INVESTIGATION OF A ROLE FOR THE α7 INTEGRIN AS A MECHANOTRANSDUCER OF HYPERTROPHIC SIGNALING IN SKELETAL MUSCLE

BY

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THESIS

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Master’s Committee:

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ABSTRACT

The α7β1 integrin is a transmembrane protein in skeletal muscle which serves as a focal adhesion at costameres of skeletal muscle, providing a critical link between the actin cytoskeleton inside the cell with laminin in the basement membrane of the extracellular matrix. Previous research has demonstrated that overexpression of the α7 integrin accelerates the muscle growth response to eccentric exercise compared to wild type controls. **PURPOSE:** The goal of this project was to elucidate a role for the α7 integrin as a mechanotransducer and intrinsic regulator of hypertrophic signaling in response to mechanical strain. **METHODS:** Primary myoblasts extracted from wild type (WT) mice were developed into myotubes in culture to determine the hypertrophic signaling response to mechanical strain. Primary myoblasts from α7 knockout (KO) mice were used as controls for these experiments. Further experimentation employed transient transfection of WT myoblasts with a α7 integrin plasmid transgene containing a muscle creatine kinase and myosin heavy chain promoter (MHCK7) to induce α7 integrin expression. Transfected myotubes were subjected to overnight incubations in wortmannin with the goal of inhibiting any potential autocrine-mediated growth factor signaling pathway through PI3K inhibition. **RESULTS:** Lack of α7 integrin was verified in KO cells. In WT, mechanical strain increased α7 integrin protein at 3 PS on both substrates compared to control (P<0.05; 1.8-fold collagen, 3-fold laminin). Phosphorylation of p70 S6K was increased 5-fold only on laminin at 3 PS (P<0.05). In contrast, no change in p70 S6K phosphorylation was observed in KO cells on either substrate. Interestingly, mTOR phosphorylation was not significantly altered in WT myotubes, and no change was observed in KO myotubes in response to strain. Transgenic overexpression of the α7 integrin in myotubes resulted in a trend toward increased activation of hypertrophic signaling compared to controls, which did not reach statistical significance. **CONCLUSION:** This study provides evidence that the α7 integrin is upregulated in response to mechanical strain and is inconclusive on whether the α7 integrin is an intrinsic regulator of strain-induced hypertrophic signaling.
ACKNOWLEDGEMENTS

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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

A) Purpose

Myofibrillar protein synthesis in skeletal muscle is greatly enhanced by the presence of nutrients, such as amino acids, and insulin/insulin-like growth factor receptor activation (Goldspink 2002; Hornberger, 2006). Growth factor induced stimulation through the Insulin/IGF-1 receptor is the most investigated, and nutrient stimulated hypertrophic signaling also has been greatly explored. While there is suggestion in the literature that myofibrillar synthesis can occur in muscle in an IGF-1 receptor-independent manner in response to mechanical strain, the mechanisms responsible for this event in muscle are not known. In addition, the primary stimulus for increased muscle growth in response to eccentric contractions has not been identified.

Integrins link the cell cytoskeleton with the extracellular matrix (ECM), thereby creating an intrinsic system capable of transmitting mechanical forces between the outside and inside of a cell (Roca-Cusachs, 2012). Overexpression of the α7 integrin in muscle using a transgenic animal approach has been used to test the hypothesis that this protein is important for muscle growth following exercise, including facilitation of fiber cross sectional area, myofibrillar content, and increased activation of hypertrophic signaling compared to wild type controls (Lueders, 2012; Zou, 2012). The purpose of this study was to determine the extent to which the α7 integrin directly regulates the growth response to eccentric exercise.

B) Mechanosensing of cells

All living things possess the ability to respond to stimuli, and thus each individual cell possesses the necessary machinery to sense stimuli. The cellular response to mechanical stimulation has been well studied. Some of the observed responses to mechanical strain include increases in size due to growth, increases or decreases in cell proliferation, changes in morphology, secretion of factors, and
alterations in alignment (Adams, 2004; Duncan, 1995; Neidlinger-Wilke, 2002; Pennisi, 2011; Wilson, 1993).

Osteoblasts and osteocytes in bone are highly responsive to loading, and this event can subsequently allow for an increase in bone formation (Duncan, 1995). Both vascular smooth muscle and skeletal muscle have been shown to release growth factors in response to strain, which cause growth in an autocrine fashion (Adams, 2004; Wilson, 1993). Both fibroblasts and myoblasts have been shown to align perpendicularly to strain, and in the presence of differentiation media, myoblasts form myotubes aligned perpendicularly to the axis of strain in vitro (Neidlinger-Wilke, 2002; Pennisi, 2011).

![Figure 1: Myoblasts align perpendicularly to the axis of strain, and differentiate perpendicularly to strain as well (Pennisi, 2011).](image)

C) The α7β1 Integrin

Integrins are transmembrane protein heterodimers consisting of α and β subunits. Integrins connect the cytoskeleton inside of the cell, with the extracellular matrix outside of the cell. They are found in various cell types to be sensors of external stimulation and transmit perceived stimuli as chemical signals into the cell (Wilson, 1995).
Eighteen different $\alpha$ subunits and 8 different $\beta$ units are expressed by a variety of mammalian cell types, resulting in the potential to form numerous unique integrin heterodimers. The $\alpha 7\beta 1$ integrin heterodimer, which is highly expressed in skeletal muscle, primarily at the neuromuscular and myotendinous junctions, acts to stabilize Z bands. The $\alpha 7$ integrin subunit binds laminin in the basal lamina, and the $\beta 1$ subunit binds the actin cytoskeleton within the muscle fiber (Bao, 1993; Boppart, 2008). The splicing of $\alpha 7$ transcripts can result in cytoplasmic domain isoforms $\alpha 7A$ or $\alpha 7B$, and extracellular domain isoforms $BX1$ and $BX2$. The predominant heterodimer is $\alpha 7BX2$ for both proliferating myoblasts, and adult skeletal muscle (Burkin, 1999). In the process of differentiation however, myoblasts predominantly express the extracellular splice variant $X1$, switching the expression to splice variant $X2$ once muscle fibers are mature (Schober, 2000). Both isoforms $\alpha 7A$ and $\alpha 7B$ are expressed in skeletal muscle as well as in terminally differentiated myotubes in vitro. However myoblasts express only the $\alpha 7B$ isoform and do not begin expressing the $\alpha 7A$ isoform until differentiation begins (Collo, 1993).
Figure 3: The α7β1 integrin acts to stabilize skeletal muscle by connecting laminin in the extracellular matrix with the actin cytoskeleton at z-bands in skeletal muscle (Used with permission, MD Boppart).

The α7 integrin plays an important structural role in skeletal muscle, providing stabilization of the fibers within the extracellular matrix. The expression of the α7 integrin is important for intramuscular health and prevention of damage during contraction (Boppart, 2006; Boppart, 2008). It plays a particularly important role in maintaining myotendinous junction integrity and is highly expressed both during development and in adult skeletal muscle. Additionally, the α7 integrin is important for the formation and proper structure of the neuromuscular junctions. Early neuromuscular junction formation is characterized by clustering of acetylcholine receptors, which is induced by the presence of laminin and the α7 integrin (Burkin, 1999).
Figure 4: The α7 integrin is highly expressed at the neuromuscular and myotendinous junctions in skeletal muscle (Burkin, 1999).

The α7 integrin allows for a compensatory role in muscular diseases where the structural integrity is compromised such as with muscular dystrophy, and the lack of the α7 integrin results in an increased accumulation of damage in muscle resulting in a similar pathology as muscular dystrophy (Mayer, 1997). In both human patients, and experimental mdx mice, α7 integrin protein is upregulated to compensate for the lack of transmembrane adhesions between the muscle fiber and extracellular matrix (Liu, 2011). In mdx mice, which serves as a model for Duchene Muscular Dystrophy, there is restoration in functionality and protection against sarcolemmal damage when bred with α7 integrin overexpressing transgenic mice so long as there is an accompanying overexpression of the β1 portion of the heterodimer due to increases in functional α7 integrin at the cell membrane (Liu, 2011). Mice lacking expression of the α7 integrin develop a progressive dystrophic phenotype, characterized the presence of damage and inflammation in muscle which is especially pronounced at the myotendinous junction (Mayer, 1997; Boppart, 2008).
In addition to the structural contribution of the α7 integrin to healthy muscle, there is evidence to suggest the α7 integrin plays a role in regulating hypertrophic signaling. The literature provides some conflicting results as to whether the α7 integrin enhances or negatively regulates the hypertrophic signaling response. In one study, activation of stress activated protein kinases c-jun NH2-terminal kinase (JNK) and p38 were reduced in transgenic mice overexpressing the α7 integrin following 30 minutes of downhill running as compared to WT controls (Boppart, 2006). Additionally, activation of hypertrophic signaling molecules mTOR and p70 S6 Kinase was reduced (Boppart, 2006). More recent studies have since demonstrated increases in whole muscle and fiber cross sectional area, force output, and activation of hypertrophic signaling in transgenic mice overexpressing the α7 integrin following both acute and multiple (3x/wk for 4 wks) bouts of downhill running (Leuders, 2012; Zou, 2012).
D) The Hypertrophic Signaling Pathway

Hypertrophy of muscle fibers can occur due to increased cytosolic content, such as increased water and glycogen, or due to increased accumulated contractile myofibrillar protein (Glass, 2005). Net protein accumulation can only occur if formation of contractile proteins occurs at a rate that exceeds degradation. Hypertrophic signaling refers to the signaling cascade that transmits extracellular signals into an increase in translational efficiency in the process of protein synthesis (Kimball, 2002; Goldspink, 1977). The four steps of protein translation include: initiation, elongation, translocation and termination. These steps are facilitated by ribosomes which are composed of a large 60S and small 40S subunit (Mahoney, 2009). In the step of initiation, initiation factors allow for the association of both mRNA and tRNA with the ribosome (Berg, 2002). In the step of elongation, mRNA moves through the ribosome, adding amino acids in a step wise fashion which elongates the peptide. Translocation is the step of translation in which tRNA moves to a different site on the ribosome, allowing for additional tRNA to bind the ribosome while the original mRNA continues to be translated until a stop codon is reached.
Termination is the final step of translation in which a stop codon is reached in the mRNA sequence, and the amino acid chain is hydrolyzed, freeing it from the ribosome (Berg, 2002).

The steps of translation that can potentially be regulated are initiation, elongation, and termination, and hypertrophic signaling in skeletal muscle primarily affects initiation (Kimball, 2002). Initiation is the rate limiting step of translation and requires a series of eukaryotic initiation factors (eIFs), which assemble together with the 60S and 40S subunits of the ribosome as well as tRNA. Initiation factors eIF1A, eIF1, eIF3, and eIF5 form the pre-initiation complex (PIC) which binds the 40S ribosomal subunit. Three initiation factors eIF4E, eIF4G, and eIF4A form a complex called eIF4F which bind the cap at the 5’ end of mRNA, facilitating binding of mRNA with the pre-initiation complex on the ribosome. Finally, eIF2 is freed from the complex through hydrolysis of GTP which is a process facilitated by eIF2B. The release of eIF2 and other initiation factors from the complex allows for the binding of the 40S and 60S subunits together to form the 80S ribosome. Phosphorylation and dephosphorylation is the regulatory mechanism for many of the proteins involved in translation. For example, eIF4F formation is a step which can be regulated by the hypertrophic signaling cascade. eIF4E-binding proteins (4E-BPs) bind eIF4E, and phosphorylation of these binding proteins releases eIF4E allowing its involvement in the formation of eIF4F (Mahoney, 2009).

Three distinct pathways have been identified (growth factor, nutrient, and mechanostimulation) all of which lead to increased translational efficiency. It is thought that the central hypertrophic signaling molecule at which these three distinct pathways conjoin is the mammalian target of rapamycin (mTOR). mTOR is made of two complexes, mTORC1 and mTORC2, with mTORC1 being shown to directly phosphorylate 4E-BP1, allowing for the formation of eIF4F, and also phosphorylate S6 Kinase via p70 S6 kinase which together allow for hypertrophy through the regulation of ribosomal biogenesis and protein synthesis (Mahoney, 2009). In growth factor signaling, mTOR is activated by phosphorylation through
AKT, however the precise mechanisms for the activation of mTOR via nutrient and mechanical stimuli are unknown.

Figure 7: Diagram of the hypertrophic signaling pathway shows three distinct and independent pathways involving nutrient, growth factor, and mechanostimulated activation of protein synthesis (Hornbeger, 2011).

The most well described pathway is growth factor signaling which begins with the insulin/IGF-1 receptor, and signals through AKT, which in addition to its involvement in protein synthesis, plays a role in regulating protein degradation and glucose uptake. The amino acid, or nutrient stimulated hypertrophic signaling pathway is less understood, but this pathway appears to be dependent on mTOR and independent of AKT (Anthony, 2000; Wang 1998). The third and least understood of these pathways is the mechanically stimulated pathway, in which a mechanotransducer transmits stress signals from outside of the cell through a signaling cascade to increase translation initiation efficiency. One of the pioneer studies which identified mechanical stimulation alone is sufficient to stimulate an increased accumulation of myofibrillar protein was performed by Vandenburg et al. in 1979. In this study, myotube cultures were stretched on frames in the absence of growth factor and amino acid uptake and total protein accumulation were measured 72 hours after the application of mechanical strain. The
results showed a marked increase in total protein in strained myotubes as compared to unstrained controls.

Figure 8: Mechanical stimulation of cultured myotubes in the absence of growth factors results in hypertrophy and increased total protein compared to unstrained controls (Vandenburg, 1979).

The use of pharmacological inhibitors is an important tool for study into the mechanisms involved in stimulating the hypertrophic signaling pathway. Wortmannin is a fungal inhibitor of phosphoinositide 3-kinase (PI3K), a signaling protein that transmits the growth factor signal from the receptor to AKT. The use of wortmannin is important in establishing the mechanism by which translation is initiated, by eliminating the ability for AKT to phosphorylate and activate mTOR. In response to mechanical loading, muscle specific forms of IGF-1, also known as MGF, can be released from the tissue, which can activate growth factor stimulated hypertrophy via autocrine and paracrine signaling (Adams, 2004; Goldspink, 2002). Inhibition of paracrine and autocrine growth factor signaling is imperative in determining the extent to which mechanical strain can stimulate hypertrophic signaling through an intrinsic mechanism. Another inhibitor, rapamycin, acts further downstream, inhibiting the
activation of mTOR. Similar to wortmannin’s use in implicating AKT involvement in signaling, rapamycin is a useful tool to implicate the involvement of mTOR in hypertrophic signaling. Miyazaki et al. demonstrated the use of these tools nicely in a study published in 2011. In this study, both wortmannin, and rapamycin inhibited activation of p70 S6 Kinase demonstrating the necessity of both mTOR and AKT in growth factor induced hypertrophic signaling. In a second experiment, Miyazaki used synergistic ablation of the gastrocnemius and soleus to cause an overload of the plantaris muscle to model load induced hypertrophy. Wortmannin and rapamycin were used to determine whether AKT and mTOR were necessary for the hypertrophic signaling response to mechanical overload. Consistent with other studies, he found that activation of p70 S6 Kinase (Thr389) by mechanical loading was AKT independent, and mTOR dependent. Interestingly, this study demonstrated a partial blunting of p70 S6 Kinase (Thr421/424) in response to overload in the presence of rapamycin. Similar results have been obtained using other approaches (Hornberger, 2004).

Figure 9: Injection of insulin alone results in the activation of p70 S6 Kinase (Thr389 & Thr421/Ser424), but this response is inhibited by both rapamycin and wortmannin. Overload of the plantaris muscle due to synergistic ablation results in activation of p70 S6 Kinase (Thr389 & Thr421/Ser424) with or without PI3K inhibition through wortmannin. Only p70 S6 Kinase (Thr389) activation due to overload is completely blocked with rapamycin, but (Thr421/Ser424) signaling is only slightly reduced.
E) Hypothesis

We hypothesize that the α7 integrin is a mechanotransducer and an intrinsic regulator of hypertrophic signaling. A series of experiments using myotubes in vitro will be employed to test the following specific hypotheses:

1. The presence of the α7 integrin is necessary for phosphorylation and activation of mTOR and p70 S6K in response to mechanical strain.

2. Adhesion of the α7 integrin to laminin, a specific ligand for the α7 integrin, provides optimal myotube adhesion and α7 integrin-mediated increases in hypertrophic signaling.

3. The α7 integrin can increase activation of mTOR and p70 S6K in a manner independent of growth factor signaling.

F) Formulating the Experimental Design

In attempting to implicate mechanotransduction as the mechanism for the increased hypertrophy of skeletal muscle observed in transgenic α7 overexpressing mice using in vitro methods, a model for mechanical stimulation of myotubes was required. Hornberger et al. previously optimized a strain protocol for use with C2C12 cells using the Flexcell System created by Flexcell International (Hornberger, 2004). In this experiment, C2C12 myoblasts were differentiated into myotubes on a collagen I treated bioflex membrane and strained uniaxially or equibiaxially at 15% strain for 10, or 60 minutes, resulting in significant increases in phospho p70 S6 Kinase (Thr 389) only with equibiaxial strain. Based on this information, myoblasts extracted from α7 integrin knockout mice were differentiated into myotubes, and in additional experiments, transient transfection was used to overexpress the α7 integrin using plasmid DNA. Current research methods have been unable to transfect terminally differentiated myotubes using liposome mediated transfection reagents, and
additional work has demonstrated higher efficiencies with the transfection of cells in suspension (Escobedo, 2003; Neuhuber, 2002). Using this information the transfection protocol developed for experiments performed in this thesis followed the established method of transfecting myoblasts during plating. The lack of a reporter in the plasmid vector brought the obstacle of determining whether transfection was successful. Previous studies provided the observation that the rat α7 integrin which is coded in the plasmid transgene, is cleaved differently in western blotting than the resident mouse integrin, which results in an additional 70 kDa band which was used to identify whether or not transfection was successful (Liu et al. 2011).

Figure 10: Cleavage of rat α7 integrin occurs during western blotting, which results in a 70 kDa band from muscles with transgenic overexpression of the α7 integrin. Due to the lack of a reporter in the vector, this information was used to determine whether transfection with the transgenic α7 plasmid was successful. (Liu, 2011)
CHAPTER II

METHODOLOGY

A) Cell culture

C2C12 Cells were grown in 50/50 DMEM/F12 (Cellgro #10-092-CV), 20% Fetal Bovine Serum (FBS) (Atlanta Biologicals #s11150 – Lot B1011) and 1% Penicillin/Streptomycin (Cellgro #30-002-CI). Primary Cells were grown in 50/50 DMEM/F12, 20% FBS 1% Penicillin/Streptomycin, with .01% recombinant Fibroblast Growth Factor (rFGF) (Invitrogen #PHG0024). Cells were thawed and plated on plastic to eliminate cells which died during freezing or thawing. After 48 hours they were detached with trypsin, counted, and plated (~150,000 cells/well) on a 6 well Bioflex silicone membrane, from Flexcell International. The membranes were treated with collagen I (Flexcell International #BF-3001C), or laminin (Flexcell International #BF-3001L) substrate. Growth media was replaced by differentiation media consisting of high glucose DMEM (Cellgro #10-017-CV) with 2% horse serum (Life Technologies #26050-088), and 1% penicillin/streptomycin and added to myoblasts when ~70% confluence was obtained.

Figure 11: The process of isolating primary myoblasts from mice using a plating method (Musaro, 2010).
When large multinucleated myotubes were more abundant than remaining myoblasts, the media was replaced with growth factor depleted stretch media consisting of high glucose DMEM and 1% penicillin/streptomycin. For PI3K inhibition experiments, 10 μM wortmannin (EMD Millipore #681675) was added to the stretch media.

**B) Transfection**

Ampicillin resistant bacteria (DH5α Amp-R) containing either plasmid DNA encoding for rat α7 integrin with a myosin heavy chain and creatine kinase promoter (pMHCK-BX2) or a vector (MHCK-CAT) was streaked onto Luria Broth (LB) + Ampicillin (AP) treated agar plates, and grown overnight at 37°C. The following afternoon, single colonies were isolated and placed into 50 mL Falcon tubes containing 5ml of LB with 100 mg/ml AP. Plasmid DNA was extracted the following morning using QIAprep Spin Miniprep Kit (QIAGEN #27104).

Transient transfection using liposome mediated delivery reagents was performed using .5 μg of DNA with either the α7 plasmid or the empty vector as a control. In order to find a working reagent, an initial experiment using FuGENE® HD (Promega #E2311), Effectene (QIAGEN # 301425), or Lipofectamine (Life Technologies # 18324010) was performed. For this test, cells were suspended in growth media and exposed to the DNA-transfection reagent mix for 24 hours while adhering to the Bioflex membrane. After deciding to use FuGENE® HD as the transfection reagent, additional experiments were performed using this reagent only.

**C) Mechanical Strain**

Mechanical strain was applied to myotubes after an 18 h incubation period in growth factor depleted stretch media with wortmannin, or media only. Strain was applied to the cells equibiaxially by deforming the silicone membrane of the Bioflex plate 18% using the Flexcell System (FX-4000)
The strain was applied in a triangle waveform with a 50% duty cycle, for 1 second, with 1 second of rest. This was repeated for 20 minutes.

Figure 12: Diagram of cell culture methods. Myoblasts were plated on bioflex membranes, differentiated into myotubes, and strained using the Flexcell system. When applicable, transfection was performed on suspended cells as they attached to the bioflex membrane.

D) Myotube Harvest

Myotubes were removed from the Bioflex dish using a cell scraper immediately post strain (0h) or three hours post strain (3h). Unstrained plates (rest) were used as controls. Cells were scraped in the stretch media (2 wells = 1 n), transferred to 15 mL falcon tubes, which were then placed on ice until they were spun at 450 g for 5 minutes. The media was aspirated, the pellet resuspended in 1 mL of cold 1x PBS, and the suspension was transferred to a 1.5 mL eppendorf tube and spun at 14,000 rpm at 2°C. The 1x PBS was aspirated off, and the pellet was frozen at -80°C until protein was extracted.

E) Protein Extraction

After harvesting the myotubes from the Bioflex dishes, protein was extracted in RIPA Buffer (Tris-Base, NaCL, NP-40, Sodium Deoxycholate, SDS Powder, PMSF, PhosSTOP (Roche #04-906-845-001) [1 pill/10 mL] and Complete Mini (Roche #04-693-124-001) [1 pill/10 mL]) on ice. Pellets were re-suspended in RIPA buffer and agitated for 45 minutes. The tubes were spun at 14,000 RPM, and supernatant containing the proteins of interest were collected. Remaining Pellets were re-suspended in
RIPA buffer and agitated for an additional 30 minutes. After a second spin at 14,000rpm, supernatant was collected and added to the supernatant collected after the first spin.

**F) Western Blot**

After protein extraction, the concentrations of protein were determined by Bradford assay. Protein was diluted with ddH₂O accordingly to reach 30ug of protein. Laemmli buffer and DTT was added to samples (reducing conditions), and boiled for 5 minutes when probing for signaling proteins. When probing for α7 integrin, Laemmli buffer without DTT was used, and the samples were not boiled (non-reducing conditions). SDS-page gels with 8% acrylamide running and 3.5% acrylamide stacking was used for both signaling proteins and α7 integrin. Precision Plus Protein Standard (BIO-RAD #161-0374) was loaded into one of the wells of the gel along with the samples and 100 volts were applied for 30 minutes. The voltage was then adjusted to 200 volts and gels were run until the 25 kDa band reached the bottom.

Protein was transferred to a nitrocellulose (GE Healthcare #RPN303D) membrane at 100 volts for 30 minutes in cold transfer buffer. The nitrocellulose membranes with protein run from reducing conditions were cut into 4 pieces. A cut above the 37 kDa ladder was made to probe for phosphorylated (Cell Signaling #4856) and total S6 (Cell Signaling #2217). A cut was made just above the 50 kDa ladder, and this piece was used to probe for α-tubulin (Cell Signaling #2144) which was used as a loading control. Another cut was made just above the 100 kDa ladder; the next piece was used to probe for Phospho-p70 S6 Kinase (Thr389) (Cell Signaling #9205) and Phospho-p70 S6 Kinase (Thr421/Ser424) (Cell Signaling #9204); with the remaining top piece used to probe for phosphorylated (Cell Signaling #2971) and total mTOR (Cell Signaling #2972). A separate gel was used to probe for α7 integrin (CDB347), which was re-probed for β-actin (Cell Signaling #4967) as a loading control. A separate gel was used to probe for phosphorylation of AKT (Cell Signaling #587F11) and total AKT (Cell Signaling #9272).
Membranes were blocked in the same solution as the primary antibody (either 5% nonfat dry milk [NFDM], or 5% Bovine Serum Albumin [BSA]). Primary antibodies were mixed in a concentration of 1:1000, all in 5% BSA except for the α7 antibody and α-tubulin which were mixed in 5% NFDM. All membranes except the membrane for α7 integrin were blocked for 1 hour, probed overnight, and incubated for 1 hour in 1:2000 secondary antibody conjugated with horse radish peroxidase (HRP) in 2% NFDM. The α7 integrin was blocked overnight, probed in the primary antibody for 1 hour, and the secondary for 1 hour at a concentration of 1:10000. After a series of washes in TBS-Tween, blots were incubated in SuperSignal West Dura Extended Duration Substrate (Thermo Scientific #34076) solution for 5 minutes in the dark, and imaged using the BIORAD Chemidoc XRS. Images were processed and quantified using Quantity One software. In order to re-probe membranes for proteins of the same size after imaging, ECL solution was rinsed off using 1x TBS-Tween, and incubated in stripping buffer for 20 minutes. The stripping buffer was then washed off 3x in 1x PBS

![Diagram of protein analysis](image)

**Figure 13:** Diagram of protein analysis. After strain, myotubes were scraped from the bioflex membrane and cytoplasmic proteins were extracted using RIPA buffer. Proteins were separated using gel electrophoresis and transferred to a nitrocellulose membrane. Antibodies were used to detect the protein of interest and imaged using the Chemidoc XRS. Protein was quantified using Quantity One software.

**G) Immunocytochemistry**

Immunocytochemistry for α7 integrin was performed after an overnight incubation in laminin-111 (Invitrogen # 23017-015) on myotubes cultured on glass chamber slides. Samples were fixed for 30
minutes in 4% paraformaldehyde and blocked for 1 hour in 10% horse serum (HS). Samples were incubated for 1 hour in the primary antibody (CDB347) which was prepared at 1:500 in 1% HS. The secondary antibody (FITC-anti rabbit 1:250 in 1% HS) incubation was also 1 hour.

**H) List of Materials**

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<tr>
<td>Phosphorylated mTOR</td>
<td>Cell Signaling</td>
<td>2971</td>
</tr>
<tr>
<td>Total mTOR</td>
<td>Cell Signaling</td>
<td>2972</td>
</tr>
<tr>
<td>α7 integrin CDB347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Cell Signaling</td>
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</tr>
<tr>
<td>Phosphorylated AKT</td>
<td>Cell Signaling</td>
<td>587F11</td>
</tr>
<tr>
<td>Total AKT</td>
<td>Cell Signaling</td>
<td>9272</td>
</tr>
<tr>
<td>SuperSignal West Dura Extended Duration Substrate</td>
<td>Thermo Scientific</td>
<td>34076</td>
</tr>
<tr>
<td>Laminin-III</td>
<td>Invitrogen</td>
<td>23017-015</td>
</tr>
</tbody>
</table>
I) Experimental design

1) Wild type vs. Knockout Primary Cells on Collagen-I or Laminin Substrate

The purpose of this experiment was to determine the effect of substrate (collagen and laminin) on α7 integrin activation and the resulting signaling response. Primary cells extracted from wild type (WT) mice or α7 integrin knockout (KO) mice were used. Protein amount and activity (α7 integrin, p70 S6K phosphorylation, total p70 S6K, mTOR phosphorylation, and total mTOR) were analyzed via western blot. The groups are as follows:

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Substrate</th>
<th>Rest</th>
<th>0h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Collagen I</td>
<td>N=6</td>
<td>N=6</td>
<td>N=6</td>
</tr>
<tr>
<td>WT</td>
<td>Laminin</td>
<td>N=4</td>
<td>N=4</td>
<td>N=4</td>
</tr>
<tr>
<td>KO</td>
<td>Collagen I</td>
<td>N=6</td>
<td>N=4</td>
<td>N=4</td>
</tr>
<tr>
<td>KO</td>
<td>Laminin</td>
<td>N=4</td>
<td>N=4</td>
<td>N=4</td>
</tr>
</tbody>
</table>

2) Laminin-111 Treatment of myotubes

In this experiment myotubes differentiated from C2C12 cells on Collagen I substrate were subjected to an overnight incubation in Laminin 111, in a high concentration (100nM) and a low concentration (50 nM). The limitation of this experiment was detachment of the tubes from the Flexcell membrane at some point during strain, particularly those tubes which had experienced an overnight incubation of laminin 111. Cells were harvested 3 hours after strain. Protein amount and activity (α7 integrin, p70 S6K phosphorylation, total p70 S6K, mTOR phosphorylation, and total mTOR) were analyzed via western blot. The groups are as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Rest no Lam 111</th>
<th>Strain no Lam 111</th>
<th>Rest Low concentration</th>
<th>Rest High concentration</th>
<th>Strain low concentration</th>
<th>Strain high concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=2</td>
<td>N=2</td>
<td>N=2</td>
<td>N=2</td>
<td>N=2</td>
<td>N=2</td>
<td>N=2</td>
</tr>
</tbody>
</table>
An additional experiment using laminin-111 was performed for immunocytochemistry (ICC) analysis. C2C12 cells were seeded onto 4 well glass chamber slides and differentiated into myotubes. Cells were then incubated overnight in laminin-111 (0nM, 25nM, 50nM, 100nM, and 200nM). ICC was then performed for α7 integrin and imaged using a fluorescence microscope.

3) Transfection optimization

The purpose of this experiment was to determine the optimal liposome mediated transfection reagent for the delivery of the pMHCK-bx2 plasmid into myoblasts coding for overexpression of the rat α7 integrin. Plasmid DNA (0.5 µg) was delivered to each well of the 6 well bioflex dish. After differentiation, myotubes were strained or left as controls, and harvested 3h post strain. Protein amount and activity (α7 integrin, p70 S6K phosphorylation, total p70 S6K, S6 phosphorylation, total S6, mTOR phosphorylation, total mTOR, α-tubulin, and β-actin) was analyzed via western blot. The limitation to this experiment was the death of all the cells which were exposed to the Effectene transfection reagent. Groups are as follows:

<table>
<thead>
<tr>
<th></th>
<th>FuGENE® HD</th>
<th>Lipofectamine</th>
<th>Effectene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest Vector (control)</td>
<td>N=2</td>
<td>N=2</td>
<td>N/A</td>
</tr>
<tr>
<td>Rest Plasmid</td>
<td>N=2</td>
<td>N=2</td>
<td>N/A</td>
</tr>
<tr>
<td>Stretch Vector</td>
<td>N=2</td>
<td>N=2</td>
<td>N/A</td>
</tr>
<tr>
<td>Stretch Plasmid</td>
<td>N=2</td>
<td>N=2</td>
<td>N/A</td>
</tr>
</tbody>
</table>

4) PI3K inhibition (Wortmannin) on Strained, and Unstrained Transfected Myotubes

In this experiment Primary Myoblasts were transfected with pMHCK-BX2 (Plasmid), or MHCK-CAT (Vector), and either incubated over night with Wortmannin (10µM) or left as a control. Half of the samples were subjected to mechanical strain, and the other half were left as controls. Cells were harvested 3h post strain. Protein amount and activity was analyzed via western blot (α7 integrin, p70
5) **PI3K Inhibition (Wortmannin) and Strain on Transfected Myotubes**

In this experiment Primary Myoblasts were transfected with pMHCK-BX2 (Plasmid), or MHCK-CAT (Vector), and either incubated over night with Wortmannin (10 µM) or left as a control. All samples were subjected to mechanical strain. Cells were harvested 3h post strain. Protein amount and activity was analyzed via western blot (α7 integrin, p70 S6K phosphorylation, total p70 S6K, S6 phosphorylation, total S6, mTOR phosphorylation, total mTOR, α-tubulin, β-actin, AKT phosphorylation and total AKT). The groups are as follows:

<table>
<thead>
<tr>
<th></th>
<th>10µM Wortmannin</th>
<th>No Wortmannin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest Vector</td>
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<td>N=3</td>
</tr>
<tr>
<td>Rest Plasmid</td>
<td>N=3</td>
<td>N=3</td>
</tr>
<tr>
<td>Strain Vector</td>
<td>N=3</td>
<td>N=3</td>
</tr>
<tr>
<td>Strain Plasmid</td>
<td>N=3</td>
<td>N=3</td>
</tr>
</tbody>
</table>
CHAPTER III

RESULTS

A) KO vs. WT Myoblasts differentiation and Myotube Phenotype

In the search for an appropriate experimental model to implicate the α7 integrin as a mechanotransducer, the first step was to form myotubes in the wells of the Bioflex culture plates. Myotubes are formed in culture by terminal differentiation and fusion of myoblasts, which have been extracted from muscles (Rudnicki, 2005). Myoblasts which were extracted from both α7 Integrin knockout (KO) and wild type (WT) mice by a previous researcher in the Molecular Muscle Physiology lab were used for this first attempt.

The 6 well Bioflex culture plates from Flexcell International can be purchased with a substrate coating. Because cells will not attach to the untreated silicone membranes, both collagen I-treated, and laminin-treated dishes were used in the first wave of experiments for this project. We also hypothesized that myotubes expressing high levels of the α7 integrin would preferentially adhere to laminin, the α7 integrin binding partner, ultimately allowing for optimal transmission of force in an outside-in manner.

As seen in Figure 14, myotubes formed from WT myoblasts formed long healthy tubes, while myotubes formed from KO myoblasts formed short tubes with unusual morphology. The unusual morphology of the KO myotubes makes this model inadequate to address the hypothesis.
Figure 14: Stunted growth of KO myotubes. Myotubes differentiated from myoblasts extracted from WT and KO mice were differentiated on collagen I treated and laminin treated Bioflex dishes.

KO and WT myotubes adherent on either collagen I or laminin were subjected to mechanical strain with groups harvested immediately post strain (0h PS) or three hours post strain (3h PS). KO and WT myotubes not subjected to strain were used as controls. Western blot analysis was used to verify the lack of α7 integrin expression in KO myotubes (Figure 15). Surprisingly, α7 integrin expression was 5-fold higher in the presence of collagen compared to laminin in the absence of strain (P < .05) (Figure 16). At 0h post strain, α7 integrin expression increased (1.6 fold on collagen, and 2 fold on laminin) in myotubes irrespective of substrate compared to substrate-matched, unstrained controls. At 3 hr post-strain, α7 integrin expression was increased 1.8-fold in the presence of collagen and 3-fold in the presence of laminin compared to unstrained controls (P < 0.05).
Figure 15: Lack of α7 expression in myotubes differentiated from KO myoblasts. Western blot analysis on myotubes shows an increase in α7 integrin expression with strain on both collagen and laminin substrates. There is no α7 integrin expression in KO myotubes.

Figure 16: Quantification of α7 integrin expression with strain. Western blotting quantification demonstrates higher basal levels of the α7 integrin on the collagen substrate than on the laminin substrate. There was an increase in α7 integrin expression in response to strain on both laminin (3 fold at 3h, P < 0.05) and collagen (1.8 fold at 3h, P < 0.05).

The hypertrophic signaling response to mechanical strain was then examined in the presence of collagen and laminin. An increase in mTOR phosphorylation was not observed in WT myotubes at any time point on either substrate (Figure 17). In contrast, p70 S6K phosphorylation was significantly
increased at 3 hr post-strain only in the presence of laminin (P < 0.05) (Figure 17) Hypertrophic signaling 
was not activated in response to strain in KO myotubes.

**Figure 17:** The presence of the α7 integrin and laminin are important for the activation of p70 S6K in response to 
mechanical strain. WT and KO myotubes were subjected to strain (18%, 1 Hz, 20 min) and phosphorylation of 
mTOR (top) and p70 S6K (bottom) were examined.
B) pMHCK-BX2 Transgenic Modification of Myotubes

The rat sequence of the α7BX2 integrin was inserted into the pMHCK vector. This plasmid DNA was transfected into WT myoblasts. The empty vector (MHCK-CAT) was transfected into cells as a control. The promoter (MHCK7) is turned on in the presence of myosin heavy chain and muscle creatine kinase. In order to find the appropriate liposome mediated transfection reagent, transfections were performed using three reagents from different companies: FuGENE HD, Lipofectamine, and Effectene. The vector does not have a reporter, so the presence of a 70k Da band on the α7 western blot, which is unique to the rat version of the α7 integrin, was used to verify a successful transfection.

All cells that were exposed to Effectene did not attach to the membrane resulting in the elimination of this group. Myotubes either remained unstrained to serve as a control, or were subjected to the same protocol previously outlined above. Tubes were collected at 3 hr post-strain. Western blot analysis yielded a higher total α7 integrin expression in samples exposed to Lipofectamine, however the 70 kDa band was only observed in samples transfected using FuGENE HD (Figure 18). Despite a trend towards increased α7 integrin protein presence in transfected myotubes compared to vector controls, significant increases were not detected (Figure 19).

![Figure 18](image-url)

**Figure 18:** FuGENE HD results in successful transfection. WT myotubes transfected with a Plasmid (P) or Empty Vector (V) were subject to strain (S) or left unstrained (R) as a control. Presence of the 70 kDa band for α7 western blot demonstrates successful transfection only with FuGENE HD.
Hypertrophic signaling in response to strain and transfection was quantified (Figure 20). No significance was found in any of the groups. However, there was a trend towards increased phosphorylation of p70 S6K (Thr389) (~30-40%) in transfected and/or strained myotubes compared to the unstrained myotubes transfected with the empty vector. A similar trend was observed in the phosphorylation of p70 S6 Kinase (Thr421/Ser424)(~50-60%). Phosphorylation of mTOR followed a trend of increase with strain compared to unstrained vector controls, as well as an increase in the unstrained plasmid group. However, phosphorylation of mTOR was not elevated in the transfected myotubes following strain. The downstream substrate of p70 S6K, S6, was not activated in response to transfection and/or mechanical strain.
Figure 20: Hypertrophic signaling response to strain in transfected myotubes. Hypertrophic signaling was quantified via western blot analysis for plasmid or empty vector, strained or untrained myotubes. No significance was found. A) p70 S6 Kinase (Thr 389) signaling response to strain in α7 overexpressing myotubes B) p70 S6 Kinase (Thr421/Ser424) signaling response to strain in α7 overexpressing myotubes C) mTOR signaling response to strain in α7 overexpressing myotubes. D) S6 signaling response to strain in α7 overexpressing myotubes.

C) Strain and PI3K Inhibition (Wortmannin) With High Transfection Efficiency in Myotubes

Previous work has shown increases in total protein in myotubes in response to strain independent of exogenous growth factors (Vandenburgh, 1979). Further work has inhibited growth factor signaling using wortmannin which blocks the activation of PI3K (Hornberger, 2004; Miyazaki, 2011). In this experiment, myotubes were incubated with wortmannin in order to ensure any possible amplifications of hypertrophic signaling in transfected samples were independent of paracrine or autocrine signaling. All myotubes were strained, and harvested 3h post strain. Half received wortmannin while half did not. In order to determine whether the wortmannin affected growth factor signaling, a western blot was performed to assess phosphorylation of AKT. As shown in Figure 21,
phosphorylation of AKT and S6 was reduced by ~60% with an overnight incubation of wortmannin with significance (P<.05) being found in the reductions of S6. Additionally there was no increase in AKT or S6 activation with strain in either group.

Figure 21: Reduction in AKT and S6 signaling demonstrates wortmannin is Effective. AKT and S6 signaling was quantified via western blot 3h post strain in myotubes either transfected with the empty vector or plasmid and in the absence or presence of wortmannin. (* P<.05)

α7 Integrin expression (Figure 22) was markedly and significantly increased 8.6 fold (P < 0.05) with transfection for the groups which did not receive wortmannin. Unfortunately, α7 integrin expression did not remain stable in the presence of wortmannin, with wortmannin increasing α7 integrin expression in myotubes transfected with the vector control and decreasing α7 integrin expression in myotubes transfected with the plasmid none of which were found to be statistically significant.
Figure 22: α7 Integrin expression with transfection and wortmannin following strain. α7 integrin expression was quantified via western blot 3h post-strain in myotubes either transfected with the empty vector or plasmid and in the absence or presence of wortmannin.

Interestingly, amplification of the phosphorylation of mTOR, p70 S6K (Thr389, Thr421), and S6 was not observed in transfected myotubes following strain compared to controls despite the 8-fold increase in α7 integrin (Figure 23).
**Figure 23:** Hypertrophic signaling response to strain and wortmannin in transfected myotubes. Hypertrophic signaling was quantified via western blot 3h post strain in myotubes either transfected with the plasmid or empty vector, after an overnight incubation in wortmannin. A) p70 S6 Kinase (Thr389) signaling response to strain, with transfection and wortmannin. B) p70 S6 Kinase (Thr421/Ser424) signaling response to strain, with transfection and wortmannin. C) mTOR signaling response to strain, with transfection and wortmannin.

**D) Wortmannin and Transfection: Rest vs. Strain**

In order to get a better understanding of the effect of wortmannin on α7 integrin expression, the previous experiment was repeated with unstrained controls. The transfection efficiency observed in this experiment was much lower than in the previous experiment (Figure 24). For samples negative for wortmannin, α7 integrin expression (Figure 24) was only slightly increased in plasmid transfected myotubes compared to vector transfected myotubes in the absence of strain (rest samples). Similarly, α7 integrin expression was not significantly increased in plasmid transfected myotubes compared to vector transfected myotubes following strain. Consistent with the previous experiment, wortmannin
altered α7 integrin expression in a manner that resulted in both the upregulation and downregulation of the protein.

**Figure 24:** α7 Integrin expression with Transfection and Wortmannin. α7 integrin expression was quantified 3h post strain, with transfection of the plasmid or empty vector, and with or without an overnight incubation in wortmannin. Transfection efficiency was found to be low.

Activation of p70 S6K (Thr389, Thr421/Ser424) was not observed either in response to strain in vector control, nor in response to low transfection of the α7 integrin. There was a significant (P < 0.05) increase in mTOR phosphorylation with strain in vector controls. No plasmid effect was observed.
Figure 25: Signaling response to strain with transfection and wortmannin. Hypertrophic signaling was quantified 3h post strain, with transfection of the plasmid or empty vector, and with or without an overnight incubation in wortmannin. A) p70 S6K (Thr389) signaling response to strain, with transfection and wortmannin. B) p70 S6K (Thr421/Ser424) signaling response to strain, with transfection and wortmannin. C) mTOR signaling response to strain, with transfection and wortmannin. D) S6 signaling response to strain, with Transfection and Wortmannin.

**E) Laminin-111 and the α7 Integrin**

Previous studies have demonstrated that injections of laminin-111 can elicit an increase in α7 integrin expression in mice (Rooney, 2009). As a preliminary step to establish whether or not laminin-111 could be used as a model of overexpressing the α7 integrin in myotubes, immunocytochemistry for α7 integrin was performed after an overnight incubation in different concentrations of laminin-111 (figure 26). There was no visible difference in α7 integrin expression until the concentration reached 100nM, and there was a vivid difference in α7 expression with 200nM laminin-111 as compared to the control which was not incubated in laminin-111.
Upon verification that there were increases in α7 integrin expression with an overnight incubation of laminin-111 using ICC, myotubes differentiated from C2C12 cells and grown on the collagen I substrate were incubated overnight in 0 nM, 50 nM, or 100 nM of laminin-111 and subjected to mechanical strain. Examination of the Flexcell dishes post strain revealed de-attachment of the myotubes had occurred at some point during strain. α7 integrin expression and signaling changes were still analyzed by western blot, but duration of strain prior to detachment is unknown. When quantified via western blot, α7 integrin expression (figure 27) was found to be slightly increased with increasing concentrations of laminin-111. There was a 20% increase in α7 expression with 100nM of laminin-111 as compared to 0 nM, but this change was not statistically significant.
Figure 27: α7 Integrin expression in response to strain and an overnight incubation in laminin-111. Myotubes (C2C12) were incubated overnight in 0 nM, 50 nM, or 100 nM of laminin-111 and subject to 20 minutes of strain. Strained myotubes detached from the bioflex membrane during strain. α7 integrin expression increased as the concentration of laminin-111 increased.

Figure 28: Hypertrophic signaling response to strain and an overnight incubation in laminin-111. Myotubes (C2C12) were incubated overnight in 0 nM, 50 nM, or 100 nM of laminin-111 and subject to 20 minutes of strain. Strained myotubes detached from the bioflex membrane during strain.
CHAPTER IV

DISCUSSION

A) Mechanical Strain

Previous work in the Molecular Muscle Physiology Lab has delineated a role for the α7 integrin in muscle hypertrophy. However, due to the complexity of the intramuscular environment it is difficult to elucidate the precise mechanism responsible for this observation in vivo. While hypertrophic signaling molecules were activated to a greater extent in transgenic mice overexpressing the α7 integrin as compared to wild type animals, the reason for enhancement of growth remains unknown (Zou, 2011). Increased adhesion of the fibers to the ECM created by transgenic overexpression of the α7 integrin can create an environment conducive to growth by limiting the amount of damage and inflammation accumulated by eccentric exercise (Boppart, 2006; Boppart, 2008; Leuders, 2011; Zou, 2011). Alternatively, the α7 integrin could be an intrinsic regulator hypertrophic signaling via mechanotransduction.

The purpose of this study was to determine whether the α7 integrin is, or is not an intrinsic regulator of hypertrophic signaling, and because of the complexity of the intramuscular environment as mentioned above, an in vivo model is not suitable to answer this question. Both primary cells and C2C12 cells were experimented with as a means to form myotubes, which is an in vitro model of muscle (Vandenburgh, 1979). In order to replicate mechanical stimulation in vivo, the Flexcell System (FX-4000) was used. Previous work Hornberger et al. had already established that 15% strain was sufficient to elicit a hypertrophic signaling response in cultured myotubes with equibiaxial strain, but not with uniaxial strain (Hornberger, 2004). The strain protocol used for the experiments in this study was based on these previous studies, with the idea that signaling responses would be directly related with α7 integrin expression, as observed in vivo. In experiments performed for our project, however, statistically significant increases in signaling with strain were extremely rare, occurring in only one trial.
as observed in Figure 17. The experiments were continued using this protocol, hoping that upon overexpression of the α7 integrin via transient transfection, there would be a statistically significant enhancement of the signaling response to support our hypothesis. However, mechanical strain did not consistently increase hypertrophic signaling even in the absence of α7 integrin overexpression, which is exemplified in Figures 7, 12A & 12B.

Future experiments of this nature call for an optimization of the strain protocol for our lab in order to establish consistent, and statistically significant increases in signaling with strain. Only after this optimization should transient overexpression of the α7 integrin be re-attempted, and strain experiments performed. Parameters of strain, such as time points of harvest, duration of strain, as well as waveform of strain and frequency are all to be compared to one another in an attempt to find a protocol which yields the greatest hypertrophic response of the myotubes to strain. Direction of strain is to be re-assessed, comparing biaxial and uniaxial strain similar to T.A. Hornberger’s experiments in 2004, as well as performing a uniaxial pre-strain on differentiating myoblasts in order to force alignment of tubes perpendicular to strain; which has been performed in the study “Uniaxial Cyclic Strain Drives Assembly and Differentiation of Skeletal Myocytes” (Pennisi, 2011). Once aligned tubes are formed, strain will be transmitted parallel to these aligned myotubes, and the hypertrophic response examined.

Because the laminin content of the pre-treated plates available through Flexcell International is a proprietary blend, it is not possible to know the concentrations of laminin, nor which isoforms are present. In order to better control substrate in future experiments, myotubes can be grown on PDMS hydrogels fabricated with an elastic modulus of 12 kPa used to mimic the stiffness of muscle, along with the addition of various concentrations of laminin (Gilbert, 2010). Signaling molecules such as JNK and p38 which are indicative of strain sensed by tissues and cells will also be analyzed by western blot (Boppart 2001, Boppart, 2006).
B) α7 Integrin Therapy

The α7 integrin has been shown previously to alleviate damage caused by muscular dystrophy, and the lack of the α7 integrin in muscle results in increased muscle damage and a muscular dystrophy phenotype (Mayer, 1997; Liu, 2011; Rooney, 2009). Increased α7 integrin through transgenic overexpression in mice has shown increased muscular growth, myofibrillar content, and increases in hypertrophic signaling (Leuders, 2011; Zou, 2011). Global gene profiles are unaltered in transgenic models with overexpression of the α7 integrin, and no toxic effects have been observed. Additionally myoblasts with elevated α7 integrin expression have been shown to proliferate faster, have better resistance to apoptosis, and adhere better laminin as compared to myoblasts with normal levels of α7, without impacting the ability for the cells to differentiate normally (Liu, 2011). In applying these established benefits of the α7 integrin towards alleviating disease or age related myopathies in humans, a safe method of manipulating the α7 integrin is necessary before this can become a therapy. Laminin has been found to be a positive regulator of α7 integrin expression and it has been shown that injections of laminin-111 increases α7 integrin expression in vivo (Burkin, 1999; Rooney, 2009).

In this study, addition of laminin-111 was found to increase α7 integrin expression in C2C12 myotubes grown on collagen I-coated Bioflex membranes (Figure 14). Despite the ability to conditionally induce α7 integrin expression, myotubes incubated in laminin-111 detached from the membrane during mechanical strain. This was likely due to preferential binding of the myotubes to laminin-111 in suspension which was not adherent to the Bioflex membrane. In future trials of this experiment, laminin-111 will be incorporated into PDMS membranes that will be attached to the Bioflex membranes. This will ensure transient upregulation of the integrin and subsequent ability to strain the myotubes without disruption.
C) Focusing the Model after Preliminary Results

Initial experiments started quite broad, examining α7 integrin expression and hypertrophic signaling 0 h post strain as well as 3 h post strain. The 0 h post strain time point was chosen to be consistent with experiments by Hornberger et al. (Hornberger, 2004). The 3 h time point chosen to examine whether the signaling changes persisted or even increased further, which was found to be the case in a study looking at signaling in vivo after downhill running (Boppart, 2006). Both the collagen I and laminin substrates were used initially as well. Collagen I was the substrate used by Hornberger et al., and we decided to use laminin as well because it is the binding partner for the α7 integrin (Collo, 1993; Hornberger, 2004; Schober, 2000). Although Hornberger et al. used C2C12 cells for his experiments, primary myoblasts were chosen for the beginning experiments because this allowed us to create KO myotubes. After this model was found to be insufficient, the decision to use C2C12 cells was made for a few experiments due to concern that the plating method used to obtain the primary myoblasts can result in a mixed cell population. After working with the C2C12 cells it was found that prior to differentiation, myoblasts rapidly proliferated and were prone to extreme overconfluence. This resulted in the presence of many undifferentiated myoblasts as well as increased risk for detachment of the C2C12 myotubes during strain. Additionally, the literature suggests that C2C12 myotubes are an insufficient compared to primary myotubes for the study of muscle physiology. C2C12 myotubes have been found to exhibit abnormal Ca\(^{2+}\) signaling and form with a heterogeneous distribution of sarcoplasmic reticulum (Lorenzon, 2002).

Preliminary experiments demonstrated higher basal α7 integrin expression on collagen I than on laminin (Figure 16). This is likely due to upregulation of α7 integrin protein on collagen in response to decreased binding. Because the binding partner for α7 is laminin, there is higher binding on this substrate, and in order to increase the chance for α7 to bind to any laminin secreted by the cells, total protein was increased on collagen. There was also an increase in α7 integrin expression in response to
strain, reaching the highest increase 3 h post strain on both substrates. Because it is unlikely that such rapid increases in protein would occur it is hypothesized that mechanical strain-induced activation of the α7 integrin and increase in binding to laminin, yields a change in integrin conformation which may allow for enhancement of primary antibody binding. Thus, the fold increase in α7 integrin expression with strain would be reflective of an increase in α7 integrin activation. In support of this hypothesis, the highest increase in α7 was observed 3 h post strain on the laminin substrate, and this was the only time point found to have a statistically significant increase in phosphor p70 S6 Kinase (Thr 389). Because of these results future experiments were performed on laminin and myotubes were harvested 3 h post strain.

D) α7 Integrin KO Myotubes as a Model

The initial experimental design to address the hypothesis was to simply have myotubes that express the α7 integrin, and to have myotubes that do not. If there was no signaling response in α7 KO myotubes, or a significantly blunted response, then the hypothesis would of course be accepted. In order to achieve this, myoblasts extracted from α7 integrin KO mice, as well as from WT mice were expanded on the bioflex membrane and differentiated into myotubes. There was absolutely no signaling response to strain in KO myotubes, as seen in Figure 17. However as observed in Figure 14, KO myoblasts did not form myotubes with normal morphology, instead forming stunted as compared to WT myotubes. It was determined that this model was unsuitable to address the hypothesis due to the formation of stunted myotubes from KO myoblasts.

The α7 integrin expression is upregulated with differentiation and binding of the α7 integrin to laminin and helps with myoblast migration during differentiation (Collo, 1993). KO myotubes could have been malformed due to the possibility that the α7 integrin is essential for differentiation of myoblasts into myotubes. The α7 integrin is important for actin stabilization at the Z-bands, and the absence of
this protein leads to a mild and progressive form of dystrophy (Mayer, 1997). Because of this unhealthy intramuscular environment, there are likely much more fibroblasts in the muscle, and a possible depletion of myoblasts as seen with muscular dystrophy (Jejurikar, 2003). This can result in the retrieval of more fibroblasts and less myoblasts during extraction, increasing the possibility of fibroblast contamination in the KO primary myoblast cultures obtained using the plating method. In order to prevent this address this concern, extracted myoblasts should be sorted by FACS prior to expansion in culture.

E) **Overexpression of α7 Integrin Using the Transient Transfection Model**

After it became apparent that the simplistic approach of comparing the hypertrophic response of mechanically strained WT myotubes to the response of KO myotubes was problematic due to the stunted morphology, overexpression of the α7 integrin via transient transfection was performed. WT Primary myoblasts were transfected with either a plasmid for rat α7 integrin (pMHCK-BX2), or the empty vector (MHCK-CAT), and subjected to strain. This technique had never before been performed in the Molecular Muscle Physiology Lab, requiring trial and error to find an appropriate reagent. In searching the literature for protocols and previously used reagents, it was determined that transfection on terminally differentiated cells yielded a very low efficiency, and transfection on suspended cells yielded a very high transfection efficiency. This required the transfection of the myoblasts during plating onto the bioflex membrane, and due to the results from KO myotubes, the issue of α7 expression affecting differentiation was considered. The worry of cells overexpressing α7 integrin with transfection, and thus affecting differentiation, was sequestered by the promoter of the vector. This promoter allows the transgene to be expressed only in the presence of muscle creatine kinase and myosin heavy chain, with the result being overexpression of α7 integrin only occurred after myotubes were mature.
The first obstacle with this model was determining transfection efficiency due to the lack of a reporter in the vector. Fortunately, the rat α7 integrin protein sequence contains a unique proteolytic cleavage site compared to mouse α7 integrin that allows for the detection of a 70 kD protein product by SDS-PAGE. (Liu, 2011). As observed in Figure 18, this was used to determine whether transfection worked, or did not work. Only comparisons of α7 integrin expression in transfected myotubes as compared to the control could be used to determine the extent to which the transfection worked. Recent access to a GFP plasmid which will allow us to co-transfect cells with GFP and the pMHCK-BX2 plasmid can be employed for future experiments. With the assumption that efficiency is the same for both plasmids, transfection efficiency can be verified and compared via fluorescence microscopy.

While transfection was successful with the FUGENE HD reagent, only one experiment yielded statistically significant increases in α7 expression with transfection of the plasmid as compared to the control (Figure 22). This transfection resulted in a similar fold increase in α7 integrin expression as is seen in vivo with the α7 transgenic mice (8-fold). When looking at the signaling changes observed in Zou et al. with this level of α7 overexpression, phosphorylation of mTOR was found to increase 50% as compared to WT controls, and phosphorylation of p70 S6K was found to increase 30%. Thus, we did not expect results to be much greater in magnitude than these. As seen in Figure 23, the hypertrophic signaling response in strained, transfected myotubes as compared to strained, control myotubes trended towards the same level of increases, however statistical significance was not found, which could potentially be due to the small sample size employed. Alternatively, insufficient β subunit availability may also limit α7 integrin function. Thus, concomitant overexpression of β1D along with α7 integrin may allow for increased translocation of α7 integrin to the membrane and increase the potential for a functional contribution over an overexpression of the α7 integrin alone (Liu 2011).
**F) Inhibition of Growth Factor Signaling**

Eighteen hours prior to straining myotubes, cell media was changed to growth factor depleted media. Despite the elimination of growth factors the potential for growth factor induced increases in hypertrophic signaling were still possible through autocrine and paracrine signaling. With strain there is a release of growth factors in from the muscle which can be released from muscle fibers themselves or other cell types in muscle such as mesenchymal stem cells (MSCs) (Adams, 2004; Goldspink, 2002; Xu, 2009). As mentioned previously, the plating method used to obtain the primary myoblasts is not a flawless method, which can result in a mixed population of cells. In order to assess mechanotransduction, wortmannin was used to inhibit PI3K, eliminating growth factor activation of hypertrophic signaling.

When protein was analyzed via western blot it was found that wortmannin had an effect on α7 integrin expression (Figure 22). For this experiment, only strained myotubes were compared to each other with and without transfection, and with and without wortmannin. In order to get a better understanding of how wortmannin was regulating protein expression and activation, the experiment was repeated and unstrained groups were included. Unfortunately, this experiment resulted in a very low increase in α7 integrin expression with transfection despite the appearance of a clear band at 70 kDa in transfected samples (Figure 24). Wortmannin again affected α7 integrin expression; however the effects were not consistent with what was observed in the previous experiment. Additionally, there was no effect of strain on hypertrophic signaling as compared to unstrained controls (Figure 25) indicating this experiment was unsuccessful.
G) Conclusion

The results of this study are inconclusive. Some of the performed experiments demonstrate trends towards the involvement in the α7 integrin in mechanotransduction. However, no statistically significant differences were observed to suggest a role for the integrin in strain-induced muscle growth. Results from PI3K inhibition were inconsistent and did not support the hypothesis. While this study yielded inconclusive results, the α7 integrin is still extremely important both for muscle repair and growth. As mentioned in the Discussion, further optimization of the experimental models used must be performed in order achieve an accurate conclusion to the hypothesis of this study.
REFERENCES


Liu, J. Milner, DJ. Boppart, MD. Ross, RS. Kaufman, SJ. (2011) β1D chain increases α7β1 integrin and laminin and protects against sarcolemmal damage in mdx mice. Human Molecular Genetics, 21: 1592-1603.


APPENDIX: Experimental Protocols

A) Procedure: Thawing, Culture, Subculture, Differentiation & Introduction of C2C12 Cell Lines

Thawing (C2C12 Cells)

1) Remove from -80°C freezer and place in bucket on dry ice
2) Immerse cryogen vial containing cells (stored in DMSO, -80°C) and warm DMEM (+FBS, Glutamine, CEE, and PSK) in 37°C water bath
3) Turn on flow hood
4) Pipette 1mL Thawed Cells in 9mL DMEM growth media in a 10mL capped tube
5) Centrifuge 5 min
6) Aspirate supernatant and add 6mL DMEM growth media to the cell pellet
7) Break the pellet to resuspend the cells and split 3 mL onto each plate
   a. If using 60mm plates keep media at 3-5mL
   b. If using 100mm plates add 7mL DMEM Growth Media (total 10mL)
8) Label dish with name & date and passage and place in incubator
9) Spray hood with 75% ETOH

Splitting Cells

1) Warm DMEM and trypsin in water bath at 37°C
2) Wash cells with 3mL 1X PBS
3) Add 1mL trypsin, incubate in CO2 incubator for 5 min. Look under microscope for detachment
4) Add 1 mL DMEM to neutralize trypsin
5) Collect cells into 10mL tube and centrifuge for 5 min
6) Aspirate supernatant
7) Resuspend cells in 5mLs of DMEM Growth Media
8) Divide onto plates

Freezing Cells

1) Follow steps “Splitting Cells” 1-6
2) Resuspend in 6mL of freeze media.
3) Add 1 mL/cryogen vial, (18 vials for 3 plates)
4) Label vials DMEM, 20% FCS, C2C12, date, passages

Counting Cells

1) Add 10uL of cells to hemocytometer
2) Count 5 fields on both sides of the meter
3) Equation:
   a. Average (Cells counted on each side ) = (Side 1+Side 2)/2
   b. (Cells counted)/5 cells/mL X 10mLs = Y Cells/mL
   c. 1 million cells for 100mm diameter plate
   d. 500,000 cells for 60 mm diameter plate
Solutions

1) DMEM Growth Media for 50mL
   a. 40 mL DMEM 50/50
   b. FBS 10 mL
   c. rFGF .005 mL
   d. PSK .5 mL

2) Differentiation Medium For 50mLs
   a. DMEM – 49 mL
   b. Horse Serum – 1 mL
   c. PSK – .5 mL

3) Freeze Medium
   a. DMEM Growth Medium (whatever the cells are grown in)
   b. DMSO (10%)

Amount of Media for each plate

35mm Plates: 3mL

60mm Plates: 5mL

100mm Plates: 10mL

Flexcell Plates (per well)

When Growing: 2.5mL

When Stretching: 3mL
B) Extraction of Protein from cells:

1) Protein is extracted at 4°C in 100-200 µl of RIPA buffer.
2) Put 200 µl of buffer in each tube, and vortex until pellet is in solution
3) Place on inverter in cold room for 30 minutes
4) Micro centrifuge at 14,000 rpm for 2-5 minutes
5) Collect supernatant in a 1.5 µl micro centrifuge tube on ice (this is your extracted sample)
6) Repeat steps 2 through 5, using 100 µl of buffer (step 2)
7) Make aliquots if necessary, and save 2 µl of each sample for the Bradford assay.
8) Snap Freeze in liquid nitrogen and store in -80

For 100 mL of RIPA buffer:

- To 50 mL ddH20 add:
  - 790 mg Tris-Base
  - 900 mg NaCL
  - pH to 7.4 with HCl
  - 1 mL NP-40 (or 10 mL of 10% NP-40)
  - 0.5 g Sodium Deoxycholate (or 5 mL of 10% sodium deoxycholate)
  - 0.1 g SDS Powder (or 1 mL of 10% SDS sol’n)
  - 0.0174 g PMSF
- Fill to 100 mL with ddH20
- Store at 4°C for 6 months or aliquot and store at -20°C long term.
- 1 pill phosSTOP and complete mini per 10mL
C) α7β1 Transgene Plasmid Extraction from Transformed DH5α Bacteria

Day 1: Seeding plates from frozen bacteria

1) Obtain vials containing bacteria (DH5α Amp-R) from -80°C - Plasmid with transgene (pMHCK-BX2), and Plasmid control (MHCK-CAT). Thaw on ice.
2) Remove Luria Broth (LB) + Ampicillin (AP) treated agar plates from the fridge and let acclimate to room temperature with the agar side of the plate facing upwards, letting the condensation fall onto the lid. Label the plates on the agar side of the plate (not the lid).
3) Turn on the Bunsen Burner (Flame should be blue).
4) Shake the plates (agar side, and lid facing downwards) in the vicinity of the flame to remove excess condensation.
5) Using the Bunsen Burner heat Loop Wire until it is visibly orange, and cool against the LB + AP treated agar plate. Be careful to lift the lid of the plate only as high as necessary to fit the loop wire between the lid and the plate.
6) Vortex the vial of bacteria and rinse with ethanol. Only open the vial in the vicinity of the flame.
7) Dip the loop wire into the vial of bacteria, and streak onto the plate (streak 1). When streaking begin on the outer edge of the plate and streak towards the center, being careful not to cross back up across a streak.
8) Repeat step 2, and streak twice over streak 1, and form a new streak in a different location on the plate(streak 2). After the second pass of streak 2 over streak 1, do not allow the loop to come into contact with streak 1 a third time.
9) Repeat step 3, this time forming a 3rd streak from streak 2.
10) Use parafilm or tape to secure the lid onto the plate, and place in the warm bacteria culture room on the third floor with the agar side of the plate facing up.
11) Allow the bacteria to grow over night, and place at room temperature the following morning.

Day 2: Inoculation liquid media with fresh bacteria

1) Obtain plates streaked with Plasmid containing bacteria and plasmid control bacteria, as well as two labeled round bottom bacteria culture falcon tubes.
2) Add 3ml of LB with 100mg/ml AP (25 ml LB + 50µl AP) into the falcon tubes.
3) Using a sterilized yellow pipette tip, pick an isolated colony, and drop the tip into the tube.
4) Place the caps on the falcon tubes, and put the tubes in the shaker in the warm bacteria culture room on the third floor.
5) Allow the bacteria to grow over night. Place in fridge the following morning
6) Do not discard the plates at this juncture. Instead wrap them in parafilm to prevent drying, and place in the fridge.

Day 3: Extraction of plasmids, and determination of concentration using a gel (taken From QIAprep Spin Miniprep Kit)

1) Using the microwave for 2 minutes, heat water to 50°C to be used in step 11.
2) In the vicinity of a flame, pour the bacteria culture into a 2ml tube. Centrifuge 3 minutes @ 9,000 RPM. Decant the supernatant and repeat until all of the culture is used.
3) Resuspend pelleted bacterial cells in 250µl Buffer P1 (from 4°C and return after use) and transfer to a micro centrifuge tube.
4) Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4-6 times. (If using LyseBlue reagent, solution turns blue). Let incubate until there is an increase in viscosity.
5) Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. (If using LyseBlue reagent, solution turns colorless).
6) Centrifuge for 10 min at 13,000 rpm in a table-top micro centrifuge.
7) Apply the supernatant (from step 6) to the QIAprep spin column by decanting or pipetting.
8) Centrifuge for 30-60s. Discard the flow-through
9) Recommended: Wash the QIAprep spin column by adding .5ml Buffer PB and centrifuging for 30-60s. Discard the flow through. (This step is only required when using endA+ or other bacteria strains with high nuclease activity or carbohydrate content)
10) Wash QIAprep spin column by adding .75ml Buffer PE and centrifuging for 30-60 s.
11) Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. After removing the spin column, dab on a Kim Wipe to ensure it is dry.
12) To elute DNA, place the QIAprep column in a clean 1.5ml micro centrifuge tube. Add 50ul water to the center of each QIAprep spin column, let stand for 3 min, and centrifuge for 1 min.

Identifying the Concentration of Plasmid after Extraction:

1) Turn on the computer and the plate reader. Open Gen5 software
2) Select Read now → Take 3 → Nucleic Acid
3) Set up the parameters in the software, Plate, microspots, dsDNA
4) Set up the plate layout: Blank, Sample, Empty
5) Blank in the reagent that the DNA is diluted in
6) Add 2ul of each sample in duplicate
7) Slowly close the cover of the DNA plate
8) Match A1 with A1 when loading the plate into the plate reader
9) Click read
10) To clean – dab with a Kim wipe (DO NOT USE LIQUID, DO NOT RUB). Repeat 2x for the cover and the microspots
11) 260/280 should be between 1.7-2 for acceptable DNA quality
D) FuGENE HD on Suspended Primary Cells

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>VOLUME (PER WELL)</th>
<th>X</th>
<th># WELLS</th>
<th>=</th>
<th>TOTAL VOLUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid:</td>
<td>4 µL (.5µG)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FuGene6</td>
<td>6 µl</td>
<td>X</td>
<td></td>
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<tr>
<td>SFM:</td>
<td>90 µL</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>100µl</td>
<td>X</td>
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</table>

PROCEDURE:

1) Grow Primary cells on 10cm dishes over night

2) Trypsinize Cells from the 10cm dish and re-plate ~150k-200k cells per well on a 6 well Flexcell dish.
   a. Resuspend in .8ml SFM media per well

3) Prepare Plasmid/Transfection Mixture (incubations at room temperature):
   a. FuGENE HD: Add FuGENE reagent in SFM media. Mix and incubate 5 minutes. Add Plasmid, mix and incubate 15 minutes.

4) Add the transfection solution to the existing media in the wells of the plate

5) Incubate the cells with the transfection solution
   a. Incubate at 37°C for 5 hr, then add 1mL growth media. Allow to incubate for an additional 17-24 hours, and change to Growth Media

6) 17-24 hours later change to differentiation medium.
### ICC of Alpha 7

**Immunocytochemistry – Alpha 7**  
1 slides (4 chambers) - 800 µl/slide

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Directions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Section Preparation</strong></td>
<td>6 µm sections placed on the slide; Allow frozen slides to thaw and dry</td>
</tr>
</tbody>
</table>
| **Fix**                    | Place all slides in *(4% Paraformaldehyde)*  
                           -30 minutes (in freezer) |
| **Wash & Rehydrate**       | 1x DPBS - 5 min, 3 times  
                           (3 ml 1x DPBS) |
| **Block**                  | 10% HS (in 1x DPBS) – 1 hr  
                           (100µl HS / 900 ml 1x DPBS)  
                           No FABS |
| **Primary Antibody**       | α7B 1:500 in 1% HS (140µl HS/ 13,860µl DPBS) - 60 min  
                           1.6 µl α7B/ 800 µl 1% HS  
                           *Don’t Put On Negative Control - Leave wash on from last step* |
| **Wash**                   | 1% HS (in 1x DPBS) - 5 min, 3 times |
| **Secondary Antibody**     | *In Dark* FITC anti-Rabbit 1:250 dilution in 1% HS - 60 min  
                           4 µl TRITC/.996 ml of 1% HS (place slides in drawer) |
| **Wash**                   | 1% HS (in 1x DPBS) - 5 min, 3 times (DAPI at wash 2) |
| **DAPI**                   | |
| **Coverslip**              | Remove Chamber using Key  
                           Add Vecta shield SPARINGLY  
                           Apply coverslip DO NOT SLIDE COVERSILP (cells will fall off)  
                           Seal with nail polish  
                           Store in refrigerator for up to 1 year |
F) **Test Phospho-mTOR (289kda) and p70 S6K (70kda) western blot**

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>DETAILS</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gels</strong></td>
<td>3.5% stacking</td>
<td>8% running</td>
</tr>
<tr>
<td><strong>Prepare Samples</strong></td>
<td><strong>Add 31mg DTT into 4xLB</strong>&lt;br&gt;Boil samples for 5 min&lt;br&gt;Centrifuge 10s</td>
<td></td>
</tr>
<tr>
<td><strong>Electrophoresis</strong></td>
<td>Start at 100V 30 min&lt;br&gt;Run 200V for 30 min</td>
<td></td>
</tr>
<tr>
<td><strong>Transfer</strong></td>
<td>Equilibrate gel/membrane/paper&lt;br&gt;Start at 100V&lt;br&gt;Transfer 100V for 30 min&lt;br&gt;(IN COLD ROOM)&lt;br&gt;Ponceau S staining 1 min&lt;br&gt;Rinse membrane with ddH₂O 3X&lt;br&gt;Take pictures&lt;br&gt;Rinse membrane with ddH₂O 2X</td>
<td></td>
</tr>
<tr>
<td><strong>Block</strong></td>
<td>5% BSA + 400ul NaN₃&lt;br&gt;2g BSA powder + 40ml TBST&lt;br&gt;1 hour</td>
<td>Cut Membrane into 2 parts at 150kda site&lt;br&gt;(2nd band from top of the ladder)</td>
</tr>
<tr>
<td><strong>Primary Antibody</strong></td>
<td>Antibody Information&lt;br&gt;<strong>Phospho-mTOR (Ser 2448) Rabbit Ab</strong>&lt;br&gt;<strong>Phospho-p70 S6K (Thr 389) Rabbit Ab</strong>&lt;br&gt;1:1000 in 2% BSA in 0.1% TBST&lt;br&gt;8ml 5% BSA (from previous step)&lt;br&gt;+ 12ml TBST&lt;br&gt;Incubation 1 hour</td>
<td></td>
</tr>
<tr>
<td><strong>Rinse</strong></td>
<td>3X Rinse with 0.1%TBS-T, 5min, each</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary Antibody</strong></td>
<td>Antibody Information&lt;br&gt;Anti-Rabbit HRP (for both)&lt;br&gt;1:2000 in 2.5% BSA in 0.1% TBST&lt;br&gt;Made with Powder (1g non fat milk + 40ml TBST), add 20ml to each&lt;br&gt;Incubation 1 hour</td>
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</table>
### Western Blot - α7β

**3 Bands - 120kDa**

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>DETAILS</th>
<th>COMMENTS</th>
</tr>
</thead>
</table>
| **Gels**  | 3.5% Stacking  
8.0% Running |         |
| **Prepare Samples** | No DTT  
Do not boil samples |         |
| **Electrophoresis** | 100V for 30 min  
200 for 30 min |         |
| **Transfer** | 100V for 30 min  
(IN COLD ROOM)  
Ponceau S staining 1min  
Rinse membrane with ddH2O 3X  
Take pictures  
Rinse membrane with ddH2O 2X |         |
| **Block** | 5% Non-fat milk + 400ml NaN3  
2.0g milk + 40ml TBST  
Incubate overnight @ 4°C |         |
| **Primary Antibody** | CDB347 1:1000 (in 2% milk)  
Take 8.0 ml 5% milk from previous step  
Add 12 ml TBST  
Incubate 60 min |         |
| **Rinse** | 3x's in 0.1% TBST, 5 min |         |
| **Secondary Antibody** | Anti-Rabbit HRP 1:10000 (in 2.5% milk)  
0.5g milk/ 20ml TBST  
Incubate 60 min |         |
| **Rinse** | 4x's in 0.1% TBST, 10 min |         |