rat muscle than we observed, for example in the Caiozzo et al., (2003) paper which found the incidence of hybrid to be 57% in the deep portion of the gastrocnemius, they still observed similar trends in that their soleus was found to have a dramatically lower percentage of hybrid fibers (13%) than the gastrocnemius (57%). Previous studies have also documented a decline in hybrid fiber proportion with age (Brummer et al., 2013, di Maso et al., 2000). Further work is required to better illuminate the specific patterns of asymmetric MHC expression in these hybrids.

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Figure Legend

Figure 1: Rat Body Mass versus Age

Figure 1 is the compiled data from Rats 1-12, grouped based on age, and their respective total-body mass. This graph shows that as the rats age, they are increasing rapidly in total body mass, especially between the 3-9 week old age groups. As the body grows, the muscles grow along with it, so the muscles are growing and increasing in length as well. This knowledge helps to narrow the window during which we would expect to see hybrids and asymmetries. As fibers are growing, they may be incorporating new MHC isoforms into the fiber, thereby forming a hybrid. The equation of the line follows:

$$y = 12.711x + 14.48$$

and the data points fit the trendline at $R^2 = 0.9159$. This is statistically significant at $p = 2.56145E-06$. (Refer to Appendix A for data.)

Figure 2: Rat Shank Flexor Complex Mass versus Age

Figure 2 shows the compiled data from Rats 1-12, grouped based on age, and their respective masses of the shank flexor. The shank flexor complex includes the soleus, gastrocnemius, and plantaris muscles. It is important to note that in Rats 2 and 8 the soleus muscle was lost during dissection. The graph shows that as the rats are growing in body mass (Figure 1) their shank flexor mass is increasing at a similar, rapid rate. Again, the greatest increase is between ages 3-9 weeks so under my hypothesis, the greatest number of asymmetries should later be found in that age group. The equation of the line follows:

$$y = 0.0859x - 0.0223$$

and the fit of the data to the trendline is $R^2 = 0.8954$, which is statistically significant at

p= 6.49493E-06. The correlation between shank mass and age is 94.6%. (Refer to Appendix A for data.)

Figure 3: Rat Bone Length versus Age

Figure 3 is the compiled data from Rats 1-12, grouped based on age, and their respective tibia and femur length in millimeters. The tibia refers to the length of the bone from the proximal end where it comprises the knee joint, to the distal end where it meets the tarsals. The femur length refers to the length of the bone from the proximal end where it meets the hip joint to the distal end where it forms the knee joint. The length of the tibia determines the length of the soleus and gastrocnemius muscles in each individual. The graph shows an increase in length over time, confirming that with an increase in mass, the rats legs are also growing in length, and the muscles will grow correspondingly. The largest increase in length for both bones is between ages 3-9 weeks of age. For the femur, it is important to note that only 11 measurements were obtained due to the disintegration of the femur of Rat 2. The equation of the line for the tibia is

$$y = 1.4771x + 15.321$$

and the fit of the data points to the trendline is $R^2 = 0.7983$, which is statistically significant at p= 1.88183E-10. The equation of the line corresponding to the femur is:

$$y = 1.3573x + 11.558$$

and the data fit the trendline at $R^2 = 0.7843$. This is statistically significant at p= 3.29479E-09. (Refer to Appendix B for data.)

Figure 4: Bivariate Fit of Soleus Hybrids by Age

This image shows statistics that have been obtained by plotting the average number of hybrids in the soleus muscles (refer to Appendix C for data) against the age of the rats. The fit of the line to the data follows the equation:

$$\log(soleus\ hybrids) = 4.6321491 - 1.1015635 * \log(age)$$

and the regression of the line is 0.784967. The decrease in the number of hybrids as the rats age was found to be significant, at $p < 0.05$

Figure 5: Bivariate Fit of Gastrocnemius Hybrids by Age

This image shows statistics that have been obtained by plotting the average number of hybrids in the gastrocnemius muscles (refer to Appendix D for data) against the age of the

rats. The fit of the line to the data follows the equation:

$$gast.hybrids = 46.5 - 2.8333333 * age$$

and the regression of the line is 0.62491. This decrease in hybrids as the rats age was found to be statistically significant, where $p < 0.05$.

Figure 6: Prevalence of Fiber Types in the Soleus Muscle Per Age Group

Shown in this graph are the proportions of MHC type I, type IIa, and the hybrid co-expression of I and IIa per age group. The observed trend is that as the rats are aging, the hybrid proportion greatly decreases, while the pure type I is increasing to above 90% by age 12 weeks. This indicates that the hybrid I/IIa fibers are transitioning toward a pure type I isoform as age increases.

Figure 7: Prevalence of Fiber Types in the Gastrocnemius Muscle Per Age Group

This graph demonstrates the level of diversity and fiber types present in the gastrocnemius. While we've demonstrated that the hybrids do decrease in proportion, co-expression is still seen in all four age groups. However, the hybrids are fiber types that do remain are of the slower fiber types, with MHC type IIb decreasing as age increases, as well as type IIx/IIb decreasing to 0% prevalence in the 12 week old rats. An interesting trend is that while the rats age, the percentage of "other" fibers increases. This represents fibers that are not of "nearest neighbor" MHC expression, typically being type I/X or the triplet co-expression of MHC I/A/X.

Figure 8: Changes in Soleus Fiber Composition as a Function of Age

Pictured are compiled images of the ATPase histochemical results of each of the age groups studied. Because these slides were non-acid pretreated, the fibers that appear light are the slow isoforms. Dark fibers represent the fast isoforms, while intermediate fibers represent intermediate-speed isoforms. It can be concluded based on these images that these rats are showing a progression of increasing population of type I fibers in their overall fiber type

makeup from age 3 weeks through 12 weeks. This pattern confirms the results that we found in our single fiber analysis. Upon counting the fibers, we found that the 3 week old rat had 62.8% slow fibers, the 6 week old rat had 69.0%, the 9 week old had 75.8%, and the 12 week old had 93.3%, showing the progression toward slow isoforms with increasing age and supporting the hypothesis.

Figure 9: Shank Cross Section with Monoclonal Antibody Labeling

The soleus is pictured on the right, separated by the gastrocnemius on the left by connective tissue. Antibodies used include: 4.74 with a green FITC secondary which indicate type IIa fibers, and 4.84 with a red TRITC secondary, which indicate slow, type I fibers. The soleus has a large population of type I fibers, as it is expected to, causing it to appear mostly red. Likewise, the gastrocnemius has a large proportion of type IIa fibers, causing it to appear mostly green. The intermediate, or yellowish colored fibers are I/IIa hybrids, (indicated with arrows) where both antibodies are co-labeling the portions of MHC isoforms that are expressed. The diameter of the fibers can also be noted: the green, IIa fibers are much smaller in diameter than the red, slow fibers. They are typically round or almost-round; where they appear oblong, it is due to oblique cryosectioning based on the angle of the tissues when they were mounted.

Figure 10: SDS PAGE Loaded with Rat 10 Deep Medial Gastrocnemius

Shown in the image of the SDS PAGE gel are the bands representing isoforms that have resolved, and below them, they have been scored. The banding pattern, from top to

bottom, is IIX>IIA>IIB>I. This particular gel shows both pure and hybrid isoforms, including multiple triplets co-expressing MHC isoforms I, A, and X.

Figure 11: SDS PAGE Expression of MHC Neonatal Isoform

A portion of the gel is shown, taken from Rat 1 soleus. The level of migration of the neonatal isoform is illustrated by the arrow, left. Shown in the far right lane is a I (lower band)/IIA (upper band) mouse soleus standard. In all six of the prior lanes, the neonatal isoform is shown in various degrees as illustrated by the intensity of the bands. The neonatal isoform is known to show up in SDS PAGE gels as a band that migrates further than the IIA isoform but not as far as the type IIb.

Figure 12: Illustrative Example: Single Mouse Fibers Labeled with Monoclonal Antibodies

Pictured above are two soleus fibers removed from a mouse during the development of the methodology for labeling individual fibers. These fibers were obtained during preliminary research and shown for illustrative purposes. The top fiber is positive for FITC (green,) which is bound to MHC type IIa, while the bottom fiber is positive for TRITC (red,) which is bound to type I. Yellow regions of the type I fiber indicate some co-expression of the IIa isoform.

Figure 13: Illustrative Example: Single Mouse Hybrid Fibers Labeled with Monoclonal Antibodies

This image shows a mouse soleus fiber, obtained during preliminary research and shown for illustrative purposes. The fiber is labeled with FITC (green) for type IIa, and with TRITC (red) for type I. In this image, red is not pictured because the type IIa and type I are co-expressed on the left hand side of the image, appearing yellow. This shows the hybrid phenotype, and the progression along the length of the fiber to pure type IIa at the right hand side illustrates fiber asymmetry.

Figure 14: Illustrative Example: Single Asymmetric Hybrid Labeled with Monoclonal Antibodies

Shown in this image is a mouse soleus fiber, dissected and labeled as a preliminary experiment and shown for illustrative purposes. The fiber is labeled for type IIa (green) and type I (red) in alternating patterns along the length of the fiber. Nuclei are labeled blue with DAPI, illustrating the multinucleate nature of these muscle cells.

Figure 15: Rat 4 Cross Section and Single Rat Fiber Labeled with Monoclonal Antibodies Exhibiting Hybrid Phenotype

Pictured in the top portion of the image is a cross section from Rat 3A, an additional 3-week-old rat that was obtained following data collection for Rats 1-12. The primary antibodies used were 4.74 (correspond to type IIx/IIa fibers and appear red due to secondary binding of Alexa Fluor 555) and 4.84 (correspond to type I fibers and appear blue due to secondary binding of Alexa Fluor 350.) The areas where they overlap, or

appear purple are hybrid fibers; both type I and IIx/IIa are co-expressed. The bottom portion of the image shows a single muscle fiber. The purple staining indicates some hybrid phenotype expression, largely along the bottom portion of the fiber. What can be seen within the fiber is the co-expression of type I and IIa. On the exterior of the fiber, the image shows the appearance of the hybrid phenotype as well as the pure type I MHC isoform. This indicates that the fiber is somewhat asymmetrical in isoform distribution.

Figure 16: Single Rat Soleus Fiber Labeled with Monoclonal Antibodies Exhibiting Asymmetry

In this image, several fibers are shown, the bottom of which is exhibiting the asymmetrical phenotype. The primary antibodies used were 4.74 (correspond to type IIx/IIa fibers and appear red due to secondary binding of Alexa Fluor 555) and 4.84 (correspond to type I fibers and appear blue due to secondary binding of Alexa Fluor 350.) The fiber with asymmetry is exhibiting hybrid phenotype where both Alexa Fluor 555 and Alexa Fluor 350 are bound. On the left side of the image, while still being a purple hybrid, the fiber appears to be more red, while along its length it transforms to appear more blue toward the right side of the image. This 'ribbon' patterning within the fiber represents that a portion of its myofibrils are expressing hybrid and asymmetrical phenotype while the remainder of the fiber is expressing pure type I.

Figure 17: Rat Cross Section Labeled with Monoclonal Antibodies

Pictured in this image is a cross section of a 3 week old rat with immunohistochemical labeling. The primary antibodies used were 4.74 (correspond to type IIx/IIa fibers and

appear red due to secondary binding of Alexa Fluor 555) and 4.84 (correspond to type I fibers and appear blue due to secondary binding of Alexa Fluor 350.) The myofibrils are easily visible within each fiber in cross section, and differences among their expressed fiber types can be observed in the hybrid fibers. The hybrids appear purple where the Alexa Fluor 555 and Alexa Fluor 350 are both bound due to the MHC isoforms that are co-expressed. The degree of labeling can be seen as well, as some of the purple hybrids appear to express more red than blue, and vice versa. Near the bottom left hand corner of the image is a hybrid fiber in partial longitudinal section. This fiber shows a blue "stripe" pattern which indicates that some myofibrils within the fiber are type pure I while others are exhibiting hybrid phenotype. This indicates that the fiber is changing in isoform, demonstrating active plasticity of the muscle.

Figures

Figure 1: Rat Body Mass versus Age

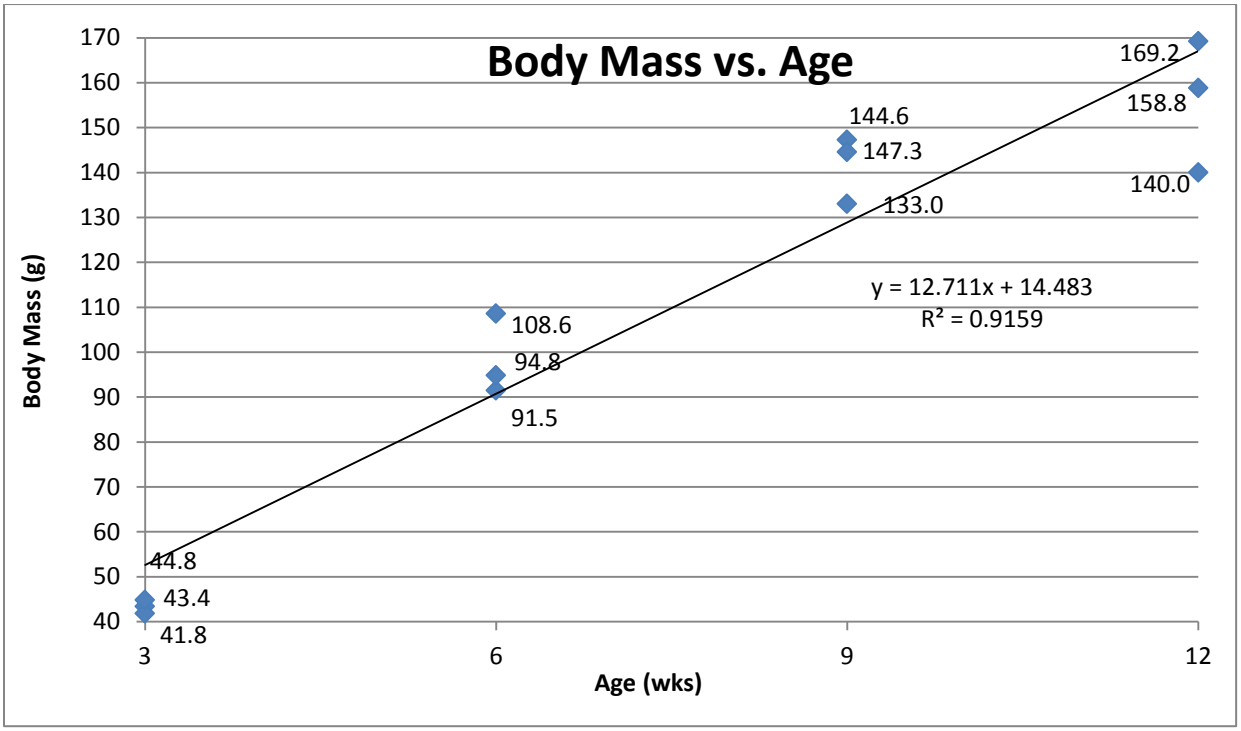


Figure 2: Rat Shank Flexor Complex Mass versus Age

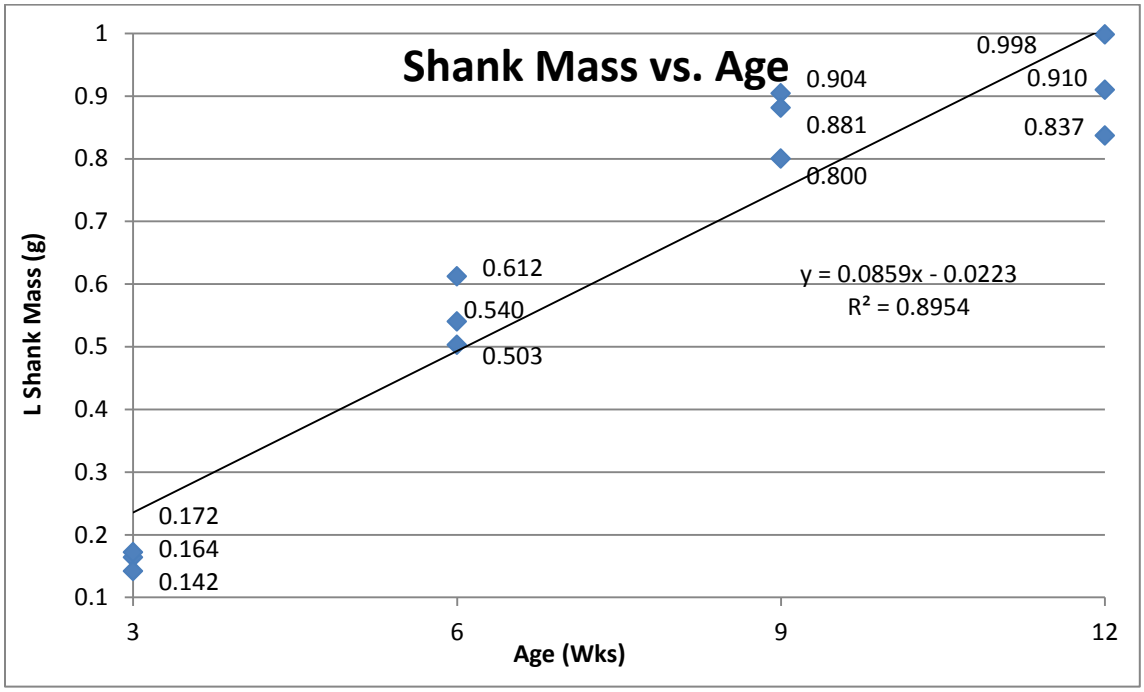


Figure 3: Rat Bone Length versus Age

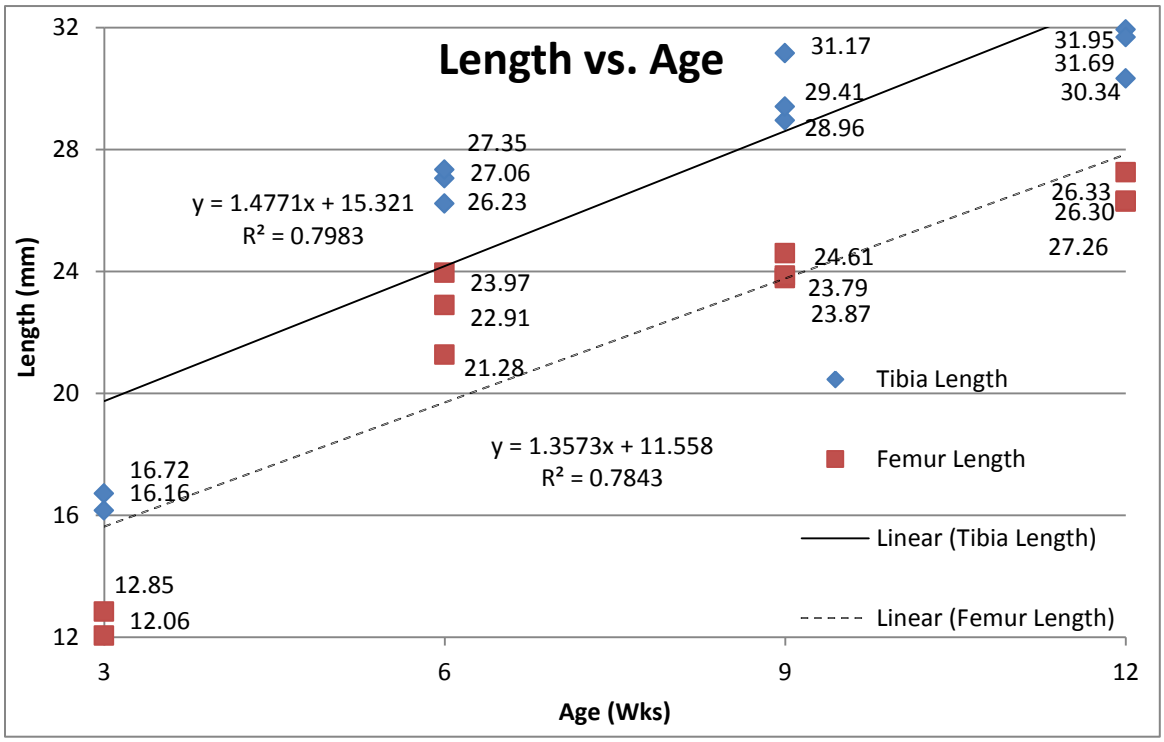


Figure 4: Bivariate Fit of Soleus Hybrids by Age

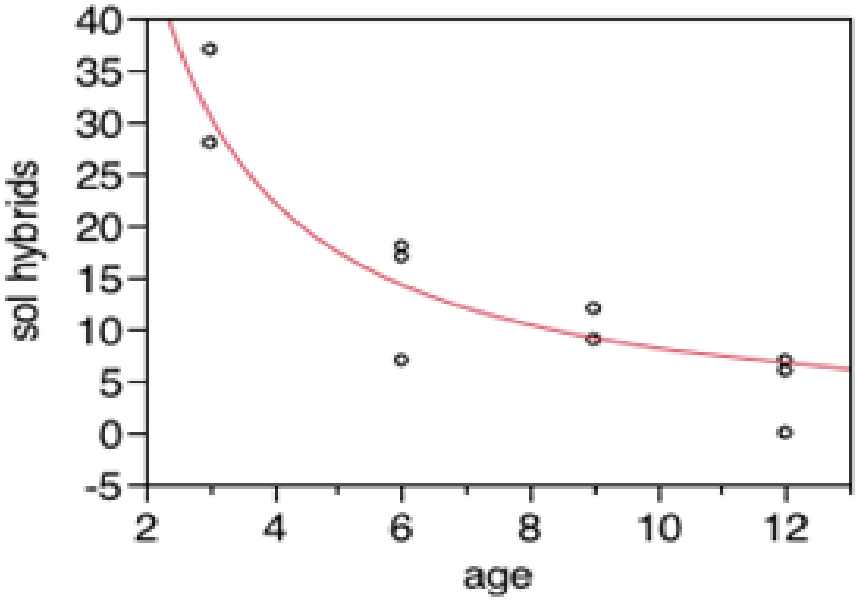


Figure 5: Bivariate Fit of Gastrocnemius Hybrids by Age

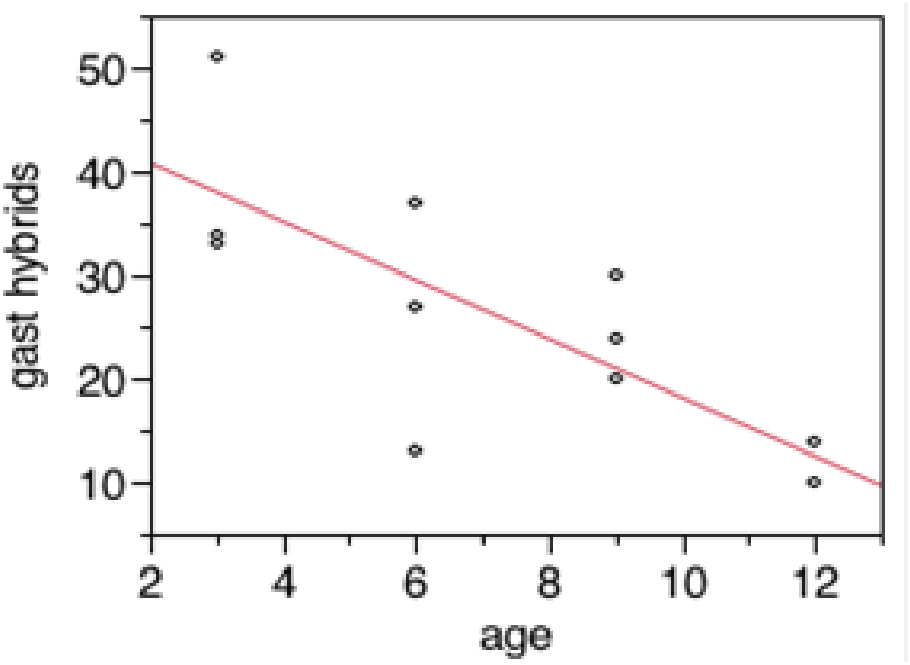


Figure 6: Prevalence of Fiber Types in the Soleus Muscle Per Age Group

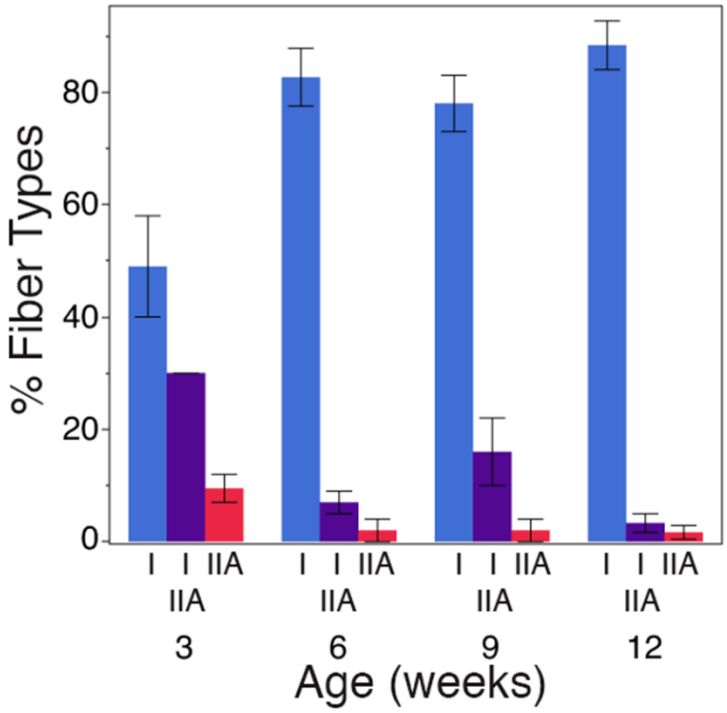


Figure 7: Prevalence of Fiber Types in the Gastrocnemius Muscle Per Age Group

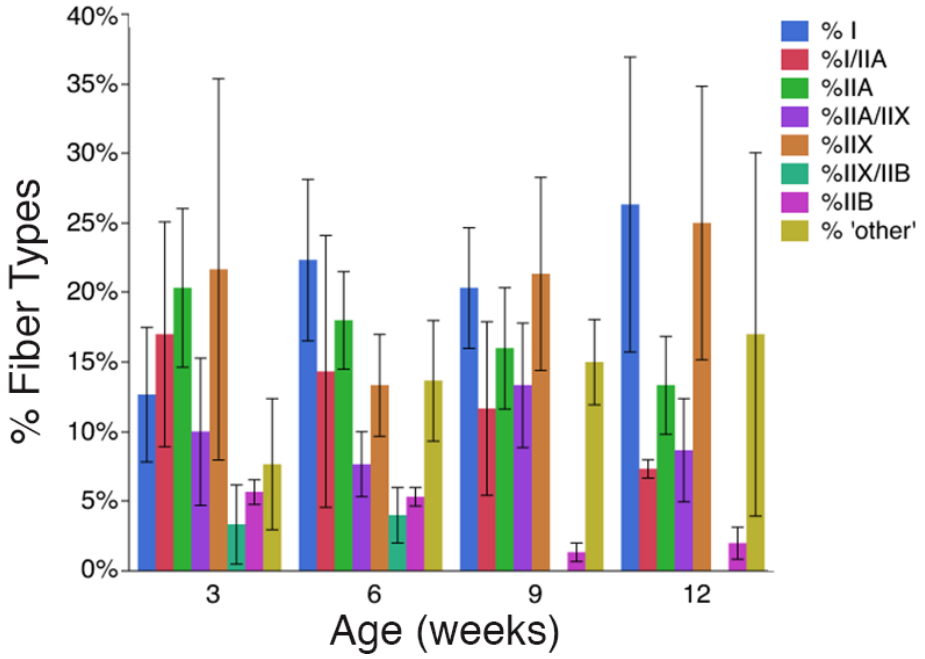


Figure 8: Changes in Soleus Fiber Composition as a Function of Age

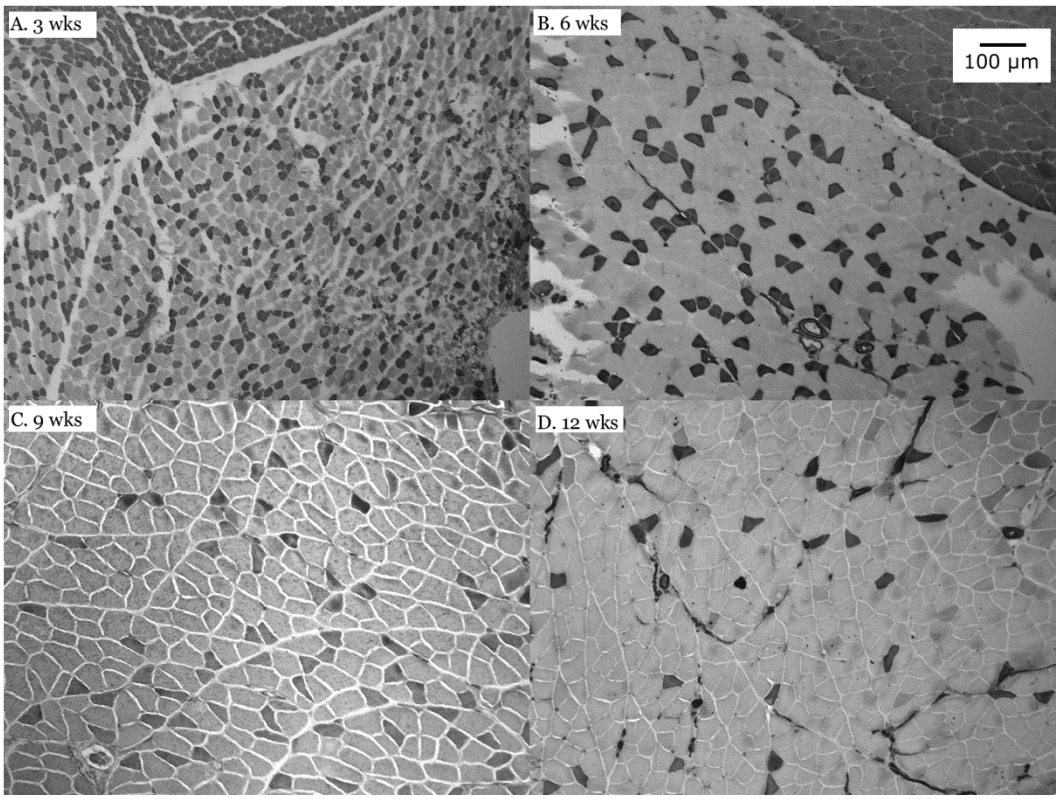


Figure 9: Shank Cross Section with Monoclonal Antibody Labeling

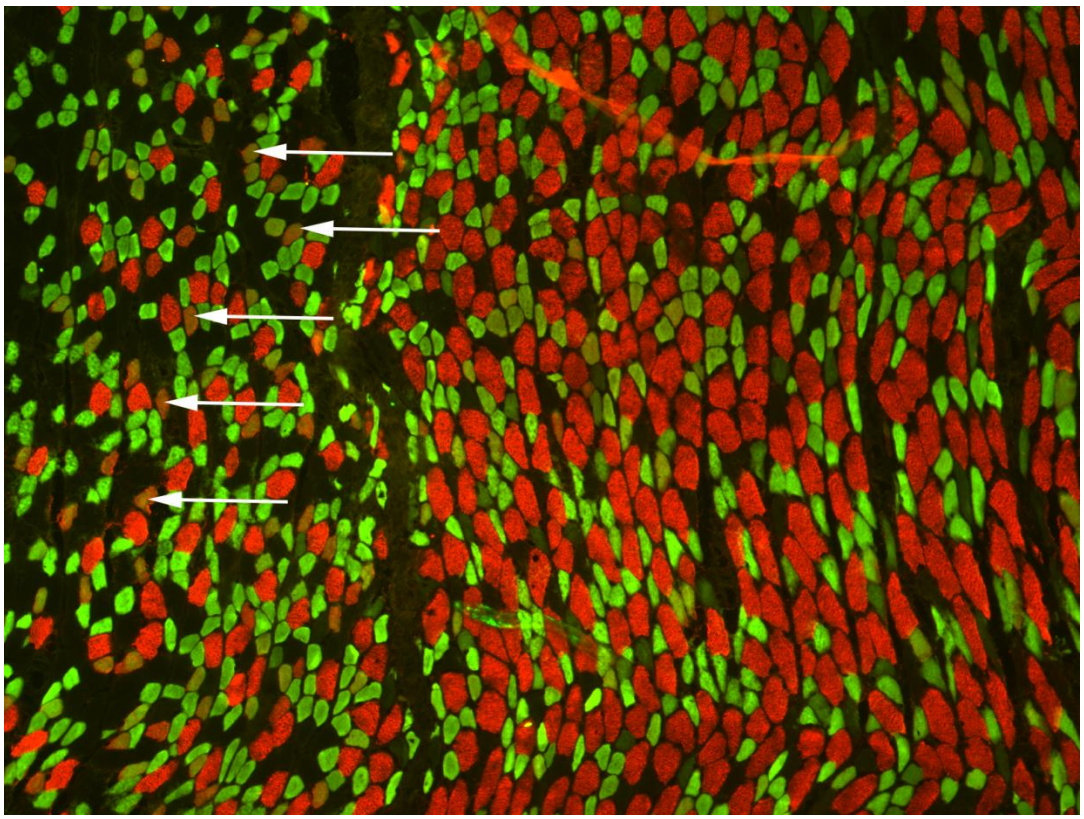


Figure 10: MHC Gel Loaded with Rat 10 Deep Medial Gastrocnemius

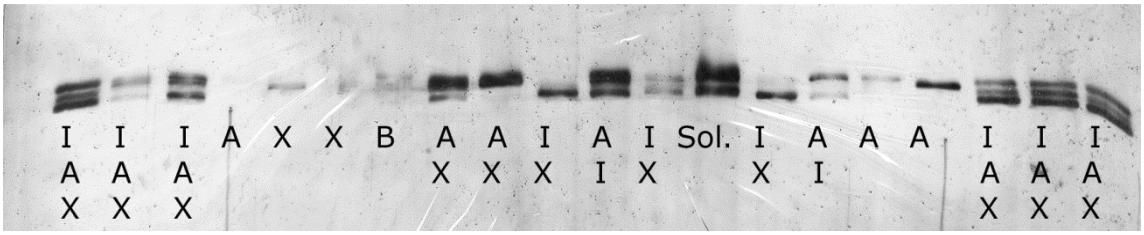


Figure 11: MHC Gel Expressing MHC Neonatal Isoform

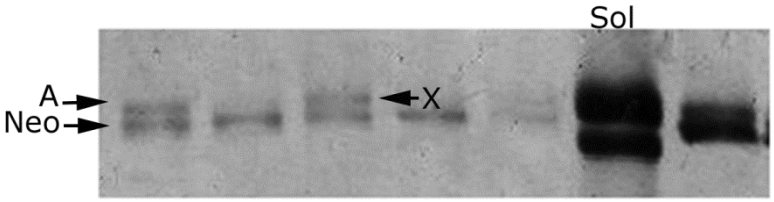


Figure 12: Illustrative Example: Single Mouse Fibers Labeled with Monoclonal Antibodies

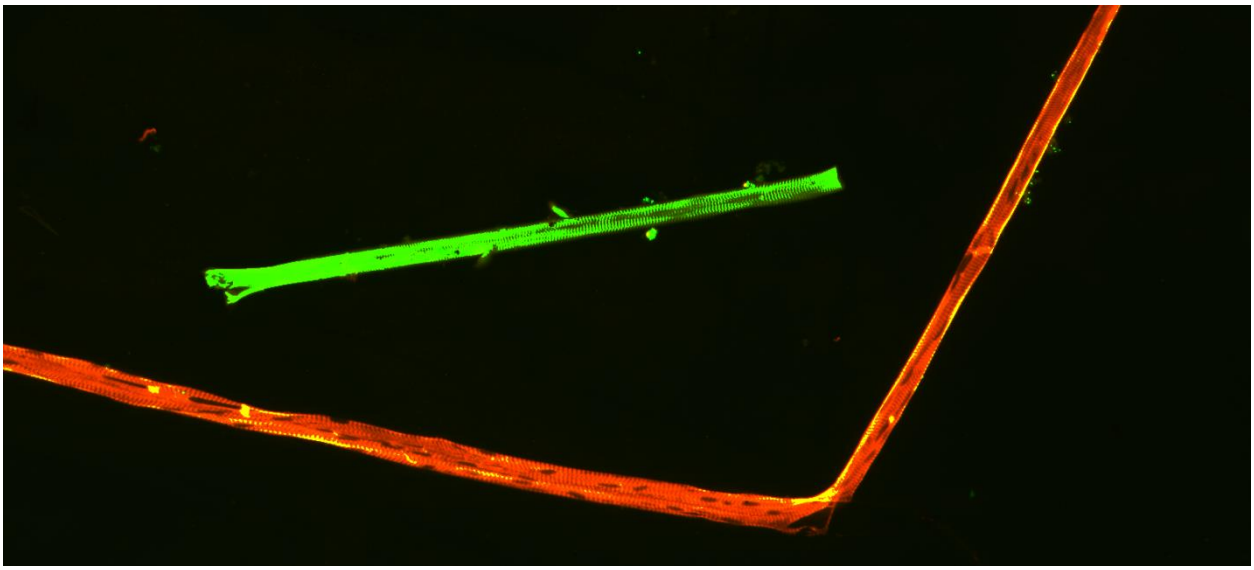


Figure 13: Illustrative Example: Single Mouse Hybrid Fiber Labeled with Monoclonal Antibodies

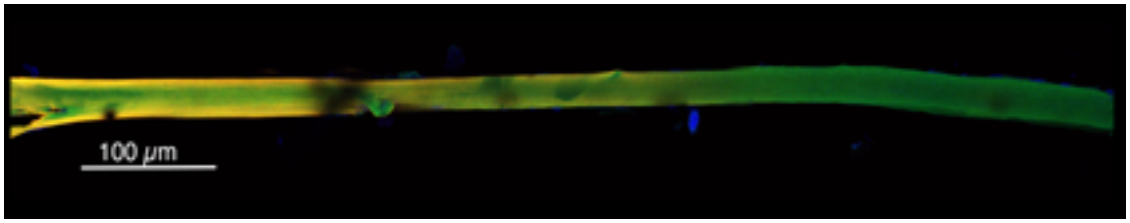


Figure 14: Illustrative Example: Single Asymmetric Hybrid Labeled with Monoclonal Antibodies

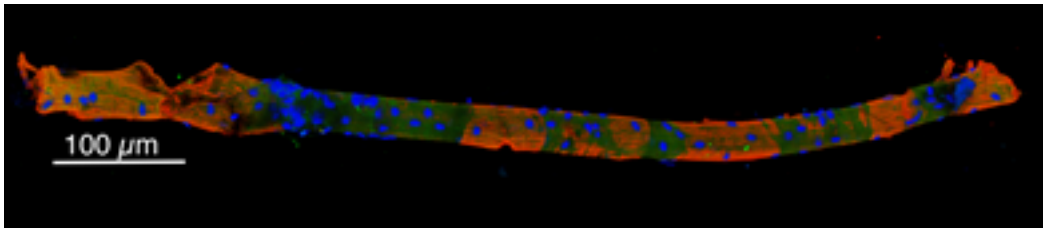


Figure 15: Rat 4 Cross Section and Single Rat Fibers Labeled with Monoclonal Antibodies Exhibiting Hybrid Phenotype

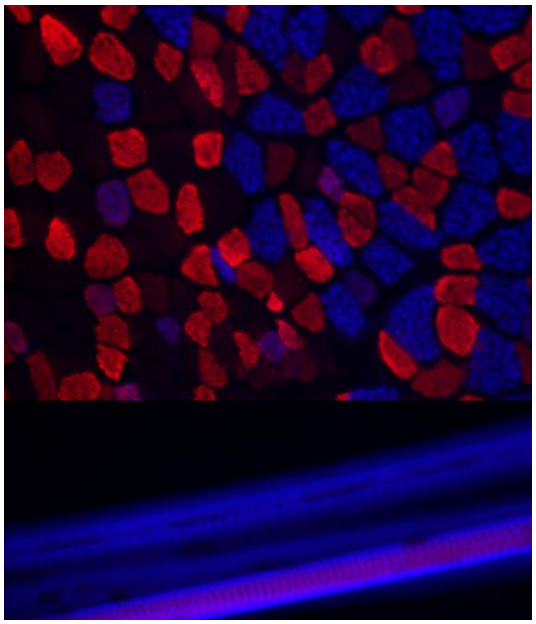


Figure 16: Single Rat Soleus Fibers Labeled with Monoclonal Antibodies Exhibiting Asymmetry

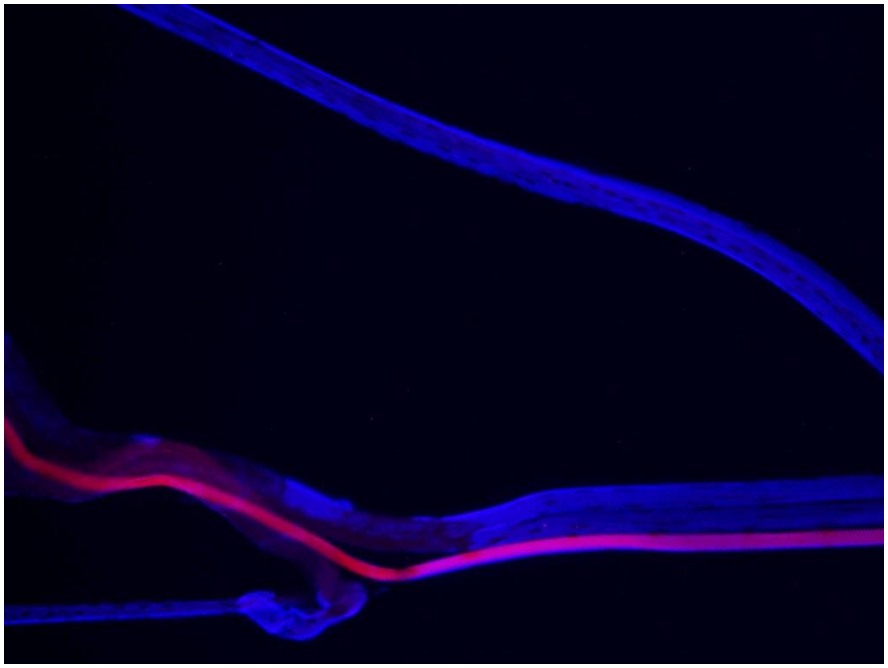
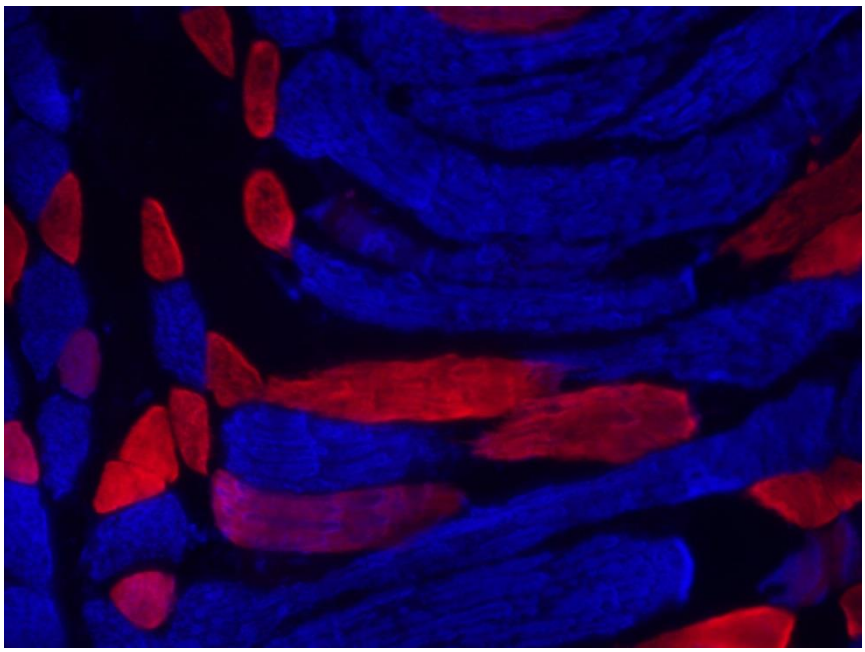


Figure 17: Rat Cross Section Labeled with Monoclonal Antibodies



Tables

Table 1: Monoclonal Antibodies Used in Immunohistochemistry

Properties of Monoclonal Primary and Secondary Antibodies used for Immunohistochemistry; University of Iowa Hybridoma Bank	
4.74; Primary	Mouse anti-human IgG_1, Specific for MHC type IIa in Rodents and Humans. Used in a 1:10 dilution.
4.84: Primary	Mouse anti-human IgM, Specific for slow MHC isoforms in Rodents and Humans. Used in a 1:10 dilution.
F18: Primary	Mouse anti-chicken IgG_1, Specific for fast MHC isoforms in Mammals and Birds, specifically IIb in rodents. Used in a 1:10 dilution.
FITC: Secondary	Goat anti-mouse-FITC (green). Used in a 1:100 dilution.
TRITC; Secondary	Goat anti-mouse-TRITC (red). Used in a 1:100 dilution.
Alexa Fluor 350: Secondary	Anti IgG_1; will bind 4.74 and F18. Used as a 1:200 dilution.
Alexa Fluor 555: Secondary	Anti IgM; will bind 4.84. Used as a 1:200 dilution.

Table 1: Properties of Monoclonal Primary and Secondary Antibodies used for Immunohistochemistry

This table is describing the primary and secondary antibodies used for immunohistochemistry and fluorescent analysis and their properties.

Appendices

Appendix A: Mass (in grams) of Rats 1-12 and their Shank Flexors					
Rat	**Body Mass**	**Mass R Sol**	**Mass R Gast**	**Mass L Shank**	**Mass R Shank**
1	43.4	0.007	0.074	0.164	0.153
2	41.8	Missing**	0.087	0.142	0.151**
3	44.8	0.011	0.085	0.172	0.199
4	91.5	0.028	0.031	0.503	0.534
5	94.8	0.055	0.067	0.540	0.551
6	108.6	0.036	0.041	0.612	0.647
7	147.3	0.084	0.083	0.904	0.894
8	133.0	Missing**	0.078	0.800	0.851**
9	144.6	0.070	0.067	0.881	0.852
10	140.0	0.071	0.068	0.837	0.845
11	169.2	0.083	0.772*	0.998	1.030
12	158.8	0.078	0.746*	0.910	0.946

***** denotes that the value was obtained by subtracting the mass of the soleus and plantaris (not shown) muscles from the total mass of the shank complex, because no value was taken for that individual gastrocnemius. Refer also to Figure 2. ** denotes that because the soleus muscle was lost during dissection, the mass shown is missing one of the muscles in the complex.

Appendix B: Age (wks) vs. Shank & Femur Length (in mm) in Rats 1-12			
Rat	**Age**	**Shank Length**	**Femur Length**
1	3	16.72	12.85
2	3	17.40	*
3	3	16.16	12.06
4	6	27.06	23.97
5	6	26.23	21.28
6	6	27.35	22.91
7	9	31.17	23.79
8	9	28.96	23.87
9	9	29.41	24.61
10	12	30.34	26.30
11	12	31.95	27.26
12	12	31.69	26.33

***** denotes that no value was obtained due to the fact that the femur had decomposed to the point that it was removed in three separate parts and could not be pieced together accurately enough to give a usable value.

Appendix C: SDS PAGE Soleus Data

												N Hybrid						%N Hybrid							
Animal	Total	I	I%	I/IIA	% I/IIA	IIA	% IIA	X	%X	B	%B	N	% N	1/N	A/N	X/N	N/B	N/B/I	1/N	A/N	X/N	N/B	N/B/I	1/x	%1/x	% Unaccounted
1	128	58	45%	36	28%	9	7%	1	1%	13	22%	1	1%	0	3	1	4	1	0%	2%	1%	3%	1%	1	1%	0%
3	145	84	58%	30	21%	18	12%	2	67%	0	0%	3	2%	7	0	0	0	0	5%	0%	0%	0%	0%	1	1%	0%
4	53	41	77%	9	17%	3	6%	0	0%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
5	46	43	93%	3	7%	0	0%	0	0%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
6	49	38	78%	9	18%	0	0%	2	33%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0%
7	109	89	82%	10	9%	0	0%	3	43%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	7	8%	0%
9	180	142	79%	22	12%	8	4%	0	0%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4%
10	68	57	84%	5	7%	3	4%	0	0%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4%
11	79	66	84%	5	6%	1	1%	2	18%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6%
12	76	74	97%	0	0%	0	0%	0	0%	0	0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3%

Shown at left are the results from the SDS PAGE analysis of individual soleus fibers of Rats 1-12. (Note that Rat 2 and Rat 8 Soleus were lost during dissection.) "Total" refers to the total number of fibers analyzed per individual. "% Unaccounted" refers to the percentage of fibers that were not able to be classified. Averages for each age group were obtained and data was used to create Figure 4.

Appendix D: SDS PAGE Gastrocnemius Data

Animal	Total	I	%I	I/IIA	%I/IIA	IIA	%IIA	IIA/IIX	%IIA/IIX	IIX	%IIX	IIX/IIB	%IIX/IIB	IIB	%IIB	I/A/X	%1/A/X	1/X	%1/X
1	77	13	17%	13	17%	19	25%	9	12%	-	0%	1	1%	5	6%	-	0%	-	0%
2	68	2	3%	2	3%	6	9%	12	18%	32	47%	6	9%	5	7%	-	0%	-	0%
3	51	9	18%	16	31%	14	27%	-	0%	9	18%	-	0%	2	4%	-	0%	-	0%
4	71	15	21%	-	0%	18	25%	5	7%	12	17%	4	6%	4	6%	3	4%	7	10%
5	49	16	33%	16	33%	7	14%	2	4%	3	6%	-	0%	2	4%	-	0%	3	6%
6	52	7	13%	5	10%	8	15%	6	12%	9	17%	3	6%	3	6%	9	17%	2	4%
7	54	15	28%	4	7%	5	9%	6	11%	12	22%	-	0%	1	2%	2	4%	9	17%
8	55	7	13%	2	4%	8	15%	12	22%	18	33%	-	0%	1	2%	5	9%	1	2%
9	45	9	20%	11	24%	11	24%	3	7%	4	9%	-	0%	-	0%	4	9%	2	4%
10	51	6	12%	4	8%	6	12%	8	16%	4	8%	-	0%	1	2%	14	27%	8	16%
11	51	24	47%	3	6%	4	8%	3	6%	13	25%	-	0%	2	4%	1	2%	1	2%
12	50	10	20%	4	8%	10	20%	2	4%	21	42%	-	0%	-	0%	-	0%	3	6%

Hybrid N						%Hybrid N								
N	%N	1/N	A/N	X/N	N/B	N/B/I	1/N	A/N	X/N	N/B	N/B/I	%Unaccounted		# of fibers
0	0%				13	-	0%	0%	0%	17%	0%	29%		4
0	0%	1		2		-	1%	0%	3%	0%	0%	0%		0
0	0%	1				-	2%	0%	0%	0%	0%	0%		0
												4%		3
												0%		0
												0%		0
												0%		0
												2%		1
												2%		1
												0%		0
												0%		0

Shown at left and continued below are the results from the SDS PAGE analysis of individual gastrocnemius fibers of Rats 1-12. "Total" refers to the total number of fibers analyzed per individual. "% Unaccounted" refers to the percentage of fibers that were not able to be classified.